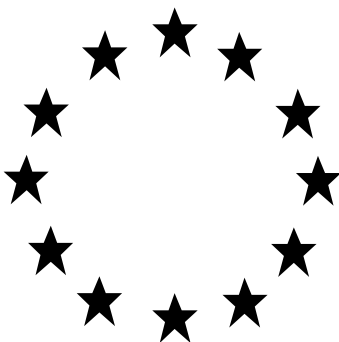


# European Union



**Draft Renewal Assessment Report prepared according to  
the Commission Regulation (EU) N<sup>o</sup> 1107/2009**

## **FLUFENACET**

**Volume 3-Annex B.9 (AS)**

**Ecotoxicology**

**RMS: Poland  
Co-RMS France**

**Summary, evaluation and assessment of the data and information examined and the list of  
studies relied upon, annotated as to the period(s) for which the particular studies are to be  
protected**

**Version History**

<b>When</b>	<b>What</b>
<b>January 1998</b>	<b>Initial DAR</b>
<b>April 2000</b>	<b>Addendum Ecotoxicology</b>
<b>April 2003</b>	<b>Flufenacet Final Addendum Ecotoxicology</b>
<b>August 2016</b>	<b>DRAR</b>

---

**Table of contents**

<b>B.9. ECOTOXICOLOGY</b>	<b>4</b>
<b>B.9.1 EFFECTS ON BIRDS AND OTHER TERRESTRIAL VERTEBRATES</b>	<b>5</b>
B.9.1.1. Effects on birds	5
B.9.1.2. Effects on terrestrial vertebrates other than birds	37
B. 9.1.3. Active substance bioconcentration in prey of birds and mammals	46
B.9.1.4. Other data on effects on terrestrial vertebrate wildlife (birds, mammals, reptiles and amphibians)	46
B.9.1.5. Endocrine disrupting properties	63
<b>B.9.2. EFFECTS ON AQUATIC ORGANISM</b>	<b>64</b>
B.9.2.1. Acute toxicity to fish.	64
B.9.2.2. Long-term and chronic toxicity to fish	100
B.9.2.3. Bioconcentration in fish	130
B.9.2.4. Acute toxicity to aquatic invertebrates	144
B.9.2.5. Long-term and chronic toxicity to aquatic invertebrates	174
B.9.2.6. Effects on algal growth	190
B.9.2.7. Effects on aquatic macrophytes	279
B.9.2.8. Further testing on aquatic organism	338
<b>B.9.3.EFFECTS ON ARTHROPODS</b>	<b>375</b>
B.9.3.1. Effects on bees	375
B.9.3.2. Effects on non-target arthropods other than bees.	398
<b>B.9.4. EFFECTS ON NON-TARGET SOIL MESO-AND MACROFAUNA</b>	<b>399</b>
B.9.4.1. Earthworm - sub-lethal effects.	399
B.9.4.2. Effects on non-target soil meso- and macrofauna (other than earthworms)	447
<b>B.9.5. EFFECTS ON SOIL NITROGEN TRANSFORMATION</b>	<b>491</b>
<b>B.9.6. EFFECTS ON TERRESTRIAL NON-TARGET HIGHER PLANTS</b>	<b>518</b>
B.9.6.1. Summary of screening data	518
B.9.6.2. Testing on non-target plants	518
<b>B.9.8. EFFECTS ON BIOLOGICAL METHODS FOR SEWAGE TREATMENT</b>	<b>541</b>
<b>B.9.9. MONITORING DATA</b>	<b>545</b>
<b>B.9.10. BIOLOGICAL ON ACTIVITY OF METABOLITES POTENTIALLY OCCURING IN GROUNDWATER</b>	<b>545</b>
<b>B.9.11. LITERATURE DATA</b>	<b>559</b>
<b>B.9.12. REFERENCES RELIED ON</b>	<b>585</b>

**B.9. ECOTOXICOLOGY**

This renewal assessment report (RAR) contains summaries of studies on flufenacet , which were not available at the time of the Annex I inclusion under Directive 91/414/EEC and were, therefore, not evaluated during the first EU review of this compound. In addition, all studies, which were already submitted for the Annex I inclusion under Directive 91/414/EEC, were re-evaluated according to the current valid test guidelines and were summarised in the RAR (study title is greyed out). In some cases where due to the study design the use of a technical substance is not possible, a solo formulation of flufenacet (Flufenacet 500 SC, Flufenacet 508.8 SC or Flufenacet 60 WG ) were used to address the intrinsic toxicity of flufenacet.

Studies which were submitted for the first EU peer-review of the active substance flufenacet but are no longer a data requirement according to the data requirements for active substances (Commission Regulation (EU)283/2013) and/or plant protection products (Commission Regulation (EU) 284/2013) are briefly summarized (text in *italic*).

In case where reliable and adequate literature was found during the literature search by Applicant , summaries are integrated in the respective sections of the RAR.

**B.9.1 EFFECTS ON BIRDS AND OTHER TERRESTRIAL VERTEBRATES****B.9.1.1. Effects on birds****B.9.1.1.1. Acute oral toxicity to birds**

The study on Bobwhite quail was submitted for the first EU approval of the active substance.

In addition, one new acute toxicity study for Canary (*Serius canaria*) and another one for Mallard duck were submitted for renewal of the active substance Flufenacet. The lowest LD<sub>50</sub> value was obtained for Canary - 434 mg a.s./kg bw/d.

All the studies were evaluated according to the representative test guidelines.

**Table B.9.1.1.1-1. Acute oral toxicity of flufenacet to bird.**

Test species	Test design	Toxicity endpoints			Reference
Bobwhite quail ( <i>Colinus virginianus</i> )	acute, oral	LD <sub>50</sub>	1608	mg a.s./kg bw/d	██████ (1992) M-003866-01-1
Mallard duck ( <i>Anas platyrhynchos</i> )		LD <sub>50</sub>	> 2000	mg a.s./kg bw/d	██████████ (1997) M-003851-01-1
Canary ( <i>Serius canaria</i> )		LD <sub>50</sub>	<b>434</b>	mg a.s./kg bw/d	██████ 2013 M-468210-01-1 KCA 8.1.1.1/03

**B.9.1.1.1.1. Acute oral toxicity of flufenacet to Bobwhite Quail**

<b>Reference:</b>	Technical FOE5043: An Acute Oral LD <sub>50</sub> with Bobwhite Quail.
<b>Author(s), year:</b>	██████████ 1992.
<b>Report/Doc. number:</b>	Study no. 102642, Reference BCS no. M-003866-01-1
<b>Guideline(s):</b>	US EPA Subdivision E, Section 71-1 (Avian oral-single test, October 1982)
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet tech. (FOE 5043), batch no: F1.036 (04.07.91), purity: 97.4%
Test species:	Bobwhite quail ( <i>Colinus virginianus</i> )
Source:	██
Number of organism:	5 males and 5 females per treatment group (housed separately) and control
Age:	Approximately 18 weeks old
Weight:	187 g – 266 g at the test initiation
Acclimation period:	14 days
Diet:	Agway Gamebird Ration, Miles Batch #7-06911 (protein: 29.2%, fat: 1.9%, 6% crude fiber) and water
Water:	Tap water
Type of test:	Acute oral toxicity
Applied concentrations:	0, 60, 125, 250, 500, 1000, 2000 mg a.s./kg b.w.
Type of application:	Capsule dosing
Time of exposure:	Single oral dose via gelatin capsules of the test material and monitored for 14 days.
<u>Test condition:</u>	
Test temperature:	21°C
Relative humidity:	10-47 %
Photoperiod	8h light/16h dark
Feed ing	Feed was provided ad libitum during acclimation and during the test, with the exception period of approximately 20 hours immediately prior to dosing, during which the birds were fasted.

Test parameter:

Observations:	<p>Mortalities and clinical signs of toxicity were recorded 3 times after dosing during the initial day. Throughout the remainder of the study the birds were observed twice daily, except the weekends, when only one observation per day was made.</p> <p>Bodyweights were observed at test initiation, on day 7 and on day.</p> <p>Food consumption for each group of five birds was recorded daily.</p> <p>Postmortem examination were performed on all birds found dead prior to the study. At test termination, post-mortem examination was carried out on birds in each group.</p>
Statistics:	<p>The LD<sub>50</sub> was calculated using a binominal probity analysis. Due to pattern of mortality (less than two concentration at which the percent dead was between 0 and 100), a probit analysis could be not accurately conducted and meaningful 95% confidence intervals could be not ascertained. Differences between control birds body weight and feed consumption and treatment level birds from day 0 to 14 was calculated using statistical analysis Annova ( <math>p \leq 0.05</math>).</p>
<u>Findings:</u>	
Mortality, toxic signs:	<p>No mortalities occurred in the control group.</p> <p>Mortality occurred only in the 2000 mg a.s./kg b.w., birds in this level were observed to be ataxic and hyperactive, and evidence of diarrhea was observed.</p> <p>No mortality occurred in the level <math>\leq 1000</math> mg/kg and only symptom of toxicity observed was diarrhea. Exposures level <math>\geq 250</math> mg a.s./kg b.w. demonstrated treatment–related effects of adult bobwhite quail.</p>
Body weight, food consumption:	<p>There was no evidence of the overall statistically significant decrease of the body weights and feed consumption by the test animals.</p>
Postmortem examinations :	<p>No gross lesions or unusual observations were recorded in the study.</p>

**Table B. 9.1.1.1.1-1: Mortality, mean bodyweights and food consumption ( ± S.D.) of Bobwhite quail following acute oral exposure.**

Nominal concentration (mg a.s./kg b.w.)	Toxicological results <sup>a</sup>	Mean body weight (g/bird) <sup>b</sup>			Mean feed consumption <sup>b</sup> (g/bird/day)
		Day 0	Day 7	Day 14	
control	0/10/0	222±23	222±23	220±22	22±8
60	0/10/0	229±15	226±20	229±15	20±3
125	0/10/0	226±11	228±13	228±13	18±2
250	0/10/*	225±14	225±14	226±15	21±5
500	0/10/*	236±16	232±12	232±12	20±4
1000	0/10/*	226±13	213±14	217±22	19±4
2000	8/10/5 <sup>1*</sup>	227±16	203±6 <sup>2</sup>	215±4 <sup>2</sup>	22±17

S.D. Standard deviation

a Number of animals which died/number of animals used/ number of animals with clinical signs

b No statistically significant different from the control ( Anova,  $p \leq 9.05$ )

\* Birds at those levels experienced diarrhea on Day 0 ( the number of birds affected is unknown)

1 Ataxia, hyporeactivity

2 Based on the body weights of the 2 surviving birds

#### Conclusion:

LD<sub>50</sub> = 1608 mg a.s./kg bw, NOEL=125 mg a.s./kg b.w.

NOAEL = 1000 mg a.s./kg b.w. (at day 0 diarrhea at dose groups  $\geq 250$  mg a.s./kg b.w.)

LOAEL = 2000 mg a.s./kg b.w.

#### **RMS comments:**

The study was conducted according to the US EPA Subdivision E, Section 71-1 (October 1982) test guideline.

The study is in general agreement with the current valid test guidelines: US EPA (OPPTS 850.2100, January 2012) and OECD 223 (July, 2010) with the following deviations:

- Relative humidity during the study was lower (being 10-47%) than recommended by U.S. EPA 71-1 guideline (should be 45-70%).
- Information on the medication is missing in the study protocol.

However, the control mortality was determined to be below 10% (being: 0%) which is in line with the validity criteria given in the OECD 223 ( 2010) guideline and US EPA guideline (OPPTS 850.2100, January 2012).

Since all validity criteria were met, these deviation are considered as having no impact on results of the study. Therefore, the study is considered acceptable.

#### **Agreed endpoints:**

LD<sub>50</sub> = 1608 mg a.s./kg bw, NOEL=125 mg a.s./kg b.w.

NOAEL = 1000 mg a.s./kg b.w.

**B.9.1.1.1.2. Acute oral toxicity of flufenacet to Mallard duck.**


---

<b>Reference:</b>	FOE 5043: Technical An Acute Oral LD <sub>50</sub> with Mallard.
<b>Author(s), year:</b>	██████████ 1997
<b>Report/Doc. number:</b>	Report no. 107700, Reference BCS no. M-003851-01-1
<b>Guideline(s):</b>	US EPA Subdivision E, Section 71-1 (Avian single-dose oral LD <sub>50</sub> ), October, 1982)
<b>GLP:</b>	Yes

---

Material and methods:

Test substance:	Technical flufenacet ( FOE 5043), batch No: 2030032/898/113008, purity: 95.6%
Test species:	Mallard duck ( <i>Anas platyrhynchos</i> )
Number of organism:	3 males and 3 females per treatment group (housed separately) and control
Age:	Approximately 20 weeks old
Weight:	Female: 901-1144 g, Male; 1063- 1386 g at test initiation
Source:	████████████████████
Acclimation period:	19 days, no mortality during acclimation period
Diet:	Agway Gamebird Ration, Miles Batch #7-059511 (protein 25,7%, fat 3.6%, 3.1% crude fiber ) and water
Water:	Tap water
Type of test:	Acute oral toxicity
Applied concentration:	0, 60, 125, 250, 500, 1000, 2000 mg a.s./kg b.w.
Type of application:	Capsule dosing f
Time of exposure:	Single oral dose via gelatin capsules of the test material and monitored for 14 days.

Test condition:

Temperature:	21°C
Relative humidity:	50 %
Photoperiod:	8h light/16h dark
Feeding:	Feed was provided ad libitum during acclimation and during the test, with the exception period of approximately 19 hours immediately prior to dosing, during which the birds were fasted.

Test parameter:

## Observations:

Mortalities, and clinical signs of toxicity were recorded 3 times after dosing during the initial day. Throughout the remainder of the study the birds were observed twice daily, except the weekends, when only one observation per day was made.

Bodyweights were observed at day-1, at the beginning of day 8 (7 days post dose) and termination (14 days post dose).

Food consumption for each group birds was recorded daily.

Necropsy examinations were conducted on all birds in the two highest dose groups with survivors and on one male and one female from the remaining dose groups and control group.

## Statistic :

The acute oral LD<sub>50</sub> was calculated using the computer program TOXCALC.

Levene's test of equal variance was performed on the data for each endpoint to determine if the dose groups had unequal variances. If the variances were not determined to be unequal, subsequent analyses were conducted using parametric techniques, otherwise, nonparametric techniques were used.

For parametric procedures, body weight and feed consumption data for all treatment levels were subjected to a standard one-way analysis of variance (ANOVA) followed by a one-tailed Dunnett's test ( $\alpha=0.05$ ).

For the nonparametric procedures, the test of equality of means was performed by ranking the data and repeating the standard ANOVA. The ranked means of the treatment groups were again compared to that of the controls using one-tailed Dunnett's test.

Findings:

## Mortalities:

No mortalities occurred in the control group.

Compound related mortalities occurred at dose  $\geq 1000$  mg a.s./kg b.w.

One female died at 2000 mg a.s./kg b.w. and two females at 1000 mg a.s./kg b.w. All died females were found dead at the first observation after dosing.

Only short lived symptoms were observed in four birds. One male in the 1000 mg a.s./kg b.w. was noted as panting, during only the first observation period on the day after dosing. One male in the 2000 mg a.s./kg b.w. was noted with diarrhea, only on the first observation period on the day after dosing. A female in the 60 mg a.s./kg b.w. was noted with hyperexcitability for two days after dosing and in the 250 mg a.s./kg b.w. was noted as hyporeactive only in the first

observation period the day after dosing. Postmortem examination of birds revealed no treatment-related findings.

Body weight,  
food consumption:

There was no evidence of the overall statistically significant decrease of the body weights and feed consumption by the test animals.

Statistical analysis showed no significant difference ( $p > 0.05$ ; Dunnett's one-tailed test) between control and dose levels for males or females.

There was a general trend for feed consumption to be reduced in dose levels 250 mg a.s./kg for day 1 following dosing. However, feed consumption in the control females and the 60 mg a.s./kg males were also reduced the day following dosing. Feed consumption was back to near normal levels in most birds on day 2. An increase in feed weight in some pans was noted on day 1 following dosing.

This was noted primarily during the first day after dosing (in a couple of birds the second day). Some birds in the higher dose levels were not eating. According to the study director because of the dose received, and the feed weight in the pans increased from one day to the next. It was determined that somehow the feed must be gaining water weight. To test this, four extra feeders were filled with feed and placed on top of the duck racks, away from any ducks but in the same room. During a five day period the feeders gained 5-6 grams in weight. The feed was dry enough, due to storage in low humidity conditions at this time of year that it was pulling moisture from the surrounding air when placed in a relatively high humidity atmosphere. Thus, all feed consumption measurements in this study were affected by the phenomenon.

However, due to the fact that the feed for all dose levels was exposed to the same conditions and to the degree of feed wastage typical in mallards, this does not affect the reliability of the results or the conclusions of the study.

Postmortem examinations:

No gross lesions or unusual observations were recorded.

**Table B. 9.1.1.1.2-1: Mortality, mean body weights and food consumption ( $\pm$  S.D) of mallard duck following acute oral exposure.**

Exposure concentration (mg a.s./kg b.w.)	Toxicological results <sup>a</sup>	Sex	Men body weight change (g/bird)		Mean feed consumption g/bird/day
			Day 1-8	Day1- 15	
control	0/6/0	female	-2 $\pm$ 14 (n=3)	-11 $\pm$ 23 (n=3)	53.4 $\pm$ 15.4 (n=3)
		male	69 $\pm$ 90 (n=3)	3 $\pm$ 60 (n=3)	87.3 $\pm$ 14.2 (n=3)
60	0/6/1*	female	15 $\pm$ 55 (n=3)	18 $\pm$ 80 (n=3)	74.8 $\pm$ 9.9 (n=3)
		male	-55 $\pm$ 49 (n=3)	-66 $\pm$ 48 (n=3)	62.0 $\pm$ 11.7 (n=3)
125	0/6/0	female	16 $\pm$ 56 (n=3)	1 $\pm$ 59 (n=3)	84.9 $\pm$ 9.9 (n=3)
		male	40 $\pm$ 34 (n=3)	5 $\pm$ 52 (n=3)	95.3 $\pm$ 19.0 (n=3)
250	0/6/1****	female	12 $\pm$ 45 (n=3)	-6 $\pm$ 37 (n=3)	84.2 $\pm$ 24.6 (n=3)
		male	-70 $\pm$ 73 (n=3)	-88 $\pm$ 75 (n=3)	74.7 $\pm$ 21.1 (n=3)
500	0/6/0	female	-32 $\pm$ 8 (n=3)	-38 $\pm$ 20 (n=3)	68.2 $\pm$ 24.8 (n=3)
		male	-7 $\pm$ 37 (n=3)	-23 $\pm$ 41 (n=3)	86.1 $\pm$ 9.3 (n=3)
1000	2/6/1***	female	-24 (n=1)	-22 (n=1)	60.6 (n=1)
		male	25 $\pm$ 142 (n=3)	-65 $\pm$ 87 (n=3)	80.9 $\pm$ 24.0 (n=3)
2000	1/6/1**	female	-43 $\pm$ 3 (n=2)	-45 $\pm$ 13 (n=2)	61.5 $\pm$ 8.7 (n=2)
		male	-9 $\pm$ 88 (n=3)	12 $\pm$ 173 (n=3)	84.0 $\pm$ 14.3 (n=3)

SD Standard Deviation

a Number of animals which died/number of animals used/ number of animals with clinical signs

\* Birds at those level experienced hyperexcitability

\*\* Birds at those levels experienced diarrhea

\*\*\* Birds at those levels experienced panting

\*\*\*\* Birds at those level experienced hyporeactivity

**Conclusion:**LD<sub>50</sub> > 2000 mg a.s./kg b.w.

NOAEL = 500 mg a.s./kg b.w (at day 0 hyperexcitability at dose groups 60 mg a.s./kg b.w. and hyporeactivity at 250 mg a.s./kg b.w)

LOEL = 1000 mg a.s./kg b.w., based on mortality

**RMS comments:**

The study was conducted according to the US EPA Subdivision E, Section 71-1 (October, 1982) guideline, which is in general agreement with the current valid test guideline US EPA (OPPTS 850.2100, January 2012) and OECD test guideline 2010.

In this study the control mortality was determined to be below 10% (being: 0%) which is in line with the validity criteria given evoked test guidelines.

The following deviations from US EPA guidelines were noted:

- In the study 6 birds per treatment (30 birds in total) instead of 10 birds/treatment were tested.

However, according to recommendations given by the current OECD 223 (2010) test guideline for acute oral test, 5 birds/treatment are acceptable.

- Information of lighting regime and medication used were missing in the study protocol.

Since all validity criteria were met, these deviations are considered as having no impact on the results of the study.

Therefore, the study was considered acceptable.

**Agreed endpoints:**

LD<sub>50</sub> > 2000 mg a.s./kg b.w./d

NOAEL = 500 mg a.s./kg b.w (at day 0 hyperexcitability at dose groups 60 mg a.s./kg b.w. and hyporeactivity at 250 mg a.s./kg b.w.

LOEL = 1000 mg a.s./kg b.w., based on mortality

**B.9.1.1.1.3. Acute oral toxicity of flufenacet to Canary (Serious canaria)**

<b>Reference:</b>	Toxicity of Flufenacet Technical during an acute oral LD <sub>50</sub> with the canary ( <i>Serinus canaria</i> ).
<b>Author(s), year:</b>	██████████ 2013
<b>Report/Doc. number:</b>	Report no. EBFOL245, Reference BCS no. M-468210-01-1
<b>Guideline(s):</b>	OCSPP 850.2100, January 2012
<b>GLP:</b>	Yes

Material and methods:

Test substance:	FOE 50439 (flufenacet/AEF133402) technical, batch no AEF133402-01-19 CAS No: 142-459-58-3, purity: 98.83%
Test species:	Canary ( <i>Serinus canaria</i> )
Source:	██████████ located in ██████████
Date of experiment	Test started on 10 September 2013 and ended on 24 September 2013.
Number of organism:	5 males/dose level and 5 females/dose level and control (housed separately)

**Age:** In adult plumage

Due to the shortage of male birds, birds from the following two lots were used for the study: 10-Jun-13 (30 females) and 12-Aug-13 (30 males), (Table B. 9.1.1.1.3-1). Male and female birds were randomly assigned to each treatment group. The randomization program distributed the birds randomly based on body weight which occurred on Day-1. Equal numbers of male (five) and female (five) canaries were assigned to each treatment group.

**Table B. 9.1.1.1.3-1: History bird information (Maryland Exotic Birds)**  
**Canary (Serinus canarius)**

Parameter	Lot - 10 June 2013	Lot-12 August
Hatch Dates	March 2010 to March 2013	March 2011 to May 2013
Age of Birds	3 months to 3 years	4 months to 2 years
Free from Diseases	Yes	Yes
Pen reared	No	No
Sex reared together	No	No
Feed	Lizz Mae	Canary Seed & Fruit
Light cycle	Daylight	Daylight
Average temperature	70F	70 F
Average humidity	56%	62%
Medical used	Antibiotics	Antibiotics

**Weight:** The range of body weight at Day -1 was between 18.3 and 24.4 g, mean 21.6 g  $\pm$  1.5 g. (Male: 19.8-24.4g with mean 22.1g; Female was 18.3- 24.0. g with mean 21.2 g).

**Acclimation period:** The range of body weights was  $\pm$  15% of the mean body weight of population 14 days.

Two mortalities occurred in the population of 248 birds. Only birds that appeared healthy were used for the study.

**Diet:** Lab Diet Advanced Protocol Small Avian Maintenance PMI Nutrition Intl, Brentwood, Missouri (protein 16%, crude fiber 2.53 %, 7.5% total fat, 8830 ppm calcium, phosphorus 7130 ppm) and water.

**Water:** Tap water

**Housing:** Cages constructed of metal bars and sheeting: 27cm (length) x33 cm (width) x 31 cm (height)

**Type of test:** Acute oral toxicity

**Applied concentration:** control 0 (empty gelatin capsules), 135, 236, 413, 723, 1265 mg a.s./kg b.w.

**Type of application:** Proper dosages of the test material were administered by coating the capsules with corn oil and then placing the gelatin capsules orally into the bird's crop.

Time of exposure: One single oral dose via gelatin capsules of the test material and monitored for 14 days.

Test condition:

Test temperature: 22.2 °C (daily average)

Humidity: 51% (daily average)

Photoperiod 8 h light/16 h dark

Light intensity 192 Lux

Feeding Feed was provided ad libitum during acclimation and during the test, with the exception period at least 4 hours immediately prior to dosing, during which the birds were fasted.

Test parameter:

Observations: The birds were observed twice daily (once on weekends/holidays and at study termination) during the treatment period for any mortalities and to detect any overt signs of toxicity or other clinical signs. The birds were observed three times on Day 0 following compound administration which occurred at approximately one, two, and three hours post-dosing.

Bodyweights were observed at test on Day -1, day 7 and 14 day.

Food consumption for each group of five birds was recorded daily. Average feed consumption (grams/day) was calculated for Day 1 to 7, Day 8 to 14, and Day 1 to 14 for each bird. Birds that died during the course of the study were subjected to gross necropsy.

Statistics: Descriptive statistics (mean and standard deviation) for body weights and feed consumption were calculated in Microsoft® Excel. All means and standard deviations were calculated using Excel. Mortality data were analyzed with CT-TOX a multi-method program that can determine the LD<sub>50</sub> and 95% confidence interval using non-linear interpolation, Binomial, Moving Average, Probit, and Spearman-Kärber methods. Normality and homogeneity of variance of the data were tested using the Chi-Square test ( $\alpha = 0.01$ ) and the Levene's test ( $\alpha = 0.05$ ), respectively. All data were normally distributed and the variances were homogenous so the data were subjected to parametric analyses. Parametric procedures involved subjecting individual body weight, body weight change, and feed consumption data to a one-tailed Dunnett's test or a Bonferroni-adjusted t-test (when sample sizes varied between groups;  $\alpha=0.05$  for both tests), where the means of the dose groups were compared to control means.

The statistical analyses on individual body weight, body weight change, and feed consumption data were conducted using TOXSTAT software.

Findings:

**Mortality, sublethal effects:** The number of bird mortalities during the study were: control (1), 135 (0), 236 (1), 413(4), 723 (9), and 1265 (10) mg a.s./kg body weight. All bird mortality occurred by Day 1 with the exception of one accidental mortality in the control group which occurred on Day 14. The death was considered accidental and no symptoms of toxicity were noted prior to this observation.

Ataxia (loss of muscular coordination), hypo-reactivity to stimuli, and/or immobility were observed in all treatment groups with the exception of the 135 mg a.s./kg bw level. No sub-lethal effects were observed in the control group during the study. Severity and prevalence of clinical observations were primarily dose dependent and all surviving birds recovered by Day 2 from the observed symptoms. The mean mortality of combined sexes and sublethal effects are presented in the Table 9.1.1.1.3-4.

**Body weight:** Individual body weight data for Day -1, Day 7, and Day 14 were subjected to hypotheses testing by treatment group. All body weight data were normally distributed and variances were homogenous therefore parametric statistical procedures were conducted with a Bonferroni t-test.

Male and female body weights were similar between the control and all treatment groups with the exception of the 723 and 1265 mg/kg bw levels in which survival was impacted.

Mean canary body weights for both sexes combined and both sexes are presented in the Table B. 9.1.1.1.3-2 and Table 9.1.1.1.3-3, respectively.

Mean body weight changes for the periods of Day -1 to 7, Day -1 to 14, and Day 7 to 14 were subjected to hypotheses testing by treatment group. All body weight change data were normally distributed and variances were homogenous therefore parametric statistical procedures were conducted with a Bonferroni t-test.

Body weight measurements (Day -1, Day 7 and Day 14) and changes in body weight (Day -1 to Day 7, Day 7 to 14) were not significantly different when treatment groups were compared to the control group. Bodyweight change was significantly lower from the control group for the 236 mg/kg b.w. group over the Day -1 to 14 interval when assessed with combined sexes (Table B 9.1.1.1.3-4:),

however no significant difference occurred for individual sexes (and Table B 9.1.1.1.3-5).

Mean feed consumption for the period of Day 1 to 7, Day 1 to 14, and Day 8 to 14 were subjected to hypotheses testing by treatment group. No significant differences were observed in daily feed consumption for males and female birds. Mean canary feed consumption data are presented in the following table for the combined sexes. (Table B 9.1.1.1.3-6).

**Table B. 9.1.1.1.3-2: Mean canary body weights - both sexes combined.**

Treatment Level (mg a.s./kg bw)	Randomization (Day -1)	Day 7	Termination (Day 14)
	Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.
Control	21.7 $\pm$ 1.6	21.3 $\pm$ 1.8	23.0 $\pm$ 1.8
135	22.0 $\pm$ 1.7	21.3 $\pm$ 1.8	22.4 $\pm$ 1.8
236	21.9 $\pm$ 1.3	20.7 $\pm$ 1.7	21.8 $\pm$ 1.5
413	21.2 $\pm$ 1.7	20.0 $\pm$ 1.2	21.7 $\pm$ 1.1
723	21.4 $\pm$ 1.7	18.2	19.5
1265	21.5 $\pm$ 1.6	-	-

$\pm$  SD standard deviation

**Table B. 9.1.1.1.3-3: Mean canary body weights - both sexes**

Treatment Level (mg a.s./kg bw)	Randomization (Day -1)	Day 7	Termination (Day 14)
	Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.
<b>Male</b>			
Control	21.9 $\pm$ 1.6	22.2 $\pm$ 0.7	23.6 $\pm$ 0.8
135	22.1 $\pm$ 1.7	21.7 $\pm$ 1.9	22.5 $\pm$ 1.8
236	22.1 $\pm$ 1.5	21.5 $\pm$ 2.2	22.9 $\pm$ 1.5
413	21.5 $\pm$ 1.6	20.4 $\pm$ 1.0	22.1 $\pm$ 1.0.
<b>723</b>	21.6 $\pm$ 1.6	-	-
<b>1265</b>	21.8 $\pm$ 1.7	-	-
<b>Female</b>			
Control	21.4 $\pm$ 1.8	20.4 $\pm$ 2.3	22.2 $\pm$ 2.5
135	21.8 $\pm$ 1.8	20.8 $\pm$ 1.9	22.2 $\pm$ 2.0
236	21.7 $\pm$ 1.3	20.0 $\pm$ 0.9	20.9 $\pm$ 1.0
413	20.9 $\pm$ 1.9	18.3-20.2	20-21.4 $\pm$ 21.4
723	21.1 $\pm$ 1.9	18.2	19.5 $\pm$
1265	21.3 $\pm$ 1.8		

$\pm$  SD Standard deviation

**Table B. 9.1.1.1.3-4: Mortality, bodyweights change ( $\pm$  S.D.) of Canary (combined sexes) following acute oral exposure.**

Nominal concentration (mg a.s./kg)	Toxicological results <sup>a</sup>	Mean body weight change		
		Day - 1 to Day 7	Day 7 to Day 14	Day - 1 to Day 14
		Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.
control	1/10/0	-0.4 $\pm$ 1.4	1.4 $\pm$ 0.2	1.2 $\pm$ 1.6
135	0/10/0	-0.7 $\pm$ 0.7	1.1 $\pm$ 0.5	0.4 $\pm$ 0.4
236	1/10/(10) <sup>1</sup>	-1.3 $\pm$ 1.3	1.1 $\pm$ 0.7	<b>-0.2 <math>\pm</math> 1.1*</b>
413	4/10/(10) <sup>1,2</sup>	-1.6 $\pm$ 0.5	1.7 $\pm$ 0.4	0.0 $\pm$ 0.8
723	9/10/(10) <sup>1,2</sup>	-1.9	1.3	-0.6
1265	10/10/(10) <sup>1,2</sup>	-	-	-

$\pm$  SD Standard deviation,

<sup>a</sup> Number of animals which died/number of animals used/ number of animals with clinical signs

<sup>1</sup> Hyporeactivity to stimuli

<sup>2</sup> Ataxia

\*Statistically significant from the control group (Bonferroni t-Test,  $\alpha=0.05$ )

**Table B. 9.1.1.1.3-5: Bodyweight changes ( $\pm$  S.D.) of Canary by sexes following acute oral exposure.**

Nominal concentration (mg a.s./kg)	Mean body weight change (g)		
	Day - 1 to Day 7	Day 7 to Day 14	Day - 1 to Day 14
	Mean $\pm$ S.D./N	Mean $\pm$ S.D./N	Mean $\pm$ S.D./N
<b>Male</b>			
control	0.3 $\pm$ 1.8/5	1.5 $\pm$ 0.3/5	1.7 $\pm$ 2.0/5
135	-0.4 $\pm$ 0.8/5	0.8 $\pm$ 0.3/5	0.4 $\pm$ 0.6/5
236	-0.9 $\pm$ 1.3/4	1.4 $\pm$ 0.9/4	0.4 $\pm$ 0.4/4
413	-1.6 $\pm$ 0.6/4	1.8 $\pm$ 0.4/4	0.2 $\pm$ 0.8/4
723	-/0	-	-
1265	-	-	-
<b>Female</b>			
control	-1.0 $\pm$ 0.7/5	1.4 $\pm$ 0.1/4	0.6 $\pm$ 0.6 /4
135	-0.9 $\pm$ 0.4 /5	1.4 $\pm$ 0.4/5	0.5 $\pm$ 0.3/5
236	-1.7 $\pm$ 1.4/5	0.9 $\pm$ 0.6/5	-0.7 $\pm$ 1.3/5
413	-2.1 - -1.3/2	1.2 - 1.7/2	-0.9 - 0.4 /2
723	-1.9 /1	1.3/1	-0.6/1
1265	-	-	-

$\pm$  SD standard deviation

Range reported when only 2 surviving birds present; value reported when only 1 surviving bird present

N number of surviving birds

**Table B. 9.1.1.1.3-6: Food Consumption of Canary (g/bird/day) combined sexes following acute oral exposure.**

Treatment Level (mg a.s./kg b.w.)	Day 1 to Day 7	Day 8 to Day 14	Day 1 to Day 14
	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
Control	5.7 ± 1.4	5.4 ± 1.3	5.5 ± 1.3
135	5.1 ± 1.0	5.0 ± 0.7	5.1 ± 0.8
236	5.5 ± 1.3	5.6 ± 1.8	5.5 ± 1.5
413	5.2 ± 0.9	5.3 ± 1.2	5.3 ± 1.0
723	4.6	3.9	4.3
1265	-	-	-

±SD      Standard Deviation

Conclusion:LD<sub>50</sub> = 434 mg a.s./kg b.w ( CL 95%: 337-560 mg a.s./kg b.w.)

NOEL = 135 mg a.s./kg b.w.

LLD = 236 mg a.s./kg b.w.

**RMS comments:**

The study was conducted in line with the provisions of the guideline US EPA (OPPTS 850.2100, January 2012) and was considered acceptable. According to USP EPA OPPTS 850.2100, January 2012, the following criteria should be met to confirm the validity of the study:

- Birds should be randomly assigned to the treatment and control pens:

In this study the randomization program distributed the birds randomly based on body weights, which were recorded on Day -1. Equal numbers of male and female canaries were assigned to each treatment group. The randomization process based on body weight was probably done because of the different age of males and females, originating from two different lots, due to the shortage of male birds.

The randomization process used in the study seems to be acceptable due to that fact that in control group as well as in treatment groups of birds weighted on Day-1 - there were individuals with lower and higher body weights (Male: 19.8-24.4g with mean - 22.1g; Female was 18.3- 24.0 g with mean - 21.2 g).

That indicated variability within the same treatment group, but at the same time in the range comparable to the mean weight of the whole test population.

However, the range of body weight of birds in the study (being 18.3-24.4g) was slightly higher than ± 10% of the mean body weight for test population (being 21.6 g ), recommended by the reference guideline US EPA OPPTS 850.2100 (January 2012). It was determined to be ± 15% with equal sex ratio. Taken into account the limited number of birds and husbandry practices it was considered acceptable.

- Mortality in the control should be <10%.

In the study only one accidental death in the control group was recorded.

- Minimum 10 birds should be used for each dose level of the test substances and the control.

In the study 10 birds/treatment were used.

- The test substance should be administered, via either capsule or gavage.

In the study the test substance was administered by capsule.

- In the definitive test a minimum of five dose levels of the test substance, plus an appropriate control should be tested

Five concentrations of test substance plus control group were tested in the study.

Generally, for passerines species including e.g.: *Serinus canaria* the specific environmental or breeding condition were not precise in the current valid guidelines – evoked US EPA guideline and OECD 223 (2010) guideline for acute oral toxicity study. However, some of them are given in the: „The Guidance for Reviewing OCSPP 850.2100 Avian Oral Toxicity Studies Conducted with Passerine Birds Memorandum”. That document was consulted by the RMS in order to evaluate the study.

The following recommendations were given in Memorandum to US EPA (OPPTS 850.2100, January 2012):

- For canaries 3 to 4 hours of fasting, sufficient to allow gut clearance, is recommended ( in the evaluated study it was 4 hours).
- The photoperiod for canaries lasting 8-10 hours of light for the non-breeding period is recommended.

In the study 8 hours of light were recorded.

-The temperature for canaries was not specified. In the study the daily average temperature - 22.1 °C, was in the range recommended by the guidelines: US EPA guideline and OECD 223 (2010) for other species (15-27°C) and was considered acceptable.

- Because of the passerines species are smaller and more prone to handling stress than other test species, it should be determined if handling during sex determination, weighting and dosing of the test item caused unnecessary stress to the animals hence affecting the quality or outcome of the study. In the study during the acclimatization period only 2 of 248 birds died.

Since all validity criteria were met, the deviations are considered as having no impact on the results of the study.

Therefore, the study was considered acceptable.

**Agreed endpoints:**

LD<sub>50</sub> = 434 mg a.s./kg b.w ( CL 95%: 337-560 mg a.s./kg b.w.)

NOEL = 135 mg a.s./kg b.w.

LLD = 236 mg a.s./kg b.w.

**B.9.1.1.2. Short-term dietary toxicity**

According to the data requirements for active substances (Commission Regulation (EU) 283/2013) and/or plant protection products (Commission Regulation (EU) 284/2013) no short-term dietary toxicity test are required to address the risk to birds. No new short-term dietary toxicity studies were submitted for the re-newal of the active substance flufenacet. The results of the short-term dietary studies summarized in the DAR (2000) are given below.

**Table B. 9.1.1.2-1: Short –term toxicity endpoints for birds.**

Test species	Test design	Ecotoxicological endpoint			Reference
Bobwhite quail ( <i>Colinus virginianus</i> )	5-day dietary	LC <sub>50</sub> LDD <sub>50</sub>	> 5317 <sup>1)</sup> > 755	ppm mg a.s./kg bw/d	█ (1994) M-003859-0 -1
Mallard duck <i>Anas platyrhynchos</i>		LC <sub>50</sub> LDD <sub>50</sub>	> 4970 <sup>2)</sup> > 949	ppm mg a.s./kg bw/d	█ (1993) M-003864-01-1

1) Highest tested concentration, two mortalities in 2 469 ppm and one mortality in 5 317 ppm group

2) Highest tested concentration, two mortalities in 4 970 ppm group

Reference: A subacute dietary LC<sub>50</sub> with mallard duck

Author: █ 1993.

Report/Doc.number: Report No.103814, M-003864-01-1

Guideline: FIFRA Guideline 71-2, OECD 205

GLP: Yes

Guidelines: FIFRA Guideline 71-2, OECD 205

**Materials and methods:**

10 day old mallard ducks (*Anas platyrhynchos*) were fed in six groups of ten birds with diets containing measured concentrations of 164, 307, 609, 1236 and 4970 mg mg a.s./kg diet for five days. The birds were observed for 8 days post dosing ( 5 days feeding+ 3 d post feeding period).

**Findings:**

Birds fed diet> 1236 mg a.s./kg diet experienced significant decreases in body weight during the exposure and/or recovery periods. Feed consumption was also apparently affected in the diet levels on day 1. A reduction in feed consumption continued through the exposure period for 4970 mg a.s./kg diet. However, feed consumption returned to control level during the recovery period. These data indicated an aversion to FOE-5043-treated feed. Birds in diet levels >164 mg a.s./kg diet experienced diarrhea on the day following administration of FOE 5043-treated feed. This was considered to be a compound-related effect but did not appear to have any adverse effect to birds. Mortality occurred at 4970 mg a.s./kg diet on day 5. Both mortalities had greatly reduced body weight at the time of death. Based on feed consumption data, mortalities may have been largely due to food-avoidance rather than due to compound-related effects.

**Results:**

LC<sub>50</sub>>4970 mg a.s./kg feed, LDD<sub>50</sub>=949 mg a.s./kg b.w.

NOEC/NOAEC =1236 mg a.s./kg feed:\*

TEC =1760 mg a.s./kg feed

(\*diarrhea, not considered an adverse effect)

Reference: FOE 50 43 technical: A subacute dietary LC<sub>50</sub> with northern bobwhite

Author: [REDACTED] 1994.

Report/Doc.number: Report No.106583, M-003859-0-1

Guideline: FIFRA Guideline 71-2, OECD 205

GLP: Yes

Guidelines: FIFRA Guideline 71-2, OECD 205

#### Material and methods:

15 weeks Bobwhite Quail (*Colinus virginianus*) in six groups of ten birds were fed with diets containing nominal concentration of 156, 312.5, 625, 2500 and 5000 mg a.s./kg diet. The birds were observed for 8 days post-dosing (5 days feeding days post feeding period).

**Findings:** Birds fed with >2469 mg a.s./kg feed exhibited symptoms of toxicity and mortality. Significant decrease in body weight and feed consumption during exposure was evident at 5317 mg a.s./kg diet.

#### Results:

LC<sub>50</sub>>5317 mg a.s./kg feed, LLC = 2469 mg a.s./kg feed

NOEC/NOAEC = 1280 mg a.s./kg feed

LLC = 2469 mg a.s./kg feed

#### B.9.1.1.3. Subchronic toxicity and reproduction to birds

Two reproductive studies on bobwhite and quail and mallard duck, were performed.

These studies were evaluated within the process of Annex I inclusion. These studies were re-evaluated and were considered still valid by RMS. The lowest NOEL was determined to be 9.4 mg a.s./kg bw/d. Details of the studies are provided briefly in the Table B.9.1.1.3-1. Short summaries of the studies are provided thereafter.

**Table B.9.1.1.3-1: Avian reproductive data of flufenacet.**

Test species	Test design	Ecotoxicological endpoint			Reference
Bobwhite quail ( <i>Colinus virginianus</i> )	22-weeks feeding, reproduction	NOEC	441	ppm	[REDACTED] (1994) M-003861-01-1
		NOEL	34	mg a.s./kg bw/d	
Mallard duck ( <i>Anas platyrhynchos</i> )	21-weeks feeding, reproduction	NOEC	88	ppm	[REDACTED] (1994) M-003858-01-1
		NOEL	<b>9.4</b>	mg a.s./kg bw/d	

Endpoints used in the regulatory risk included in bold

**Table B. 9.1.1.3.1. Effect of a subchronic dietary exposure of FOE 5043 tech. on Bobwhite quail including effects on reproduction and health.**

<b>Reference:</b>	Effect of a subchronic dietary exposure of FOE 5043 tech. on Bobwhite quail including effects on reproduction and health.
<b>Author(s), year:</b>	██████████ 1994
<b>Report/Doc. number:</b>	Report no. SXR/REP 03, Reference no. M-003861-01-1
<b>Guideline(s):</b>	US-EPA Subdivision E, & 71-4, Section 71-4, 1982 (Avian reproduction study)
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Technical flufenacet (FOE 5043), batch no: FL.036 from 04.07.1991, purity 97.7%
Test species:	Bobwhite Quail ( <i>Colinus virginianus</i> ).
Source:	██
Number of organism:	20 pairs of birds with one male and one female per pen
Age:	Approximately 23 weeks of age at study initiation
Weight:	174-283 g at the study initiation
Type of test:	Reproductive toxicity
Nominal Dietary concentration:	Control (untreated diet), 100 ppm, 447 ppm, 2000 ppm a.s.
Mean Measured Concentration:	Control (untreated diet), 109 ppm, 441 ppm, 1890 ppm a.s.
Type of application:	Test substance mixed in the diet, prepared weekly
Phases of the study:	Acclimation (pre-treatment): 2 weeks
Pre-egg laying phase:	Weeks :1-7
Induction phase:	Weeks: 8-10
Egg-laying phase :	Week: 11-22
Time of exposure:	Test birds were fed treated diet ad libitum for approximately 22 weeks during the pre-egg laying period and the egg production period. Control birds were fed untreated basal diet throughout.

Test condition:

Temperature/Relative humidity	Adult housing: 20-22°C /55-75%
	Egg storing: 16°C/
	Incubation: 37.5±0.5 °C /50-65%
	Hatching: 35-38°C, 1 <sup>st</sup> week, 30-32°C the 2 week/ 70-75%

Lighting:	<p>Weeks 1-7: 7 h Light/17 h Dark</p> <p>Week 8 onwards: photoperiod increased to 17 hours of light per day and was maintained at that length until the adult birds were euthanized.</p> <p>A 30 minute "dawn/dusk" cycle was used to simulate the light/dark transition period.</p>
Feeding:	<p>All adult birds and their offspring were given feed and water ad libitum during acclimation and testing.</p> <p>The basal diet for adults contained 16.5% of raw protein, 3.5% raw fat , 6% crude fibres.</p> <p>The basal diet of hatchlings a(Quail-Rearing Diet (██████████0711), contained at least 29 % protein and 3.3 % fat and 5.5% crude fibres.</p>
<u>Test parameter:</u>	
Observation:	<p>Adult birds were observed at least once daily throughout the adult phase for signs of toxicity, or abnormal behavior.</p> <p><u>Adult body weight:</u> Adult body weights were measured at study initiation, then every second week until week 8 and at termination of the study. Body weights were not measured during egg laying because of the possible adverse effects handling may have on egg production.</p> <p><u>The average weight of the hatchlings</u> was recorded after removal from the hatcher determined by individual weighing.</p> <p><u>The average weight of the 14 days survivors</u> taken after removal from the hatcher determined by individual weighing.</p> <p><u>Adult feed consumption:</u> Feed consumption was measured weekly per pen throughout the adult phase of the study.</p> <p><u>Egg parameter:</u> Egg were collected a daily. Records were maintained for each cage and each week of the numbers of eggs that were laid, cracked, abnormal, set and hatched and the numbers of viable and live three-week embryos.</p> <p>Eggshell thickness was recorded for each cage and week as appropriate.</p> <p><u>Hatchling parameter:</u> Records were kept of the numbers of hatchling and offspring surviving for 14 days (14-days survivors) per pen, per week.</p> <p><u>Necroscopy:</u> Adult birds that died or were euthanized during the course of the study were subjected to a gross necropsy. At the end of the</p>

exposure period, all surviving adult birds were euthanized with carbon dioxide gas and necropsied.

Statistic:

All data on reproductive performance have been calculated over 11 weeks of treatment on a "per pair" basis. Prior to analysis, all ratio data (i.e., percentage data) were transformed using a square-root arcsin transformation. Bartlett's test of equal variance was performed on a 0,1% level of significance for each parameter to determine if the dose groups have equal variances. If the variances were equal, subsequent analyses were conducted using parametric methods; otherwise, non-parametric methods were used. Parametric procedures involved subjecting study parameters to a standard oneway analysis of variance (ANOVA;  $\alpha \leq 0.05$ ). If significant differences among the means were indicated, a Multiple Range Test, e.g. a LSD (Least Significant Difference - Test) on a 5% Level ( $\alpha \leq 0.05$ ), was used to determine which treatment groups differed significantly from the controls.

For nonparametric procedures, the Mann-Whitney U test for independent samples was used, which allows a comparison of unpaired groups of different sizes.

All described statistical procedures are components of "Statgraphics Plus"-Software.

Findings:

Analytical results:

Analytical results showed average concentrations of 109, 441 and 1890 mg flufenacet/kg diet for the 100, 447 and 2000 ppm diet level respectively. Therefore, the measured concentrations were within 80% of the nominal concentration.

Flufenacet was homogeneously distributed within the diet by the employed mixing procedure (rel. SD. of the measured values: < 2%), as verified by analysis of representative samples from the lowest (100 ppm) test concentration as well as from a 2500 ppm concentration.

For analytical verification of stability in the food batches, samples from a single food batch were analysed over a maximum storage period of 24 hours in deep frozen state, followed by a 14 d storage under conditions comparable to those prevailed during the feeding study. This approach simulated representatively the practice of diet handling during the

treatment period. Measured values of active substances at the endpoint of storage showed no inadmissible deviation (91.8% at 100 ppm level / 94.3% at 2500 ppm level) from the initial a.s. concentration.

This verifies a stability of FOE 5043 in diet under the chosen storage conditions.

Biological effects:

Mortality and symptom of toxicity:

During the 22-week study period, a total loss of 16 out of 80 birds was recorded. Most of them (9 females / 5 males) were removed from cages (10 alive and 6 dead) due to severe injuries as a consequence of mate interactions. Six of these birds originated from the control group (1 dead from injuries, 4 alive), 3 females from the 109 ppm group, two females from the 441 ppm group and 3 birds from the 1890 ppm group. No symptoms were evident prior to death / removal, except for female from 441 ppm group which showed an apathetic behaviour over 3 days in the week prior to death.

Additional two birds females from one from control group and one from 1890 ppm were found dead in breeding cages without any visible injuries. While female from control group showed no behavioural impacts before death, female from 1890 ppm showed a strong apathy on the day before death.

*Postmortem examination*

The postmortem examination of both birds showed no lesions or injuries for female from 1890 ppm group, but a hemorrhage inside the right auditory tube of female from control group. Further observations apart from less severe and only transitory visible harms due to mate interactions as e.g. feather losses from females' head were apathetic behaviour of female from control group (14 days), male from 109 ppm group (5 days) and female from 441 ppm (one day). A strong apathy of female from 441 ppm for one day (laying on its belly) was followed by tumbling and disorientation during the following day. Some negligible signs of indisposition were observed for males from control (two birds) and female 109 ppm group (slight apathy), for female 109 ppm and male 109 ppm (slight tumbling), and for female 1890 ppm (slight tremble).

All these symptoms lasted only for less than a week and birds recovered completely before end of exposure.

Body weight/food consumption

Statistical analyses of body mass and feed consumption of adults revealed a significant decrease of male body mass in the 1890 ppm group ( $\alpha \leq 0.05$ ) at the beginning of the test period and also during 3, 7 and 9 weeks. Males from the lower treatment group - 109 ppm differed significantly from control group only in 7<sup>th</sup> week.

At the end of the test phase, however, no significant differences were observed in males body mass.

The body mass of treated female birds developed comparable to the weights of untreated birds throughout the whole exposure period.

A statistically significant decrease in feeding rates relative to the controls was recorded in the 109 ppm group during 7 weeks within the short day period. While birds of the 441 ppm group showed also reduced food consumption for 4 short-day weeks, the 1890 group level had a statistically significant lower feed consumption only during one week. The pattern of feeding reduction as observed during the short day period which is negatively correlated with the treatment level of the test substance, does not allow conclusive assumptions concerning potential causative factors. Feeding intensity was also reduced in single weeks during the long-day test phase, but again was not unequivocally correlated with the treatment level.

Reproductive parameter

Concerning reproductive parameters, no statistically significant differences were observed between the 109 and 441 ppm group and the controls, based on the mean values over 11 weeks. In contrast, the 1890 ppm treatment group showed statistically significant differences to control birds for several reproductive parameters. The mean number of cracked eggs per hen and their percentage on laid eggs was significantly increased in the 1890 ppm group. This effect was associated with a significant reduction of egg weights.

A further effect at the 1890 ppm level was a decrease in hatchability.

Hatchlings and offspring body weight

The mean body mass of the hatchlings from the 1890 ppm group was significantly below that of control hatchlings.

Observations on health and vitality of hatchlings during the 14 day post-hatching period showed no significant differences between treatment groups and controls.

**Table B 9. 1.1.3-1: Mean group body weight from a Bobwhite quail dietary reproduction study.**

Test concentration a.s. ppm	Sex	Group mean body weight (g)					
		Week 1	Week 3	Week 5	Week 7	Week 9	Test end
Control	Female	229	239	241	248	250	266
	Male	235	246	245	253	255	261
109	Female	226	235	237	243	245	249
	Male	227	236	235	<b>240*</b>	243	249
441	Female	226	237	237	245	248	274
	Male	225	235	235	<b>238*</b>	<b>238*</b>	255
1890	Female	230	238	239	242	246	268
	Male	<b>223*</b>	<b>233*</b>	234	<b>237*</b>	<b>242*</b>	244

\* significant different from control at  $\alpha \leq 0.05$

**TableB 9. 1.1.3-2: Mean food consumption ( g/pair/day) from a Bobwhite quail dietary reproduction study.**

Week No	Control	109 ppm	441 ppm	1890 ppm
1	30.2	<b>28.4*</b>	29.3	28.8
2	33.2	31.8	32.2	33.8
3	38.4	<b>35.1*</b>	<b>35.5*</b>	37.4
4	38.8	<b>35.8*</b>	37.3	38.8
5	37.5	<b>34.4*</b>	35.3	37.6
6	38.4	<b>33.4*</b>	<b>35.0*</b>	<b>36.1*</b>
7	36.7	<b>31.4*</b>	<b>32.3*</b>	36.2
8	35.5	<b>30.8*</b>	<b>32.3*</b>	35.2
9	33.8	32.0	31.9	34.2
10	34.2	32.3	35.5	35.6
11	34.5	33.6	36.2	34.1
12	33.9	31.8	33.4	33.5
13	36.7	<b>32.7*</b>	<b>33.3*</b>	<b>33.1*</b>
14	35.7	33.6	33.1	32.7
15	40.2	38.7	37.1	<b>36.9*</b>
16	40.3	39.3	40.1	39.5
17	38.9	38.2	41.1	40.4
18	42.1	42.2	43.7	43.8
19	44.3	44.1	43.3	44.6
20	44.8	43.4	42.8	43.7
21	47.2	44.5	44.9	45.5
22	44.8	46.3	46.3	46.5

\*statistically significant from control on 5% level

**Table B 9. 1.1.3-3: Summary from reproductive results in the Bobwhite quail.**

Reproductive parameter	Test concentration mg a.s./kg diet			
	Control	109 ppm	441 ppm	1890 ppm
Eggs laid/hen/in 11 week	49.8	48.1	43.5	41.1
Eggs cracked /hen/in 11 week	1.0	1.1	1.3	<b>2.0*</b>
Eggs set/ hen/in 11 week	45.6	43.6	39.2	36.6
Fertile eggs/ hen/11 in week	37.3	39.6	35.3	33.4
Vialable embrion d 18 /hen	35.9	36.9	33.7	31.5
Live Hatchlings/ hen	26.3	25.3	21.7	18.6
14-day old survivors /hen	22.6	21.3	18.7	14.8
% of cracked rel. to eggs laid	2.0	2.2	3.1	<b>4.9<sup>a</sup></b>
% of fertile eggs on Day 11/Eggs Set	81.9	90.8	89.9	91.5
% of viable embr. on d 18 /Eggs Set	78.7	84.6	85.9	86.2
% of self hatched Chicks, /Eggs set	57.8	58.0	55.3	50.8
% of self hatched Chicks,/Fertile eggs	70.5	63.9	61.5	55.5
% of self hatched Chicks, /viab. 18 d -embryos	73.3	68.6	64.4	<b>58.9*</b>
% of 14 day old survivors /Eggs set	49.5	48.9	47.7	40.5
% of 14 day old Survivors self hatched chicks	85.7	84.2	86.2	79.8
Total number of eggs laid	798	821	790	659
Total number of hatchling	422	433	394	316
Total number of survivors	326	364	340	253
	<b>Control</b>	<b>109 ppm</b>	<b>441 ppm</b>	<b>1890 ppm</b>
Weight for eggs	10.2	10.0	10.1	<b>9.4*</b>
Body mass for hatchling	7.1	7.0	6.9	<b>6.6*</b>
Body mass of 14 day old chicks	35.9	37.9	37.2	35.1
Shell thinness (mm)	0.21	0.22	0.22	0.21

\*statistically significant different from the control ( $\alpha < 0.05$ )

### Conclusion

During a 22 - week dietary exposure of FOE 5043 technical a.s. at concentrations of 109, 441 and 1890 ppm, no dose - dependent and thus treatment related adverse effects on behavior, survival rate or body mass changes of adult bobwhite quail were observed.

Concerning reproductive parameters, adverse effects were observed and were found to be statistically significantly different from the controls ( $\alpha < 0.05$ ) in the 1890 ppm treatment group.

Based on the mean values over 11 weeks, treatment - related adverse effects were:

- an increased rate of cracked eggs,
- reduced egg weights,
- a reduced hatching rate,
- reduced body mass of hatchlings.

The NOEC concerning reproductive performance is 441 ppm.

The LOEC for reproduction is 1890 ppm, based on the above described effects.

#### Comments RMS:

The bird reproduction study was conducted according to and US EPA Subdivision E, Section 71-4 test guidelines.

Based on the validity criteria stated in the guideline OECD 206 (1984) the study was considered acceptable.

The mortality of adults in the control was < 10% (being 5%) at the end of the test.

The average number of 14-day old survivors per Bobwhite quail hen in the controls was at least 14 (being: 22.6).

The average egg shell thickness for the control group was at least 0.19 mm (being: 0.21 mm).

The concentration of the substance being tested in diet was >80% of nominal concentration throughout the test period.

The following deviations from the current OECD 206 (1984) guideline were noted:

- Humidity data of egg storing was missing,
- Lighting regime was not reported in the study.

Since all validity criteria were met, the deviations are considered as having no impact on the results of the study.

Based on the results of the study the NOEC was determined to be 441 ppm. Based on an average daily feed consumption of 18.45 g/bird/d and an average body weight of 241.08 g a daily dose of 34 mg a.s./kg bw/d was determined (according to EFSA GD for birds and mammals, 2009).

**NOEL = 34 mg a.s./kg b.w./d**

#### B.9.1.1.3.2. Effect of technical FOE 5043 on Mallard reproduction.

<b>Reference:</b>	Effect of technical FOE5043 on Mallard reproduction.
<b>Author(s), year:</b>	██████████ 1994
<b>Report/Doc. number:</b>	Report no. 106594, Reference no. M-003858-01-1
<b>Guideline(s):</b>	FIFRA Subdivision E, Section 71-4b (Avian Reproduction study)
<b>GLP:</b>	Yes

#### Material and methods:

Test substance:	Technical flufenacet ( FOE 5043), Batch No: 2030032, purity 95.0 %
Test species:	Mallard duck, ( <i>Anas platyrhynchos</i> )
Number of organisms:	15 pairs of birds with one male and one female per cage
Age:	Approximately 18 weeks of age at study initiation

Weight:	934-1488 g at the test initiation
Type of test:	Reproductive toxicity
Nominal	Dietary concentration: Control (untreated diet), 100 ppm, 250 ppm, 625 ppm a.s.
Mean Measured Concentration:	Control (untreated diet), 88 ppm, 211 ppm, 544 ppm a.s.
Analytics:	<p>Concentration, homogeneity and stability of the test substance in the diet were verified by analytical methods.</p> <p>Samples of both control and treated diets were taken weekly, immediately after mixing, and frozen. Samples taken on weeks 1, 5, 10, 15, and 20 were analyzed for FOE 5043 diet concentration. All other samples were retained in the freezer until analytical results were received. Diet homogeneity was determined by analyzing samples from the first feed mixing from the 100 ppm and 625 ppm diet levels. Three samples were taken from three different locations in the mixing bowl for a total of nine samples. Test material stability (freezer and ambient) was performed during the pilot study.</p>
Type of application:	Dietary application. Test substance mixed in the diet, prepared weekly
Phases of the study:	<p>Acclimation (pre-treatment): 6.5 weeks</p> <p>Pre-photostimulation: 8 weeks</p> <p>Pre-egg laying (with photostimulation): 2 weeks</p> <p>Egg-laying: 10 weeks</p> <p>Post-adult termination (final incubation, hatching, and 14-day offspring rearing period): 6 weeks</p>
Time of exposure:	Test birds were fed treated diet ad libitum for approximately 21 weeks during the pre-egg laying period and the egg production period. Control birds were fed untreated basal diet throughout.
<u>Test condition:</u>	
Temperature/Relative humidity	<p>Adult housing: 21°C / 54%</p> <p>Egg storing: 15°C / 64%</p> <p>Incubation: 37.4°C / 50%</p> <p>Hatching: 37°C / 74%</p> <p>Offspring management: 21°C / 44%</p>
Lighting:	<p>Weeks 1-7: 7h Light: 17h Darkness</p> <p>Week 8 onwards: photoperiod increased to 17 hours of light per day and was maintained at that length until the adult birds were euthanized.</p> <p>A 40 min dawn/dusk cycle was used in light/dark transmission.</p>

	<p>Illuminance: average min. 5 foot candles (approximately 54 lux).</p> <p>The photoperiod for the hatchlings was maintained at 14 hours light per day.</p>
Housing	<p>Adult birds were housed indoors in a single room in pens were measured approximately 81cm (length) x 61cm (width) x 51 cm (height).</p> <p>Pens were constructed of stainless steel wire grid and stainless steel sheeting.</p>
Feeding	<p>All adult birds and their offsprings were given feed (Agway Game Bird and/ adults, Teklad DU-11 Duck Starter/offsprings), water ad libitum during acclimation and testing.</p> <p>The basal diets contained at least: 23% protein, 2.6% fat and <math>\leq 5\%</math> crude fibre (2.02% of crude fibre in diet offspring's).</p> <p>In addition, calcium and phosphorus were added the basal diet for the adults.</p>
<u>Test parameter:</u>	
Observation:	<p><u>Adult birds</u> were observed at least once daily throughout the adult phase for signs of toxicity, injuries or illness.</p> <p><u>Hatchlings</u> were observed once daily throughout the 14 day period for signs of toxicity, injuries, or illness.</p> <p><u>Adult body weight</u>: Adult body weights were measured at study initiation, at the beginning of weeks 3, 5, 7 and 9, and at terminal adult sacrifice (beginning of week 21). Body weights were not measured during egg laying because of the possible adverse effects handling may have on egg production.</p> <p><u>Hatchling bodyweight</u> were measured and recorded at hatch and on day 14.</p> <p><u>Adult feed consumption</u>: Feed consumption was measured weekly by cage throughout the adult phase of the study.</p> <p><u>Hatchling feed consumption</u> was not monitored.</p> <p><u>Egg parameter:</u></p> <p>Egg were collected twice a daily (once on the weekends and holidays). Records were maintained for each cage and each week of the numbers of eggs that were laid, cracked, abnormal, set and hatched and the numbers of viable and live three-week embryos.</p> <p>Eggshell thickness was recorded for each cage and week as appropriate.</p> <p>Egg strength measured at one point on the waist of the egg, analyzed on a per-hen basis.</p>

Hatchling parameter: Records were kept of the numbers of hatchling and offspring surviving for 14 days (14-days survivors) per cage per week.

Necroscopy: Adult birds that died or were euthanized during the course of the study were subjected to a gross necropsy. At the end of the exposure period, all surviving adult birds were euthanized with carbon dioxide gas and necropsied.

Statistic:

Dunnett's method was used to determine statistically significant differences between the control group and each of the treatment groups. Sample units were the individual pens within each experimental group, except adult body weight where the sample unit was the individual bird.

Findings:

Analytical results:

Analytical data showed average diet concentrations of flufenacet to be 88, 211, and 544 ppm for the 100, 250, and 625 ppm diet levels. Therefore, the measured concentrations were within 80% of the nominal concentration.

Flufenacet-treated diets were also determined to be homogeneous. Homogeneity was resampled during the week 5 feed mixing as the 100 ppm level sampling from week 1 had a coefficients of variation of 12%, which is slightly higher than normal. The coefficients of variation for the 100 ppm level at the resampling was 4%.

Biological effects:

*Mortality and symptom of toxicity*

Two adult mortalities occurred during the course of the adult in-life phase of the study. Both mortalities were females in the control group. One female was found dead during week 4 of the study. The second one was found dead two days later during week 4. Upon postmortem examination both birds appeared healthy. However, one of the birds appeared to have a broken neck. The study director explained that as a result of cage mate aggression or by injuries caused by trying to free its head from the cage unit.

*Body weight/feed consumption*

There were no statistically significant differences ( $p > 0.05$ ) in body weights or body weight change of males during the study. Although not statistically significant, males in the 544 ppm treatment group had a mean weight loss (-7) over the course of the study, while control males gained an average of 45 g. Mean body weight for females in the 544 ppm treatment group was significantly

reduced ( $p \leq 0.05$ ) compared to controls at weeks 3, 5, and 9 and at termination. For the lower rate 211 ppm a.s. the mean body weight also significantly reduced ( $p \leq 0.05$ ) compared to controls at week 3 and at termination.

Weight change in the 211 and 544 ppm treatment groups was significantly less ( $p \leq 0.05$ ) than controls. There appeared to be a dose-related reduction in body weight change at the termination.

#### *Postmortem Examination*

Postmortem observations of study birds showed no treatment related findings, other than approximately half of the females in the 544 ppm treatment group with regressed ovaries.

This supported the very low egg laying rate in these females throughout the reproductive period.

One male in the 544 ppm treatment group had clear fluid-filled sacks on the posterior edge of both testes. Postmortem examination of male in the 544 ppm treatment group, that had exhibited opisthotonos, revealed a stiff, crooked neck consistent with the clinical observation. Three females, two in the 544 ppm and one in the 88 ppm treatment groups, had white or cream-colored bumps on the proventriculus or gizzard wall. As only three birds exhibited this findings and there was no dose dependent trend, it was not considered treatment related.

The mean group body weight and mean food consumption were presented in the Table B 9.1.1.3.2-1 Table B 9.1.1.3.2-2 and below:

**B.9.1.1.3.2-1: Mean group body weight from mallard duck dietary reproduction study.**

Test concentration ppm	Sex	Group mean body weight (g/bw/d)						Change
		Week 1	Week 3	Week 5	Week 7	Week 9	Test end	
Control	Female	1080	1069	1105	1128	1126	1316	229
	Male	1242	1224	1222	1222	1214	1275	45
88	Female	1078	1045	1089	1122	1085	1266	188
	Male	1227	1198	1236	1254	1230	1251	24
211	Female	1064	1018*	1061	1101	1070	1194*	130*
	Male	1224	1177	1217	1247	1209	1261	38
544	Female	1081	953*	1024*	1088	1034*	1106*	25*
	Male	1266	1147	1209	1231	1176	1259	-7

\* significantly different from control at  $p \leq 0.05$ .

**B.9.1.1.3.2-2: Mean food consumption on mallard duck dietary reproduction study.**

Mean Dietary concentration (ppm)	Mean food consumption ( g/bird/day)
Control	116 ± 15
88	125 ± 16
211	132 ± 14
544	141 ± 16

*Reproductive parameters:*

The following treatment–related effects, statistically significant ( $p \leq 0.05$ ), were recorded for reproductive parameters:

- Reduction of eggs laid per hen, hatchlings per hen, 14-day survivors per hen, viable eggs of eggs set, hatchlings of viable eggs, and hatchlings of three-week embryos at 544 ppm test concentration.
- Reduction of hatchlings per hen, hatchlings of viable eggs, and hatchlings of three-week embryos were also at the 211 ppm test concentration.

*Hatchling and offspring body weights*

- Reduction of mean hatchling weight at the 544 ppm test concentration.
- Reduction of 14-day survivor body weight both the 211 and 544 ppm test concentration.

*Egg shell thickness*

- Reduction in eggshell strength in the 544 ppm treatment group.

The reproductive parameters were presented in the Table B 9.1.1.3.2-3 below:

**B.9.1.1.3.2-3: Summary of reproductive results in the mallard duck dietary reproduction study.**

Reproductive parameter	Test concentration (mg a.s./kg feed)			
	Control	88 ppm	211 ppm	544 ppm
Total eggs laid	630	745	654	229
Eggs cracked	6	17	5	6
Eggs removed for thickness/strength	47	51	45	18
Eggs defective	10	15	13	11
Eggs set	567	662	591	194
Fertile eggs	514	589	527	87
Live 3-week embryos	513	586	525	84
Hatchlings	475	531	433	37
14-day old survivors	475	520	428	36
Eggs laid/hen in 10 weeks	48	50	44	15*
14-day old survivors/hen	37	35	29	2*
Mean eggshell thickness measurements [mm]	0.375	0.365	0.371	0.359
Egg strength (kg)	3.04	3.07	3.08	2.72*
Mean hatchling bodyweight [g]	39.6	38.8	37.6	32.2*
Mean 14-day old survivor bodyweight [g]	240.8	226.2	217.2*	201.2*
Eggs cracked/eggs laid [%]	1	2	1	3

Reproductive parameter	Test concentration (mg a.s./kg feed)			
Viable embryos/eggs set [%]	91	88	90	42*
Live 3-week embryos/viable embryos [%]	100	99	100	97
Hatchlings/live 3-week embryos [%]	92	88	82*	35*
Hatchling of viable eggs (%)	92	88	82*	34*
Hatchlings per hen	37	35	29	2*
14-day old survivors/hatchlings [%]	100	99	98	98
Hatchlings/eggs set [%]	84	80	73	19
14-day old survivors/eggs set [%]	84	79	72	19

\* significantly different from control at  $p \leq 0.05$

### Conclusion

Based on reproductive parameters, adult female body weights and hatchling 14-day survivors body weights, the no- observed-effect concentration (NOEC) was 88 ppm a.s. and LOEC=211ppm a.s.

For eggshell strength, the NOEC was 211 ppm a.s.

Postmortem examination of the study birds showed no treatment findings, other than approximately half of females in the 544 ppm a.s./kg feed treatment group with regression ovaries.

### **Comments RMS:**

The bird reproduction study was conducted according to US EPA, Subdivision E, Section 71-4 test. Based on the validity criteria stated in the guideline OECD 206 ( 1984) the study is considered acceptable.

The mortality of adults in the control was below 10% at the end of the test.

The average number of 14-day old survivors per hen in the controls was at least 14 for mallard ducks (being: 37).

The average egg shell thickness for the control group was at least 0.34 mm (being: 0.375 mm). The concentration of the substance being tested in the diet was >80% of nominal concentration throughout the test period.

The following deviations from OECD 206 guideline were noted:

-The age of the birds at the beginning of the test (being 18 weeks) was not in the range as proposed according to the OECD 206 guideline (9 – 12 months).

In addition, the following deviations from recommendation given by OECD 206 (1984) and US EPA (January 2012) guidelines is noted:

- Food consumption of offspring was not recorded.

These deviations are not considered to affect the validity of the study.

Based on the results of the study the NOEC was determined to be 88 ppm. Based on an average daily feed consumption of 125 g/bird/d and an average body weight of 1173.38 g a daily dose of 9.4 mg a.s./kg bw/d was determined (according to EFSA GD for birds and mammals, 2009).

### **Agreed endpoint:**

**NOEL = 9.4 mg a.s./kg bw/d**

**B.9.1.1.4. Metabolites**

In plants flufenacet is degraded via glutathionate conjugation resulting in metabolites containing either the fluorophenyl acetamide-or the thiadone-moiety of the parent compound.

Plant metabolite studies in wheat (please refer to Volume 3 (C.A.), Section B.6) indicated two plant metabolites which exceed 10% of radioactive residues in edible crop parts in wheat: FOE-oxalate (M01), with fluorofenyl moiety and trifluoroacetate metabolite (TFA, M05) with is formed by break-down of thiadone (M09).

For these metabolites no toxicity endpoints to birds are available.

The weight of evidence approach was performed by RMS and presented in this RAR (please refer to Volume 3 (CP)).

**B.9.1.2. Effects on terrestrial vertebrates other than birds****B.9.1.2.1. Acute oral toxicity to mammals**

The acute oral toxicity of flufenacet to mammals was examined and results are presented in the several study reports evaluated for the previous authorization of the compound in the EU. These studies were re-evaluated for the purpose of the current assessment found valid and summarised in Section 6 of the RAR. Additionally the Applicant submitted one new study aimed the toxicity of TFA –metabolite. The study was also evaluated and summerised in the Section Toxicology of this RAR.

A summary of acute toxicity of flufenacet to mammals and its metabolite TFA is given in the Table B.9.1.2.1-1.

**Table B.9.1.2.1-1: Acute toxicity of flufenacet and its metabolite TFA to mammals.**

Test species	Test design	Ecotoxicological endpoint			Reference
Flufenacet					
Rat	acute oral	LD <sub>50</sub>	♂ 1617 ♀ 589	mg a.s./kg bw/d	<div></div> (1993) M-004865-02-1 and M-004865-02-1
TFA					
Rat	acute oral	LD <sub>50</sub>	> 2000	mg met./kg bw/d	<div></div> (2013) M-444479-01-1

The endpoints from the acute oral toxicity study with rats (■■■■■, 1993) for active substance flufenacet were 1617 mg a.s./kg b.w. for males and 589 mg a.s./kg b.w. for females, respectively.

As difference between male and female was > 25%, the lower endpoint was used in the risk assessment for the active substance.

The acute oral toxicity endpoint for the metabolite determined in the limit dose test (■■■■■, 2013) >2000 mg met./kg b.w. and this endpoint was used in the acute risk assessment.

#### B.9.1.2.2. Long term and reproduction toxicity to mammals

The two-generation study for rat, developmental studies for rat and rabbit and dietary study for rat were examined for the previous authorization of the compound in the EU. These studies were re-evaluated for the purpose of the current assessment and found valid and summarised in Section 6 of the RAR. The new studies aimed the toxicity of TFA –metabolite were performed for the renewal process.

In addition, the Applicant presented two statements concerning estimation of toxicity ecotoxicology endpoints to flufenacet and its metabolite-TFA, to mammals:

The key results of flufenacet and its metabolites to mammals relevant to use in the risk assessment is given in the Table B.9.1.2.2-1.

**Table B.9.1.2.2-1. Long- term toxicity of flufenacet and its metabolite TFA to mammals.**

Test species	Test design	Toxicity endpoints			Reference
Flufenacet					
Rat	Two-generation reproduction	NOAEL <sub>ecotox</sub>	500 37.4 <sup>1</sup>  Parental: There was compound related reduction in body weight for P generation females ( 5-7%) during the pre mating phase, in the P and F generation adults increased absolute and relative liver weights and histopatological changes in the liver. No effects on reproductive and litter parameters.	ppm mg a.s./kg bw/d	<div></div> (1995) (M-004984-03-1). Diesing (2014) (M-476600-01-1)
TFA					
Rat	90 d feeding study	NOAEL <sub>ecotox</sub>	1600 ♂ 98 <sup>2</sup> /♀ 123  Based on: no decrease bw at dose 98 mg /kg, Liver weight↑.	ppm mg met./kg bw/d	<div></div> (2007) M-283994-01-1 Diesing (2014) M-477154-01-1

<sup>1</sup>Endpoints evaluated in Diesing (2014, M-476600-01-1) and in Section B.6, Volume 3 (CA), point B.6.6.1.

<sup>2</sup> Endpoints evaluated in Diesing (2014, M-477154-01-1) and in Section B.6, Volume 3 (CA), point B.6.3.2.

Justification of NOAEL<sub>ecotox</sub> is presented below the Table B.9.1.2.2-1.

The Applicant presented two statements concerning estimation of ecotoxicology endpoints to flufenacet and its metabolite-TFA, to mammals.

**The Applicants's proposal (Diesing (2014, M-476600-01-1) of the chronic toxicity endpoints to mammals for active substance flufenacet is as follows:**

In the scope of the last EU review of flufenacet an official ecotoxicological endpoint addressing the reproductive and long-term risks for wild mammals has not been set. Below the relevant reproduction and developmental toxicity data available for flufenacet are summarized and an appropriate no-observed-adverse-effect-level (NOAEL) is proposed that should be used for the wild mammal risk assessment.

### Assessment

Flufenacet has been tested for adverse effects on fertility and reproduction performance in a two-generation rat study. Developmental toxicity studies addressing embryotoxic and teratogenic effects of flufenacet were performed in rats and rabbits. The studies were done in accordance with the testing requirements valid at that time. An overview on the dose levels tested is given in the following table.

Reproduction study							Reference
Species	ppm	0	20	100	500		
rat	mg/kg bw/day (premating ♂ / ♀)	0	1.4 / 1.5	7.4 / 8.2	37.4 / 41.4		██████████ (1995) (M-004984-03-1).
Developmental studies							Reference
rat	mg/kg bw/day	0	5	25	125		██████████ (1995) M-004976-02-1
rabbit	mg/kg bw/day	0	5	25	125	200	██████████ (1995) M-004979-01-1

An overview on the toxic effects induced by flufenacet is shown in the table below. The treatment-related findings from reproduction and developmental toxicity studies are listed in a dose-dependent way.

### Dose-effect relationship in reproductive toxicity studies

	dose level ppm	dose level mg/kg bw/day ♂ / ♀	Findings
Reproduction main study	20	1.3 / 2.4	NOEL
Reproduction main study	100	7.4 / 8.2	liver weight ↑, hepatocellular hypertrophy; NOAEL
Reproduction pilot study	100	~10	NOAEL
Developmental rat		25	NOAEL

	dose level ppm	dose level mg/kg bw/day ♂ / ♀	Findings
Developmental rabbit		25	NOAEL
Reproduction main study	500	37.4 / 41.4	bw ↓ (5 - 7%); <b>NOAEL<sub>ecotox</sub></b>
Reproduction pilot study	400	~40	bw ↓ (<10%); pup weight ↓
Developmental rat		125	bw (↓), food consumption (↓); fetus weight ↓; delayed ossification, no. of variations ↑
Developmental rabbit		125	soft feces; fetus weight ↓; delayed ossification, no. of variations ↑
Reproduction pilot study	1600	~160	bw ↓ (12 – 23%); litter size ↓; pup weight ↓↓
Developmental rabbit		200	soft feces, bw ↓ (<5%); fetus weight ↓; delayed ossification, no. of variations ↑
Reproduction pilot study	3000	~300	bw ↓↓ (16 – 37%); litter size ↓, pup weight ↓↓, pup viability ↓

↓: decrease; (↓): slight decrease; ↓↓: strong decrease      bw: body weight

The following assessment can be made from this:

- The overall reproduction performance was adversely impaired only at rather high dose-levels; the number of pups per litter was lower at  $\geq 160$  mg/kg bw/day and pup viability was decreased at 300 mg/kg bw/day. As indicated by the substantially decreased body weights, severe somatic toxicity was apparent in mother animals at these dose levels.
- Lower birth weights of pups/fetuses were found to be the most sensitive treatment-related effect with possible direct ecotoxicological relevance: At 125 mg/kg bw/day fetuses were ~3% lighter in rabbits and ~6% lighter in rats; at 200 mg/kg bw/day the rabbit fetuses were ~10% lighter. For both rodent species a clear NOAEL for lower fetus weights was established at 25 mg/kg bw/day in the developmental toxicity studies.
- In the rat reproduction study no adverse effects on pup weights were detectable up to the highest dose tested. Birth weights of pups from the 500 ppm dose group were identical to that of the control group and also during the lactation period pups gained similar weight in all dose groups.
- In the main reproduction study body weight development of high dose females was slightly retarded in comparison to the control group. Beginning at week 6 of the premating period body weights of parent females were ca. 4 – 7 % lower at 500 ppm. This difference was maintained throughout the gestation period but at the end of the lactation period the difference to the control group was less than 3%. No body weight effects occurred in male rats at 500 ppm.
- The marginally lower body weights in females are considered to be of no ecotoxicological relevance as viability, fertility and reproduction performance were not adversely impacted at this dose level. In addition, also the food intake was lower at 500 ppm, so that reduced palatability of the feed may have contributed to the retarded body weight gain of females.

- The morphological findings in the liver characterised by organ weight increase and hypertrophy of hepatocytes have no relevance for the wild mammal risk assessment; they are to be seen as physiological adaptation of the organ to an increased metabolic burden and not as adverse toxic effect.

#### The Applicant's conclusion:

The wild mammal long-term/reproductive risk assessment for flufenacet should be based on the ecotoxicological NOAEL obtained in the rat reproduction study (██████████ (1995), M-004984-03-1) 500 ppm, equivalent to 37.4 mg/kg bw/day.

Additionally on the request of RMS the Applicant performed the evaluation of 90- day dietary study for rat.

The evaluation of this study, carried out by Applicant, is presented below:

The following table 1 provides an overview on the result of the 90 feeding study in rats.

**Table 1: Findings in the subchronic feeding study in rats**

Study type / guideline	90-day feeding study in rats / OECD 408
Authors / year / Bayer no.	██████████ / 1995 / M-004999-01-1
Dose levels / ppm mg/kg bw/day	<b>0 – 100 – 400 - 1600 – 3000 ppm</b> ♂: 0 – 6.0– 24.3 – 109.1 – 191.2 mg/kg bw/day ♀: 0 – 7.2 – 28.8 – 127.2 – 224.5 mg/kg bw/day
General findings	<b>body weight ↓</b> ♂: 1600 ppm: -7.0%; <b>3000 ppm: -12.7%</b> ♀: 100 ppm: -3.3%; 400 ppm: -4.8%; <b>1600 ppm: -11.8%; 3000 ppm: -18.6%</b> feed consumption ↑ at ≥1600 ppm (increased spillage because of poor palatability) <b>hematology</b> ≥100 ppm: hemoglobin ↓; leucocytes ↑ ≥400 ppm: erythrocytes ↓; hematocrit ↓ ≥1600 ppm: reticulocytes ↑ 3000 ppm: platelets ↑ <b>clinical chemistry</b> ≥100 ppm: T4 ↓; cholesterol ↑ ≥400 ppm: T3 ↓ (♂); glucose ↓; triglycerides ↓ 3000 ppm: T3 ↑ (♂) <b>organ weights</b> ≥400 ppm: liver ↑; thyroid ↑; 3000 ppm: spleen ↑ <b>histopathology</b> ≥400 ppm: hepatocellular swelling, degeneration & necrosis of single hepatocytes; spleen: pigment accumulation; kidneys: mild injury of proximal tubuli

Study type / guideline	90-day feeding study in rats / OECD 408
<b>overall NOAEL</b>	<100 ppm, equivalent to 6.0 / 7.2 mg/kg bw/day, based on marginally lower body weights in females, mild effects on blood cells, on liver and kidney at 100 ppm
<b>Ecotox relevant findings</b>	lower body weights at 1600 ppm in females and at 3000 ppm in males
<b>Ecotox NOAEL</b>	= 400 ppm, equivalent to 24.3 / 28.8 mg/kg bw/day based on distinctly lower body weights at 1600 ppm in females

#### Applicant's assessment including 90 day subchronic feeding studies for rat

Table 2 provides an overview on the dose-effect relationship of toxicological findings seen with flufenacet in relevant studies. Findings from the subchronic rat study are included now. As outlined above, the mild haematological and liver findings at 100 and 400 ppm are not expected to be relevant for the wild mammal risk assessment as such effects would not impair survival and reproduction behaviour in any way. But distinctly lower body weights in females at 1600 ppm (equivalent to 127 mg/kg bw/day) may indicate systemic toxicity of possible ecotoxicological relevance (although lower feed intake because of poor palatability may have contributed to this finding). Overall, the wild mammal endpoint that can be supported for flufenacet on basis of the 2-generation rat reproduction study is not jeopardised by findings in the subchronic feeding study.

NOAEL<sub>ecotox</sub> = 500 ppm, equivalent to 37.4 mg/kg bw/day.

**Table 2: Dose-effect relationship in the reproductive toxicity studies supplemented by findings from the subchronic rat study.**

	dose level ppm	dose level mg/kg bw/day ♂ / ♀	Findings
reproduction study	20	1.3 / 2.4	NOEL
subchronic rat	100	6.0 / 7.2	bw (↓) ♀: 3.3%; hemoglobin ↓; leucocytes ↑; T4 ↓; cholesterol ↑
reproduction study	100	7.4 / 8.2	liver weight ↑, hepatocellular hypertrophy; NOAEL
developmental rat		25	NOAEL
developmental rabbit		25	NOAEL
subchronic rat	400	24.3 / 28.8	bw (↓) ♀: 4.8% erythrocytes ↓; hematocrit ↓; T3 ↓ (♂); glucose ↓; triglycerides ↓; liver weight ↑; thyroid weight ↑; hepatocellular hypertrophy, degeneration & necrosis of single hepatocytes; pigment accumulation in spleen; mild injury of proximal renal tubuli
reproduction study	500	37.4 / 41.4	♀: bw ↓ (5 - 7%); NOAEL <sub>ecotox</sub>
subchronic rat	1600	109.1 / 127.2	bw ↓ ♂: 7.0%, ♀: 11.8%; reticulocytes ↑

<i>developmental rat</i>		125	<i>bw (↓), food consumption (↓); fetus weight ↓; delayed ossification, no. of variations ↑</i>
<i>developmental rabbit</i>		125	<i>soft feces; fetus weight ↓; delayed ossification, no. of variations ↑</i>
<i>subchronic rat</i>	3000	191.2 / 224.5	<i>bw ↓ ♂: 12.7%, ♀: 18.6%; platelets ↑; T3 ↑ (♂); spleen weight ↑</i>
<i>developmental rabbit</i>		200	<i>soft feces, bw ↓ (&lt;5%); fetus weight ↓; delayed ossification, no. of variations ↑</i>

**RMS comments:**

RMS agrees with the value of  $NOAEL_{ecotox}=37.4$  mg a.s./kg b.w./d proposed by the Applicant.

An effect of the substance on the body weight of parents and fetuses and on the development of fetuses have been observed at 109.1-127.2 mg a.s./kg bw/d in the 90d feeding study with rats and at 125 mg a.s./kg bw/d in the developmental study with rats (and rabbits to a lesser extent).

In these studies, no effect has been observed at 24.3-28.8-25 mg a.s./kg bw/d.

An intermediate dose (37.4-41.4 mg a.s./kg bw/d) has been tested in the 2 years reproduction study with rats.

Only a 6-7% reduction (not statistically significant) in body weights of females is reported at 37.4-41.4 mg a.s./kg bw/d). Other effects occurring in the P and F1 generation adults included increased absolute and relative liver weights and histopathological changes in the liver were observed. The  $NOAEL$  of 37.4 mg a.s./kg bw/d for reproductive toxicity was determined.

**Agreed endpoint:**

**$NOAEL = 37.4$  mg a.s./kg b.w./d for the long-term risk assessment to mammals**

**TFA metabolite:**

For the metabolite TFA a full reproduction toxicity study was not available.

Two dietary studies for rats (██████████ (2005, 2007) and one developmental study for rats (██████████, 2010) evaluated in the Section Toxicology, were indicated by the Applicant (as the basis for estimating  $NOAEL_{ecotox}$  for mammals from the exposure to TFA metabolite.

The evaluation of these studies was presented in the statement provided by Diesing (2014, M-477154-01-1,) and is reported below:

**Developmental toxicity study for rats (██████████, 2010, M-411209-01)**

TFA sodium salt was administered to female rats from Day 6 to Day 19 of pregnancy at dose levels of 0 (control), 37.5, 75 and 150 mg/kg b.w./d. Following caesarean section on Day 20, fetuses were evaluated for visceral and skeletal alterations.

Findings: Doses up 150 mg /kg b.w./d were well tolerated and had no adverse effect on maternal, reproduction and offspring parameter. Also no indications of embryotoxic or teratogenic effects were obtained up to high dose level.

According to the findings from the developmental study the value of NOEAL was estimated as 150 mg TFA/kg b.w./d

**The 28 days feeding study in rats (██████████, 2005, M-259106-01-1)**

The study was conducted according to OECD guideline 407.

TFA sodium salt was administered continuously for 4 weeks to groups of 5 male and 5 female Wistar at diet concentrations of 0 (control), 600, 18000, 54000 and 16000 ppm. Resulting dose levels were 0, 50, 149, 436 and 1315 mg/kg b.w. in males and 0, 52, 157, 457 and 1344 mg a.s./kg b.w. in females.

Findings: Up to the highest test dose no treatment-related mortalities and no clinical signs of toxicity occurred; there were no effects on body weight and food consumption. Slight liver weight increases and mild changes of liver-related clinical chemistry parameters were seen at feed concentrations >5400 ppm. These findings, however, were not considered as toxicologically adverse and they have no ecotoxicological relevance.

Following subacute administration of TFA, the ecotoxicologically relevant no-observed adverse effect level (NOAEL<sub>ecotox</sub>) was estimated by Diesing (2014, ) as 16000 ppm, equivalent to 1315 mg/kg bw/day in males and 1344 mg/kg bw/day in females.

In turn, a **90-day feeding study** with TFA sodium salt in rats (██████████, 2007, M-283994-01-1) was performed according to the design of OECD Guideline 408. The compound was administered continuously for 3 months to groups of 10 male and 10 female Wistar rats at diet concentrations of 0 (control), 160, 1600 and 16000 ppm.

The resulting dose levels were 0, 9.9, 98 and 1043 mg/kg bw/d in males and 0, 12.2, 123 and 1216 mg/kg b.w./d in females.

**Findings:** Up to the highest test dose no treatment-related mortalities and no clinical signs of toxicity occurred. Body weights were slightly lower at the end of the treatment period in animals of the high dose group; males: -10%, females: -4.3%. The extent of body weight retardation reached ecotoxicological relevance only in males at 16000 ppm. The slight effects on red blood cell parameters (mainly decreases of haemoglobin and haematocrit) at dose levels >1600 ppm are of no relevance for the wild mammal risk assessment as a pathological dimension was not reached. This holds true for changes of certain clinical chemistry parameters observed at >1600 ppm: bilirubin ↓, glucose ↓, alkaline phosphatase ↑, liver transaminases ↑. The liver findings at 1600 ppm (mainly hepatocellular hypertrophy and organ weight increases) are to be seen as physiological adaptation of the organ to the enhanced metabolic burden rather than as an adverse toxic effect so that an ecotoxicological relevance can be excluded.

**Conclusion by Diesing (2014):** Following subchronic administration of TFA, the ecotoxicologically relevant no-observed adverse effect level (NOAEL<sub>ecotox</sub>) is 98 mg/kg bw/day in males and 123 mg/kg bw/day in females.

**RMS comments:**

According to the evaluation of 90 day dietary study in the Section Toxicology ( please refer to Volume 3 (CA.), B.6. Point B.6.3.1 and Point B.6.3.2), the NOAEL based on changes in haematological and clinical chemistry parameters, organ weights and histopathological liver findings was established at 160 ppm for both sexes after 90-day exposure to sodium trifluoroacetate (TFA) which is equivalent to 10 mg/kg bw/day and 12 mg /kg bw/day for males and females, respectively. Taking into account that at the higher dose of 1600 ppm, no decrease of body weight nor any signs of toxicity to mammals were reported, RMS agrees with the value of NOAEL<sub>ecotox</sub>=98mg met./kg bw, proposed by the Applicant as ecotoxicologically relevant endpoint to be used in the long-term risk assessment to mammals for TFA metabolite.

**Agreed endpoint:**

NOAEL<sub>ecotox</sub> = 98 mg TFA/kg b.w./d

**B. 9.1.3. Active substance bioconcentration in prey of birds and mammals**

Substances with a high bioaccumulation potential could theoretically bear a risk of secondary poisoning for birds if feeding on contaminated prey like fish or earthworms. For organic chemicals, a log Pow > 3 is used to trigger an in-depth evaluation of the potential for bioaccumulation. As the log Pow of the active substance flufenacet (but not for its metabolites) is above the trigger (>3), evaluation of secondary poisoning is needed.

**Table 9.1.3-1. Log Pow values**

Substance	log P <sub>ow</sub>
Flufenacet	3.5
FOE oxalate (M01)	0.80
	pH-dependent
	-2.0 (pH 5)
	-2.2 (pH 7)
FOE sulfonic acid (M02)	- 2.4 (pH 9)
	Not pH-dependent
FOE methylsulfide (M05)	- 2.72
	2.6 (pH 5)
	2.6 (pH 7)
	2.6 (pH 9)
FOE methylsulfone (M07)	1.7 (pH 5)
	1.7 (pH 7)
	1.7 (pH 9)
	pH-dependent
FOE-thiadone (M09)	1.92 (pH 4.3)
	0.62 (pH 7)
	- 0.90 (pH 9.4)
	pH-dependent
FOE 5043-trifluoroethanesulfonic acid (M44)	-3.0 (pH 5)
	-2.95 (pH 7)
	-3.16 (pH 9)
	pH-dependent
trifluoroacetic acid (TFA) (M45)	-2.5 (pH 5)
	-2.6 (pH 7)
	-2.8 (pH 9)

**B.9.1.4. Other data on effects on terrestrial vertebrate wildlife (birds, mammals, reptiles and amphibians)**

**B.9.1.4.1. Studies conducted for the use in the risk assessment for birds and mammals****9.1.4.1.1. Determination of residues of Flufenacet SC 500 g/L in *Poecilus cupreus* L. (Coleoptera, Carabidae) in an extended laboratory test**

<b>Reference:</b>	Determination of residues of Flufenacet SC 500 g/L in <i>Poecilus cupreus</i> L. (Coleoptera, Carabidae) in an extended laboratory test
<b>Author(s), year:</b>	Jans, D., 2010
<b>Report/Doc. number:</b>	Report No., Reference BCS no: M-368306-01-1
<b>Guideline(s):</b>	None; test method according to Heimbach et al. (2000)
<b>GLP:</b>	Yes

The purpose of this study was the determination of residues of Flufenacet SC 500 g/L in the carabid beetle (*Poecilus cupreus*) on the day of application and after different periods of aging under extended laboratory conditions.

**Materials and Methods**

A suspension concentrate of Flufenacet SC 500 g/L was tested, specified by sample description: TOX 08540-00; specification no.: 102000007779; batch ID: EFKF000636 [analysed content of active ingredient: Flufenacet: 42.1 % w/w]; density: 1.193 g/mL.

The test item was applied at a rate of 600 g as/ha (91.9 % of nominal in stock solution) on *Poecilus cupreus* beetles, the food (*Musca domestica* pupae) for the first three days and the substrate (natural soil). After spray application of the test item, the beetles were maintained in the laboratory on natural soil substrate. After defined time intervals, beetles were removed from their exposure units, deep frozen and submitted to residue analysis. Beetles maintained in separate exposure units were used as a blank control.

The samples were analysed for residues of flufenacet according to method 01160. This method describes the determination of residues of flufenacet in/on insects. Flufenacet was extracted from the sample material using a mixture of acetonitrile/water (1/1, v/v). After filtration, an aliquot of the extract was diluted with methanol/water (2/8, v/v) and mixed with an internal standard solution. The residues were quantified by reversed phase HPLC with electrospray and MS/MS-detection.

The test was performed in a controlled environment room at a temperature of 19.5 - 20.5 °C and a relative humidity of 63 – 87 %. The climatic conditions were continuously recorded with thermohygrographs. The light / dark cycle was 16 : 8 h with a light intensity of 301 - 707 Lux (measured once per week using a Luxmeter).

**Findings:**

The samples were analysed for residues of flufenacet according to method 01160 (Analytical Method 01160 for the Determination of Flufenacet (FOE 5043) Residues in/on Insects by HPLC-MS/MS, P602094719, MR-09/089, R. Schöning, P. Köster).

Flufenacet was extracted as described above. The results of the analytical analysis of the control and the treated beetle samples are given in the tables below.

**Table B 9.1.4.1.1-1: Flufenacet residue values in treated beetle samples**

No. unit used	Sampling time	No. of Beetles	Residues Flufenacet [mg as/kg fresh weight]
37 + 38	DAA 0; 10:43 a.m.	12	<b>5.0<sup>#</sup></b>
39 + 40	DAA 0, 19:05 p.m.	12	<b>0.88</b>
41 + 42	DAA 1, 10:40 a.m.	12	<b>0.25</b>
43 + 44	DAA 2, 10:40 a.m.	12	<b>0.12</b>
45 + 46	DAA 3, 10:45 a.m.	12	<b>0.33</b>
47 + 48	DAA 4, 10:50 a.m.	12	<b>0.16</b>
49 + 50	DAA 5, 10:50 a.m.	12	<b>0.14</b>
51 + 52	DAA 6, 10:50 a.m.	12	<b>0.11</b>
53 + 54	DAA 7, 10:40 a.m.	12	0.09
55 + 56	DAA 8, 10:50 a.m.	12	0.10
57 + 58	DAA 9, 10:45 a.m.	12	0.10
59 + 60	DAA 10, 10:45 a.m.	12	0.11
61 + 62	DAA 11, 10:55 a.m.	12	0.10
63 + 64	DAA 12, 10:50 a.m.	12	0.07
69 + 70	DAA 13, 10:50 a.m.	12	0.08
71 + 72	DAA 14, 10:50 a.m.	12	0.08

LOQ = 0.10 mg/kg, LOD = 0.025 mg/kg

DAA = Days after application

<sup>#</sup> Values printed in **bold** are included in DT<sub>50</sub> calculation (residue concentrations > LOQ)

The DT<sub>50</sub> value for residue dissipation of flufenacet from the carabid beetles was calculated based on the measured residues over the sampling days 0 to 6 after application. Afterwards the measured residues fluctuated around the LOQ (0.10 mg/kg) and inclusion of the data in the calculation was not considered meaningful.

Based on single 1<sup>st</sup> order (SFO) calculation the DT<sub>50</sub> for residue dissipation of flufenacet from the carabid beetles was determined at 0.15 days. However, the curve fit was poor as were the distributions of residuals. The Chi<sup>2</sup> error value was just above the 15% specified under FOCUS (2006).

Using best fit calculation (FOMC) gave better curve fit and distribution of the residuals. The DT<sub>90</sub> for residue dissipation of Flufenacet from the carabid beetles was estimated at 0.73 days. Where an SFO DT<sub>50</sub> is needed for

calculation of Time-Weighted Average (TWA) residue concentrations, a usable and conservative approximation can be calculated according to FOCUS (2006) as  $DT_{50} = DT_{90} / 3.32$ , i.e.  $DT_{50} = 0.22$  days.

#### DT<sub>50</sub> Evaluation

**Table B 9.1.4.1.1-2. DT<sub>50</sub> Evaluation early data SFO (first order)**

DT <sub>50</sub> (days)	0.1535
DT <sub>90</sub> (days)	0.5099
Chi <sup>2</sup> error	15.10%
P	<0.001
Visual fit	Fair
Residual fit	poor

**Table B 9.1.4.1.3. DT<sub>50</sub> Evaluation all data from FOMC (best fit)**

DT <sub>50</sub> (days)	0.0645
DT <sub>90</sub> (days)	0.732
DT <sub>50</sub> (from DT <sub>90</sub> ) (days)	0.221
Chi <sup>2</sup> error	8.80%
P	$\alpha$ 0.005; $\beta$ 0.11
Visual fit	Good
Residual fit	Good

#### Conclusions:

Based on single 1st order (SFO) calculation the DT<sub>50</sub> for residue dissipation of Flufenacet from the carabid beetles was determined at 0.15 days. With best fit evaluation, a usable and conservative approximation can be obtained as  $DT_{50} = 0.22$  days.

#### **RMS comments:**

The kinetic analysis of the results was verified and found acceptable compliant with the current guidelines and common scientific approach in that area. Therefore, the  $DT_{50} = 0.22$  days is considered acceptable.

**9.1.4.1.2. Determination of the residues of flufenacet in/on winter barley and winter wheat after spray application of Flufenacet & Diflufenican SC 600 in Germany, the Netherlands, and Belgium.**

<b>Reference:</b>	Determination of the residues of flufenacet in/on winter barley and winter wheat after spray application of Flufenacet & Diflufenican SC 600 in Germany, the Netherlands, and Belgium.
<b>Author(s), year:</b>	Noss, G.; Diehl, P.; 2012
<b>Report/Doc. number:</b>	Study no: 11-2950, Reference BCS no:M-443138-01-1
<b>Guideline(s):</b>	EU Guidance working document 7029/VI/95 rev 5, (1997-07-22), US EPA OCSPP Guideline No. 860. 1500.SUPP
<b>GLP:</b>	Yes

The purpose of the study was to determine the magnitude of flufenacet residues in/on green material of winter barley and winter wheat at an early growth stage (24-25 BBCH) of the plants after one spraying application with Flufenacet & Diflufenican SC 600 specified by sample description: FAR 01538-00; specification no.: 102000007948-03; batch ID: EV56002670 [analysed content of active ingredients: Flufenacet: 32.7% w/w; Diflufenican: 16.4% w/w]; density: 1.246 g/mL. The product is a suspension concentrate formulation containing 200 g/L diflufenican and 400 g/L flufenacet. The purity of the test substance - flufenacet was 99.6%.

The study included four supervised residue trials conducted in northern Europe (the Netherlands, Germany and Belgium) during the 2011 season. No trials of Southern Europe were submitted by Applicant. At each trial site there was one untreated plot in addition to treated plot. The treated and untreated plot were cultivated in the same manner.

Description of the trial locations and cropping information on treated plots are presented in the Table B 9.1.4.1.2-1 below.

**Table B 9.1.4.1.2-1: The trial locations and cropping information on treated plots.**

<b>Trial number</b>	<b>11-2950-01</b>	<b>11-2950-02</b>	<b>11-2950-03</b>	<b>11-2950-04</b>
Trial location	53913 Swistall Miel	NL 9981 TM Withulzen	513339 Burscheid	6210 Vilers-Perwin
Country	Germany	The Netherlands	Germany	Belgium
Area of application	field	field	field	field
Plot size (m <sup>2</sup> )	67.5	80	216	45
Type of soil	Sandy loam	Clay	Sandy loam	Silt loam
pH –value of soil (in water)	-	6.8	-	6.4
pH-value of soil (in CaCl <sub>2</sub> )	6.2	-	6.3	-

Trial number	11-2950-01	11-2950-02	11-2950-03	11-2950-04
Content of organic carbon (C %)	1.0	2.0	1.3	1.8
Test system	Winter barley	Winter barley	Winter wheat	Winter wheat
Variety	Lomerit winter	Malabar	Harmann winter wheat	Julius
Seed rate	180	140	197	150
Date of sowing	2010-09-27	2010-10-01	2010-10-11	2010-10-23
Start of flowering	2011-06-15	2011-06-01	2011-05-26	2011-05-02
End of flowering	2011-06-21	2011-06-15	2011-06-03	2011-06-10
Date of commercial harvest	2011-08-01 to 2011-08-14	2011-07-20 to 2011-08-01	2011-06-30 to 2011-07-31	2011-08-02 to 2011-08-15

Table 9.1.4.1.2-2. Climatic data of trial site.

Trial site	Data	Activity	Mean temperature (°C)	Rainfall (mm)	Sunshine (h)
11-2950-01, Swisttal Miel/ Germany	Meteo Station DWD Koeln-Bonn (25 km away)				
	2011-03-16	Treatment, sampling	9 (14)*	0	6
	2011-03-17	sampling	6	0	0
	2011-03-19	sampling	7	4	9
	2011-03-21	sampling	6	0	10
	2011-03-29	sampling	9	0	11
	March 2011		7	16	24
11-2950-02, NL9981TM Withuizen The Netherlands	Meteo Station, Lauwersoong (30 km away)				
	2011-04-04	Treatment, sampling	9 (13*)	0	8
	2011-04-05	sampling	10	0	0
	2011-04-07	sampling	10	0	7
	2011-04-09	sampling	8	0	13
	2011-04-18	sampling	11	0	12
	April 2011		12	8	274
11-2950-03 Burscheid Germany	Meteo Station, Versuchsgut,				
	2011-03-25	Treatment, sampling	11 (13*)	0	9
	2011-03-26	sampling	8	0	2
	2011-03-28	sampling	8	0	11
	2011-03-30	sampling	9	1	3
	2011-04-08	sampling	12	0	11
	March 2011		7	17	217
	April 2011		14	42	233

Trial site	Data	Activity	Mean temperature (°C)	Rainfall (mm)	Sunshine (h)
11-2950-04 6210 (Villers –Pervin) Belgium	Meto station, Redebel				
	2011-03-24	Treatment, sampling	10 (16*)	0	10
	2011-03-25	sampling	11	0	10
	2011-03-27	sampling	9	0	10
	2011-03-29	sampling	9	0	10
	2011-04-07	sampling	16	0	11
	March 2011		7	13	238
	April 2011		13	19	299

\*Air temperature during application ( from trial site)

The actual application data are presented in the B 9.1.4.1.2-3.

**Table B 9.1.4.1.2-3. Application of Flufenacet & Diflufenican SC 600 in trial sites.**

Trial no. Country	Sample material	Formulation	Application						
			Appl. mode	No. of appl.	Growth stage (BBCH code)	Test item rate (L/ha)	Water rate (L/ha)	Active substance	Appl. rate (kg a.s./ha)
11-2950-01 Germany	Winter barley	Flufenacet & Diflufenican SC 600	Spraying	1	25	0.6	300	Diflufenican	0.12
								Flufenacet	<b>0.24</b>
11-2950-02 Netherlands	Winter barley	Flufenacet & Diflufenican SC 600	Spraying	1	25	0.6	300	diflufenican	0.12
								flufenacet	<b>0.24</b>
11-2950-03 Germany	Winter wheat	Flufenacet & Diflufenican SC 600	Spraying	1	25	0.6	300	diflufenican	0.12
								flufenacet	<b>0.24</b>
11-2950-04 Belgium	Winter wheat	Flufenacet & Diflufenican SC 600	Spraying	1	24	0.6	200	diflufenican	0.12
								flufenacet	<b>0.24</b>

#### Results:

The analyses were conducted according to the following analytical method:

**Table B 9.1.4.1.2-4: Summary of analytical method criteria relevant to this study.**

Active substance	Analytes	Method number	Limit of quantitation [mg/kg]	Measurement principle
Flufenacet	flufenacet	01300	0.01	HPLC-MS/MS

Results of procedural recovery are presented in the Table B.9.1.4.1.2-5 below.

**Table B.9.1.4.1.2-5: Recovery data for flufenacet in green material of winter barley.**

Sample Material	FL (mg/kg)	Single values (%)	Mean value (%)	RSD (%)	LOQ (mg/kg)
Winter barley/green material	0.01	77, 84, 88,	83	6.7	0.01
	0.10	92, 92, 96	93	2.5	
		Overall recovery (n=6)	88	7.7	

FL: Fortification level, RSD=Relative standard deviation, LOQ=Practical limit of quantificationFortified with flufenacet, determined as flufenacet and calculated as flufenacet

The matrix green material of winter barley is also representative for green material of winter wheat.

The limit quantitation (LOQ) for flufenacet is 0.01 mg/kg. No residues above the LOQ were found in the control samples. Results were not corrected for concurrent recoveries.

The average recoveries were within the acceptable range of 70–110%. RSD values were well below 20%.

The storage period of deep frozen samples for flufenacet analysis and metabolites, ranged between 445-478 days.

This is covered by the storage stability studies for flufenacet and metabolites ( see B.7.CA).

The level of residues of flufenacet in the treated samples are summarised in the Table B.9.1.4.1.2-6 below:

**Table B.9.1.4.1.2-6: Residue summary in/on winter barley and winter wheat**

Trial No. Country	Sample material	DALT (days after treatment)	Residues [mg/kg]
			a.s.- flufenacet
11-2950-01 Germany	green material winter barley	0	7.5
		1	5.8
		3	1.4
		5	0.96
		13	0.27
11-2950-02 Netherlands	green material winter barley	0	9.1
		1	6.5
		3	4.1
		5	3.1
		14	0.091
11-2950-03 Germany	green material winter wheat	0	8.9
		1	6.7
		3	7.1
		5	5.1
		14	0.050
11-2950-04 Belgium	green material winter wheat	0	12
		1	9.9
		3	5.9
		5	4.8
		14	0.084

**Conclusion:**

The supervised residue trials in young plants of winter barley (two trials) and young plants of winter wheat (two

trials) are available, where the decline of residues from day 0 after application to 13/14 days after application was determined. Residues of flufenacet were in range of 7.5-12 mg/kg on the day of application, 13/14 days after application residues had declined to 0.27 to 0.050 mg/kg.

**RMS comments:**

The trials were conducted at BBCH 24-25 growth stage in winter cereals, which is not in line with proposed GAP (BBCH 10-13 for autumn application to winter cereals at rates between 160-240 g a.s./ha and BBCH 0-22 for autumn and spring application to winter cereals at rate 120 g a.s./ha).

It should be noted that application of DFF+FFA SC 600 was done in the spring ( March–April) when the plants were in mid–tillering stage. At the end of the study the growth stage was 30-31 BBCH.

The increase in biomass of winter cereals growing at spring, when starting from the growth stage BBCH 24-25, may be faster comparing to the would-be increase of the same parameter for the same crop but in autumn, when the crop is at the growth stage of BBCH 10-12. That may result in the dilution of the residue of uptaken Flufenacet. However, that dilution is not expected to be significant.

Therefore, small difference in biomass and tested growth stages does not represent a principal deficiency of the study that would justify a complete rejection of the data. In cereals at early growth stages (until end of tillering) plant size ( biomass) is not considered to represent the most crucial parameter for the decline of residues.

The following facts support this statement:

- At the study start ( day 0 after application) active substance concentration on the plant are related only to the use rate ( compound per ha) and thus are independent from the actual growth stage
- The process of residue decline ( $DT_{50}$ ) is independent from the concentration at start of the study
- It is correct that plant growth may lead to a kind of,, residue dilution ‘but the sampling intervals were rather short in this study (one to two days until day 5), the contribution from this effect is rather limited.
- More than the growth stage at the study start, actual temperatures during the study period have the potential to influence plant growth. It is noted that the mean temperatures during sampling period of this study ( ~6 to ~11°C in mid March to early April) are considered to be representative also for the climatic conditions that can be found at autumn uses in October to November.

**Therefore, the study is considered acceptable.**

**B. 9.1.4.1.3. Statement on residue dissipation of flufenacet in treated foliage of monocotyledonous plants: kinetic evaluation.**

**Reference:** Scherr, F.; Ellerich, C.; 2013

<b>Author(s), year:</b>	Statement on residue dissipation of flufenacet in treated foliage of monocotyledonous plants: kinetic evaluation.
<b>Report/Doc. number:</b>	M-451178-01-1
<b>Guideline(s):</b>	None
<b>GLP:</b>	No

**RMS Summary:**

The aim of the study was to kinetically examine the results obtained in the study by [Noss and Diehl; 2012] (Report No. 11-2950, study reference number M-443138-01-1) aimed on the determination of the residues of Flufenacet in and on winter cereals – barley and wheat after application of the test compound in form of the formulation Flufenacet and Diflufenican 600 SC.

The data used in the experiment are presented below in the Table B.9.1.4.1.3-1 below:

**Table B: 9.1.4.1.3-1: The residue data used in the experiment**

Results obtained in the trial R01		Results obtained in the trial R02		Results obtained in the trial R03		Results obtained in the trial R04	
Time point [days]	Residue concentration in/on plant [mg/kg]	Time point [days]	Residue concentration in/on plant [mg/kg]	Time point [days]	Residue concentration in/on plant [mg/kg]	Time point [days]	Residue concentration in/on plant [mg/kg]
0.0	7.50	0.0	9.10	0.0	8.90	0.0	12.00
1.0	5.80	1.0	6.50	1.0	6.7	1.0	9.90
3.0	1.40	3.0	4.10	3.0	7.10	3.0	5.90
5.0	0.96	5.0	3.10	5.0	5.10	5.0	4.80
14.0	0.27	14.0	0.091	14.0	0.050	14.0	0.084

The data presented in the table above were subjected to a multistep evaluation procedure performed in line with the recommendations of FOCUS Kinetics Guidelines [FOCUS; 2006]. It consisted of the following steps:

- **Step 1:** Processing of the raw input data;
- **Step 2:** Kinetic evaluation of the processed data using SFO kinetic model and KinGUI 2 as a modelling tool.
- **Step 3:** Evaluation of the results of kinetic examination of the data;
- **Step 4:** Reporting of the obtained results, in particular kinetic parameters (not normalised) recommended for modelling.

The raw input data, presented above in the table 9.1.4.1.1.3 -1 were processes following the recommendations given by FOCUS. In general terms it looked as follows:

- Measured and reported true replicates were taken into account singularly;
- The data sets were checked for their consistency and clear outliers. In case the outliers were found and removed, that was clearly indicated.

The processed data were kinetically examined using KinGUI ver. 2 modelling tool, developed by Bayer. The optimisation was performed using IRLS (Iteratively Reweighed Nonlinear Least Squares) algorithm. The fitting procedure was carried out using one 1<sup>st</sup> order kinetic model – SFO. Other 1<sup>st</sup> order kinetic models routinely used in the kinetic examination of the data – FOMC, DFOP and HS, were not used.

The obtained results of the kinetic analysis of the data were evaluated by the Applicant. That was done by means of a detailed statistical analysis, comprising the following components:

- visual assessment of the fit;
- $\chi^2$ -error statistics;
- t-test significance;
- correlation analysis.

Characterising the whole procedure the Applicant stated at the beginning that it is generally preferred to select the simplest kinetic model with a high goodness of fit.

Characterising the adopted approach to the visual assessment of the fit, considered to be the first step in the evaluation, the Applicant stated that it focused on the following features:

- the conformity of the fitted decline curve with measured residue concentrations;
- the distribution of the residuals, which should be random and not systematic;
- level of residuals, which should be as small as possible – in such case even if their distribution is rather systematic, the fit may be still qualified as acceptable.

Based on these criteria the fit could be classified as:

- **good fit**, when the conformity of the kinetic curve and measured residues was good, levels of residuals were low, they were randomly scattered and no obvious systematic deviation in residual plot was visible;
- **acceptable fit**, when the conformity of the kinetic curve and measured residues was acceptable, levels of residuals were medium and they were more-or-less randomly scattered, and the absolute level of residuals was low;
- **poor fit**, when the fitted decline curve significantly deviated from the measured residues and did not match the observed pattern, the level of residuals was high and they were clearly not randomly scattered around zero line.

Characterising the next component of the assessment –  $\chi^2$ -error statistics, the Applicant indicated that its level should correspond to experience and expectation of the measurement uncertainty associated with the specific study. However, according to the provisions of the FOCUS Kinetics GD, the level of the  $\chi^2$ -error should not be greater than 15%.

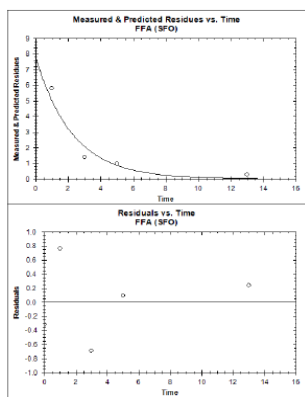
Finally, characterising the t-test, the Applicant stated that the t-test probability of 0.05 was sufficiently small and should be used as acceptability criterion, in case however of degradation products, or the results of field dissipation studies the *prob* > *t* value of 0.10 or even higher may be still acceptable.

The graphical and numerical results of the kinetic examination of the data and their evaluation are presented below. The graphical results of the fitting – the visual fits, are presented on figure B.9.1.4.1.3-1. And the numerical results in the Table B.9.1.4.1.3-2. Additionally the derived kinetic endpoints – DT<sub>50</sub> and DT<sub>90</sub> values for the test crop in each trial are given in the table B 9.1.4.1.3-3. RMS analysing the obtained results stated that in case of the results obtained in the trial R03 it was not possible to obtain the reliable fit – the visual fit was poor.

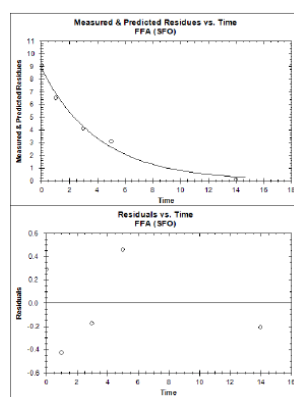
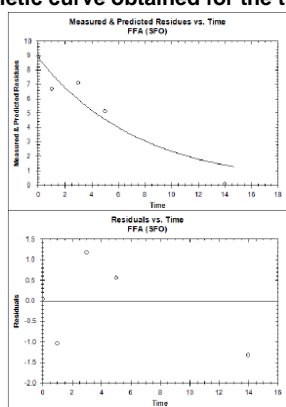
For that reason the kinetic endpoints obtained in that fit were presented in the table B.9.1.4.1.3-3 in italics and were not used to calculate the geomean DT<sub>50</sub> and DT<sub>90</sub> values.

The kinetic curve obtained for the trial R01

The kinetic curve obtained for the trial R02



The kinetic curve obtained for the trial R03



The kinetic curve obtained for the trial R04

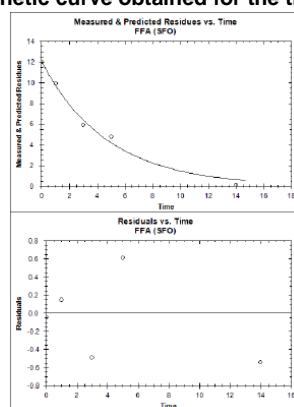


Figure B. 9.1.4.1.3-1: The graphical results of the kinetic examination of the data

Table B 9.1.4.1.3-2: The numerical results of the kinetic examination of the data.

Trial	Kinetic model	Model parameters	Statistical evaluation of the parameter					Evaluation of the fit	
			Value	Error	Confidence intervals		Prob. > t	$\chi^2$ % error	Visual assessment
					Lower	Upper			
R01	SFO	$M_0$	7.818	0.597	6.648	8.988	4.81 E-4	12.45	Acceptable
		$k$	0.4397	0.0799	0.2831	0.596	5.91 E-3		
R02	SFO	$M_0$	8.812	0.371	8.084	9.540	8.2 E-5	5.794	Good
		$k$	0.2411	0.0257	0.1906	0.292	1.29 E-3		
R03	SFO	$M_0$	8.849	0.962	6.962	10.736	1.36 E-3	13.63	Poor
		$k$	0.1333	0.0429	0.0492	0.217	2.65 E-2		
R04	SFO	$M_0$	12.043	0.475	11.113	12.973	6.71 E-5	5.284	Good
		$k$	0.2111	0.0218	0.1684	0.254	1.17 E-3		

Table B 9.1.4.1.3-3: The kinetic endpoints determined in the experiment.

Determined parameter	Trial			
	R01	R02	R03	R04
DT <sub>50</sub> [days]	1.576	2.875	5.101	3.284

DT <sub>90</sub> [ days]	5.236	9.551	17.277	10.907
--------------------------	-------	-------	--------	--------

**Conclusion of RMS :**

The determined final kinetic parameters resulting from the study – geomean values, are following:

DT<sub>50</sub> = 2.46 days, DT<sub>90</sub> = 8.17 days.

The determined final kinetic parameters resulting from the study –arithmetic values, are following:

DT<sub>50</sub> = 3.20 days, DT<sub>90</sub> = 10.74 days.

The determined final kinetic parameters resulting from the study – the longest DT<sub>50</sub> values, are following:

DT<sub>50</sub> = 5.101 days, DT<sub>90</sub> = 17.277 days.

**RMS comments:**

The determined final kinetic parameters resulting from the study – geomean values, are following:

DT<sub>50</sub> = 2.46 days, DT<sub>90</sub> = 8.17 days.

The determined final kinetic parameters resulting from the study –arithmetic values, are following:

DT<sub>50</sub> = 3.20 days, DT<sub>90</sub> = 10.74 days.

The determined final kinetic parameters resulting from the study – the longest DT<sub>50</sub> values, are following:

DT<sub>50</sub> = 5.101 days, DT<sub>90</sub> = 17.277 days.

The DT<sub>50</sub> geomean value proposed by the Applicant is not appropriate to use in the risk assessment for winter cereals at stage 10-13 BBCH for the autumn application in winter cereals. That is due to the fact that the experiment was performed at spring using plants that were at higher growth stages, having therefore different characteristic (higher biomass and growth rate than 1-3- leaves seedlings in autumn). In addition, it should be indicated that data were obtained only from four sites ( Germany, Belgium and Netherlands with one replicate).

In RMS's opinion the longest **DT<sub>50</sub> value** of **5.101** days should be used to cover uncertainties related to differences between the growth stages indicated in the representative GAP – BBCH 10-13, and tested – BBCH 24-25 for Winter cereals and application rate 240 g Flufenacet/ha, as well as those resulting from the small data set.

**Agreed endpoints:**

**DT<sub>50</sub> = 5.101 days**

**B. 9.1.4.1.4. Residue of FOE 5043 on avian forage and the availability of forage plants in corn fields.**

<b>Reference:</b>	Residue of FOE 5043 on avian forage and the availability of forage plants in corn fields
<b>Author(s), year:</b>	Toll P.A., 1995
<b>Report/Doc. number:</b>	Report No:107101
<b>Guideline(s):</b>	No guideline available
<b>GLP:</b>	Yes

#### Materials and methods:

The purpose of the study evaluated in the DAR was to determine the magnitude of flufenacet residues in/on green material of winter wheat plants after one spraying application with Flufenacet formulation -FOE5043 60DF at specified by sample description: batch ID: 5030056/5033013 (analysed content of active ingredient: Flufenacet: 60% w/w). The winter wheat four plots used in the residue data collection were located in Stilwell/Kansas/USA.

One application of 200 and 600 g a.s./ha were made. The foliage were collected from winter wheat plots on the day of application, and on the days 1, 3, 7 and 14 post application. Samples from the winter wheat plots were made up of both wheat and weed foliage if present. Method of validation was conducted at 0.1 and 10 ppm. Average recovery was 71.7±4.4% during method validation. Concurrent recoveries averaged 85.7±8.1%.

#### Results:

**Table 9.1.4.1.4-1 The mean residues ( mg/kg ) of FOE 5043 applied to winter weed.**

Plot No	Application rate	Day 0	Day 1	Day 3	Day 7	Day 14
1	200	4.5	5.3	3.3	0.1	0.5
2	200	5.3	4.6	4.0	0.2	0.5
3	400	18.4	20.2	3.9	0.2	0.8
4	400	13.0	12.8	1.5	2.0	1.0

The DT<sub>50</sub> for foliage was estimated to be approximately 3 days.

#### **RMS comments:**

The kinetic analyses of the results obtained in this study was performed by RMS in line with the general recommendation of the FOCUS Kinetics Guidance document. The tool used in the that analysis was CAKE3. The analysis of the obtained kinetic fits showed that none of them met acceptability criteria-the visual fits were poor and they were statistically not reliable. For that reason RMS decided not take these results into account in the evaluation. RMS also decided not to present the results of the kinetic fitting in the report, in order not overburden

item.

They may however be provide on request.

The value of  $DT_{50} = 3$  days proposed by Applicant for dissipation flufenacet was valid based on analysis made by using Cake Tool.

#### B.9.1.4.2. Effects on reptiles and amphibians

##### B.9.1.4.2.1. Toxicity of Flufenacet Technical to the African Clawed Frog (*Xenopus laevis*) under Static Conditions.

<b>Reference:</b>	Toxicity of Flufenacet Technical to the African Clawed Frog ( <i>Xenopus laevis</i> ) Under Static Conditions.
<b>Author(s), year:</b>	████████████████████ 2013
<b>Report/Doc. number:</b>	Study No: EBFON083., Reference BCS No. M-471899-01-1
<b>Guideline(s):</b>	No formal English guideline exists for this test protocol. Methodologies from USEPA, OPPTS Guideline 850.1075, USEPA-FIFRA, 40 CFR, Part 158, Guideline No. 72-1, and OECD Guideline 203, were considered in the development of this protocol. Scientific discretion was implemented where guideline parameters do not fully converge.
<b>GLP:</b>	Yes

#### Material and methods:

Test substance:	Flufenacet Technical, Batch No: NK61BX0367, Purity: 97.49 %,
Test species:	African Clawed Frog ( <i>Xenopus laevis</i> )
Holding:	Test medium: Dilution water (dechlorinated blended soft water) All test tadpoles were held and observed for 5 days prior to testing and were acclimatized to the actual test system for 24 hours (without food) prior to test commencement. Mortalities less than 5% during holding period.
Environmental condition:	
Temperature:	21.1°-21.9 C
Photoperiod:	16 hours light and 8 h darkness. Light intensity: 655 to 936 Lux.
Number of organisms:	Ten tdpoles per test concentration and controls, three replicates
Organism length:	17.5 ± 0.55 mm, measured at experimental start

Type of test:	Static, 48 hours
<u>Applied concentrations:</u>	
Nominal:	Control (0), Solvent control (0), 0.63, 1.25, 2.5, 50, and 10 mg a.s/L
Measured (mean):	Control (0), Solvent Control (0), 0.68, 1.19, 2.4, 4.5, and 8.7 mg s.a./L
Solvent:	DMF, 100 µL/L
<u>Test conditions:</u>	
Water quality:	Dilution water (blended spring water), Hardness: 54-62 mg/Las CaCO <sub>3</sub> , alkalinity: 44-54 mg/Las CaCO <sub>3</sub>
Conductivity:	186-210 µmhos/cm
Temperature:	Test start: 21-22.6 °C Test end: 21.9-22.1 °C
pH:	8.0-8.1
O <sub>2</sub> content:	Test start: 8.5-8.6 mg/L. Light intensity: 535-854 Lux Test end: 7.9 mg/L corresponding to 90 -98 %air saturation
Light regime:	16 hours light and 8 h darkness
Feeding:	The tadpoles were not fed throughout the duration of the tests.
Methods:	The test was carried out in glass aquaria of 8.3 litter capacity. Test and control tanks were set up using 7 L final volumes of dilution water. Tanks were not aerated during the test.  Tadpoles were impartially distributed, two at a time, to each test chamber in order of randomization until ten tadpoles were in each test tanks.
Test parameters:	All test vessels were monitored for mortality and sub-lethal effects after 6 h, 24, and 48 hours.  Dissolved oxygen and pH were measured daily for each test concentration; Day 0 measurements were performed on the batch test solutions, with the exception of the control and solvent control, which were measured from composite samples of the replicates, Day 1 and Day 2 measurements.  were performed on composite samples of the replicates. Hardness, alkalinity, and conductivity were measured on Day 0 and Day 2 for each test concentration; Day 0 measurements were performed on the batch test solutions, with the exception of the control and solvent control, which were measured from composite samples of the replicates and Day 2 measurements were performed on composite samples of the replicates.

Analytical measurements: Test solution samples were collected for analysis on study day 0 and day 2. On day 0, samples were collected from the batch test solutions; except the control and solvent control, which were collected as a composite of replicates. On day 2, composites of replicates at each concentration were collected for HPLC analysis.

Statistic: No statistical calculations were necessary to determine the  $LC_{50}$  for this study. The NOEC and LOEC were empirically determined based upon observation data including lethal and sublethal effects.

#### Findings:

Analytical data: The mean measured recovery of solutions analyzed on day 0 and day 2 ranged from 87 to 107% of the nominal concentrations. Since the concentration of the test solution was stable and within 20% of the nominal concentrations, the results of the study are based on the nominal test concentration.

Biological effects: There were no sublethal effects noted during the test

**Table B. 9.1.4.2.1-1: % Mortality and sub-lethal effects on African Clawed Frog (*Xenopus laevis*) exposed of flufenacet.**

Nominal concentration (mg a.s./L)	(%)/ (no. of dead fish / no. of treated organism) <sup>a</sup>		
	6 h	24 h	48 h
Control	0/0/30	3.3/1/30	3.3/1/30
Solvent control	0/0/30	3.3/1/30	3.3/1/30
0.63	0/0/3	3.3/1/30	3.3/1/30
1.25	0/0/30	0/0/30	3.3/1/30
2.5	0/0/30	0/0/30	0/0/30
5.0	0/0/30	0/0/30	3.3/1/30
10	3.3/1/30	3.3/1/30	3.3/1/30

Conclusion: The mortality remained below 4% for any given test concentration and does not follow a dose response trend. There were no sublethal effects noted during the test, therefore, these mortalities are considered incidental and not indicative of a toxic response.

Hence, the NOEC was 10 mg a.s./L and lowest-observed-effect-concentration (LOEC) was >10 mg a.s./L. Based on the mortality data collected and the nominal concentration of flufenacet the 96-hour  $LC_{50}$  > 10 mg a.s./L.

**Comments RMS:**

No formal guideline exists for this test protocol. Methodologies from USEPA, OPPTS Guideline 850.1075, USEPA-FIFRA, 40 CFR, Part 158, Guideline No. 72-1 and OECD Guideline 203 were considered in this study. Taking into account the validity criteria given in OECD 203 and US EPA 850.1075 test guidelines the acute study is considered acceptable. The mortality in the controls was <10 % (being 3.33%). The dissolved oxygen throughout the test was >60% of the air saturation (being 90-98% saturation). The test condition parameters during the test were in line recommended in OECD 203 guideline.

**Agreed endpoints:**

48-hour  $LC_{50} > 10$  mg a.s./L, based on nominal test concentration

48 hours NOEC = 10 mg a.s./L, based on nominal test concentration

**B.9.1.5. Endocrine disrupting properties****Wild Mammals**

Mechanistic studies submitted for evaluation of Flufenacet demonstrated that effects on thyroid hormone levels and minimal changes in thyroid gland histopathology are secondary to increased T4 clearance by the liver.

Flufenacet itself does not possess endocrine disrupting properties.

Therefore, based on a complete toxicological data set ( please refer to Volume 3, (CA), B.6. Section Toxicology) there is no evidence of any endocrine disrupting potential of Flufenacet in mammals.

**Birds**

The population relevant effects of Flufenacet on birds were studied in reproductive toxicity studies on bobwhite quail and mallard ducks. No statistically significant effects on adult birds, offspring or reproductive parameters were found at 88 mg Flufenacet/kg diet in mallard ducks and 441 mg Flufenacet/kg diet in bobwhite quails. However, in the mallard reproduction study at the highest tested dose, half of females experienced regressed ovaries and a very low egg laying rate were observed. This effects may be linked to the observed reduced body weight increase.

Reduced hatching success and delayed body weight development of hatchlings were the most prominent effects observed in both species.

There are currently no defined criteria for indentifining endocrine distrutors or interpreting the significance of any effects in ecotoxicology studies under the Commission Regulation (EU) No. 2009/1107.

Therefore, it is difficult to conclude that endocrine distrutive effects are/are not taking place.

### Amphibians and Reptiles

Currently no test methods are established to assess the population relevant effects of chemicals to amphibians or reptiles. While an amphibian metamorphosis test exists, this test was developed to evaluate the potential effect on the thyroid system, and not to measure population relevant effects. Therefore no further studies can be suggested at this time for these groups of organisms.

There are currently no defined criteria for indentifining endocrine distruotors or interpreting the significance of any effects in ecotoxicology studies under the Commission Regulation (EU) No. 2009/1107.

## B.9.2. EFFECTS ON AQUATIC ORGANISM

### B.9.2.1. Acute toxicity to fish.

For flufenacet four acute toxicity studies with four different fish species were performed.

An acute GLP study with *Lepomis macrochirus* (Bluegill sunfish) has ben performed according to OECD No. 203 and US-EPA, Subdivision E§ 72-1 guidelines. This study was already submitted and accepted during the first EU evaluation of flufenacet. The study is still valid and appropriate for the re-evaluation of flufenacet. The 96 hour LC<sub>50</sub> was estimated to be 2.13 mg a.s./L.

A study on acute effects of the two metabolites of flufenacet: FOE sulfonic acid and FOE-Thiadone were submitted for *Oncorhynchus mykiss* species and were accepted during the first EU evaluation of flufenacet for Annex I inclusion. The study is still valid and appropriate for the re-evaluation of flufenacet.

In addition, for FOE-Thiadone, two acute toxicity GLP studies were conducted with *Lepomis macrochirus* and for *Cyprindon variegatus* species. These studies were conducted according to the US EPA test guideline OPPTS 850.1075 (1985) and resulted in an lower toxicity endpoints for tested species in comparison to *Oncorychus mykiss*, being the most sensitive to exposure of FOE -Thiadone metabolite, with LC<sub>50</sub> value of 9.1 mg met/L.

Another acute toxicity study was performed for trifluoroacetatic metabolite (TFA) using Zebra fish (*Brachydario Rerio*) species. No sublethal effects and mortality were observed for the the highest tested rate, resulting in an LC<sub>50</sub> >1200 mg TFA-Na/L.

Due to the fact that algae and *Lemna* sp are the most sensitive to parent compound, for remaining metabolites included in residue definition for aquatic risk assessment, in most cases testing limited to those organism.

Acute toxicity data of flufenacet and its metabolite to fish are summerised in the Table B.9.2.1-1.

Table B.9.2.1-1: Acute toxicity data of flufenacet and metabolite to fish.

Organism	Test substance	Endpoint (type of the test)	Value (mg a.s./L)	Reference
<b>Fish</b>				
Oncorhynchus mykiss (Rainbow trout)	Flufenacet	96 h LC <sub>50</sub> (static-renewal, mortality)	5.84 mm	██████████ (1995) M-002379-01-1
Lepomis macrochirus (Bluegill sunfish)	Flufenacet	96 h LC <sub>50</sub> (static-renewal, mortality)	2.13 mm	██████████ (1995) M-002378-01-1
Cyprinus carpio	Flufenacet	96 h LC <sub>50</sub> (static-renewal, mortality)	10-12 nom >sat.con	██████████ (2010) M-361666-03-1
<i>Cyprinodon variegatus</i> (Sheepshead Minnow)	Flufenacet	96 h LC <sub>50</sub> (static-renewal, mortality)	3.31 mm	██████████ (1994) M-002422-01-1
Oncorhynchus mykiss (Rainbow trout)	FOEsulfonic acid	96 h LC <sub>50</sub> (static-renewal, mortality)	>86.7 nom	██████████ (1995) M-004932-01-1
Oncorhynchus mykiss (Rainbow trout)	FOE-Thiadone	96 h LC <sub>50</sub> (static, mortality)	9.1 mm	██████████ (1998) M-005388-01-1
Lepomis Macrochirus (Bluegill)	FOE-Thiadone	96 h LC <sub>50</sub> (static, mortality)	18.6 mm	██████████ (1999) M-016583-01-1
Sheepshead minnow (Cyprinodon variegatus)	FOE-Thiadone	96 h LC <sub>50</sub> (static, mortality)	15.3 mm	██████████ (1999) M-009684-01-1
Brachydanio rerio (Zebra fish)	TFA-Na	96 h LC <sub>50</sub> (static, mortality)	>1200 nom	██████████ (1992) M-247889-01-1

## Studies on flufenacet

**B.9.2.1.1. Acute Toxicity of FOE 5043 technical to the Rainbow Trout (*Oncorhynchus mykiss*) under static-renewal condition.**

Reference:	Acute Toxicity of FOE 5043 technical to the Rainbow Trout ( <i>Oncorhynchus mykiss</i> ) under static-renewal condition.
Author(s), year:	██████████ 1995
Report/Doc. number:	Study No: 106673, Reference BCS No: M-002379-01-1
Guideline(s):	US EPA/FIFRA 72-1 Guideline: Acute toxicity test for freshwater fish.
GLP:	Yes

Material and methods:

Test substance:	FOE 5043 technical, Batch No.: 3030057, Purity: 98.88% (analysed)
Test species:	Rainbow trout ( <i>Oncorhynchus mykiss</i> , formerly known as <i>Salmo gairdneri</i> )
Holding of fish:	Test medium: Soft blended water All test fish were held and observed for 17 days prior to testing in the laboratory. Feeding of fish during acclimatization period: Daily ad libitum. Photoperiod: 16 h L/ 8 h D. During the 48-hours immediately prior to initiation of the 96-hour exposure period the fish were held under the test conditions, i.e. $22 \pm 1^\circ\text{C}$ and 16-hour daylight photoperiod. The fish were not fed and no mortalities were observed during this period.
Number of organisms:	20 fish per test concentrations and controls, one replicate
Age, length, weight:	Age: not given. Length (23-36 mm, 30.3 mm (mean length), weight 0.18-0.59 mm g wet weight, (0.39 g (mean wet weight)
Loading:	0.46 g/L fish loading per test vessel
Type of test: static-renewal:	Semi-renewal, 96 hours

Applied concentrations:

Nominal (mean measured): Control (0), Solvent control (0), 0.44 (0.40), 0.88 (0.84), 1.75 (1.60), 3.5 (3.38), 7.0 (7.14) mg a.s./L

Solvent: Acetone, 500 µl/L

Test conditions:

Water quality: Dilution water, hardness: 48-52 mg/L as CaCO<sub>3</sub> (mean, 51 mg/L as CaCO<sub>3</sub>)  
alkalinity: 38-42 mg/L as CaCO<sub>3</sub> (mean, 40 mg/L as CaCO<sub>3</sub>)

Conductivity: Test start: 143-148 µmhos/cm  
Test end: 143-144 µmhos/cm

Temperature: Test start: 11.3-12.0°C  
Test end: 11.8-12.3 °C

pH: Test start: 7.2  
Test end: 6.9-7.0

O<sub>2</sub> content: Test start: 9.9-10.2 mg /L corresponding to 94-97%  
Test end: 6.5-7.7 mg/L corresponding to 61.9-73.33%  
Throughout the study the dissolved oxygen was > 60%

Light regime: Light/dark cycle of 16/8, Light intensity: 50-60 foot candles corresponding to 538-654 Lux.

Feeding: The fish were not fed throughout the duration of the tests.

Methods: The test was carried out in glass aquaria of ca. 23 liter capacity. Test and control tanks were set up using 17 L final volumes of dilution water and a depth 23 cm. The dilution water was aerated prior to tank preparation. Tanks were not aerated during the test. Fish were transferred to freshly prepared tanks. The test solutions were renewed after 48 hours.

Test parameters: All test vessels were monitored for mortality and sub-lethal effects after 24, 48, 72 and 96 hours. At the termination of the definitive test, the length and weight of each fish in the control tank was recorded.

Dissolved oxygen, temperature, conductivity and pH were measured in the control, solvent control, low, middle and high concentrations containing surviving fish at beginning (day 0), on day 2 and on day 4.

Water hardness and alkalinity were measured were measured in the control, solvent control, low, middle and high concentration containing surviving fish (day 0) at the beginning and on the 4 day.

Water samples were collected from all test chambers, including controls, on

Day 0, Day 2 and Day 4 to measure actual exposure concentrations.

On Day 2, the renewed test solutions were analyzed. Test solution samples were extracted on the same day as collected and were analyzed by HPLC technique.

Statistic:

Binomial probability, moving average, angle, and probit.

The slope of the toxicity curve was determined by linear regression.

Findings:

Analytical data:

The analytical findings of flufenacet determined in all test levels on day 0 ranged between 87 and 103% of nominal, on day 2 ranged between 95 and 104% of nominal and on Day 4 analyzed concentrations ranged between 83 and 98 % of nominal concentrations. The mean measured concentration of flufenacet concentration during the test period ranged from 91-102% of the nominal concentration.

Biological effects:

Behavioral/sublethal effects were observed at the 7.14 and 3.38 mg a.s./L test concentrations. The effects observed were: darkened coloration, abnormal position at the surface and at the bottom of the test chamber, loss of equilibrium, labored respiration, quiescence, lethargy and erratic swimming. There were no behavioral/sublethal effects in the controls and 0.40, 0.84 and 1.60 mg a.s./L test concentrations.

**Table B. 9.2.1.1-1: Mortality and sub-lethal effects.**

Mean measured concentration [mg a.s./L]	Mortality [%] (no. of dead fish / no. of treated fish)				
	0 h	24 h	48 h	72 h	96 h
Control	0 (0/20N)	0 (0/20 N)	0 (0/20N)	0 (0/20N)	0 (0/20N)
Solvent control	0 (0/20N)	0 (0/20N)	0 (0/20N)	0 (0/20N)	0 (0/20N)
0.400	0 (0/20N)	0 (0/20N)	0 (0/20N)	0 (0/20N)	0 (0/20N)
0.840	0 (0/20N)	0 (0/20N)	0 (0/20N)	0 (0/20N)	0 (0/20N)
1.60	0 (0/20N)	0 (0/20N)	0 (0/20N)	0 (0/20N)	0 (0/20N)
3.38	0 (0/20N)	0 (0/20) <sup>OB, DC, Q</sup>	0 (0/20) <sup>L, AS, LR, Q, OB, LR, DC</sup>	0 (0/20) <sup>L, Q, OB, DC, LR</sup>	0 (0/20) <sup>L, Q, DC, LR</sup>
7.14	0 (0/20N)	25 (5/20) <sup>OB, DC, LR, AS, LE</sup>	50 (10/20) <sup>OB, LR, DC, Q, E</sup>	55 (11/20) <sup>LR, DC, Q, LE</sup>	75 (15/20) <sup>LR, E, DC, LE, OB</sup>

Observations: AS = At surface, DC = Darkened coloration, E = Erratic L = Lethargy, LE = Loss of equilibrium LR = Laboured respiration OB = On bottom, Q = Quiescent, N=Normal

**Conclusion:** No mortalities or sublethal effects were recorded at the test concentration 1.60 mg a.s./L over 96 h period. Therefore, NOEC of 1.60 mg a.s./L and lowest-observed-effect-concentration (LOEC) of 3.38 mg a.s./L were determined. Based on the mortality data and the mean measured flufenacet concentrations the 96-hour LC<sub>50</sub> of 5.84 mg a.s./L (95% CI: 3.38 to 7.14 mg a.s./L) was estimated.

**RMS comments:**

The study was conducted according to the EPA/FIFRA 72-1 guideline, equivalent to OECD test guideline 203 (1984). Taking into account validity criteria given in the test guidelines OECD 203 (1992) and US EPA (1996) the study is considered acceptable.

The mortality in the control was <10 % (being 0). The dissolved oxygen throughout the test was >60% of the air saturation ( being 61.9-97%). The loading rate of fish was 0.39 g fish/L.

The following deviations from OECD 203 (1992) test guideline were noted:

-The dissolved oxygen, pH, and conductivity on Day 2 in the old solution from the control, solvent control, and low test concentration were not measured. The new test solutions were added (renewed) prior to water quality measurements

It should be noted that in the old test solution on Day 2 at the middle and high test concentrations environmental parameters were measured and were at the acceptable level: the dissolved oxygen was 61% of the air saturation, conductivity and pH were determined to be 141µmhos/cm and 6.7, respectively.

All water quality parameters measured in the new test solutions on Day 2, as required.

- No age of fish was given in the study report.

- Water temperature was ranged between 11.3 and 12.3°C (for rainbow trout temperature in range of 13-17°C is recommended by OECD the guideline and in range of 12 ±2 by US EPA test guideline).

- Mean length of fish was 3.1 cm, ranged between : 2.3-3.6 cm (according to OECD the guideline preferred length is 5±1cm).

In opinion of RMS is not expected that slight deviations in length of tested organisms could significantly influence study results.

Indicated deviations are, however are considered as having no impact on the study results, since all validity criteria were met. The study is considered acceptable.

**Agreed endpoints:**

LC<sub>50</sub> = 5.84 mg a.s./L (based on mean measured concentration)

NOEC = 1.60 mg a.s./L (based on mean measured concentration)

**B.9.2.1.2. Acute toxicity of flufenacet to Bluegill (*Lepomis macrochirus*) to under static-renewal condition.**

<b>Reference:</b>	Acute Toxicity of FOE 5043 technical to Bluegill ( <i>Lepomis macrochirus</i> ) to under static-renewal condition.
<b>Author(s), year:</b>	██████████ 1995
<b>Report/Doc. number:</b>	Study No: 106674, Reference BCS No: M-002378-01-1
<b>Guideline(s):</b>	US EPA/FIFRA 72-1 guideline: Acute toxicity test for freshwater fish.
<b>GLP:</b>	Yes

Material and methods:

Test substance:	FOE 5043 Technical, Batch No: 3030057, Purity: 98.8%
Test species:	Bluegill ( <i>Lepomis macrochirus</i> )
Holding of fish:	Test medium: Soft blended water All test fish were held and observed for six weeks prior to testing and were acclimatized to the actual test system for 48 hours prior to test commencement. Feeding fish during the acclimatization period: Daily with live ( <i>D.magna</i> , <i>C.dubia</i> , brine shrip) and/or commercial food. The antibiotic treatment ended 23 days prior to the study. No significant mortality (<0.01%) occurred in the test after treatment ended. During the 48-hours immediately prior to initiation of the 96 hour exposure period the fish were held under the test conditions, i.e. 22 ± 1°C and 16-hour day light photoperiod. The fish were not fed and no mortalities were observed during this period.
Number of organisms:	20 fish per test concentrations and controls
Age, length, weight:	Age not reported. Length: 17-28 mm (mean length, 21.6 mm, ±2.5 SD), weight: 0.09-0.49 (mean wet weight, 0.23 g ±0.09 SD)
Loading:	0.27 g/L fish loading per test vessel
Type of test:	Static-renewal, 96 hours

Applied concentrations:

Nominal (mean measured):	Control (0), Solvent control (0), 7 (7.06), 4.2 (4.25), 2.5 (2.39), 1.5 (1.53), 0.91 (0.91) mg a.s./L
Solvent control:	Acetone, 500 µl/L

Test conditions:

Water quality:	Dilution water, hardness: 50-54 mg/L as CaCO <sub>3</sub> (mean, 53 mg/L as CaCO <sub>3</sub> , alkalinity: 38-44 as CaCO <sub>3</sub> , (mean, 41 mg/L as CaCO <sub>3</sub> ,)
Conductivity:	138 µmhos/cm
Temperature:	Test start: 21.1-22.3 °C The temperature decreased to 20.7°C for four hours between the 24 and 48 hour of the study period Test end: 21.3-22.4 °C
pH:	Test start: 7.3-7.5 Test end: 7.2-7.4
O <sub>2</sub> content:	Test start: 7.8 mg/l corresponding to 89% Test end: 5.4-6.2 mg/l corresponding to 62 -72% Throughout the study the dissolved oxygen was > 60%
Light regime:	16 h light/8 h dark. Light intensity ranged from 538 to 861 Lux.
Methods:	The test was carried out in glass aquaria of ca. 23 litter capacity. Test and control tanks were set up using 17 l final volumes of dilution water and a depth 23 cm. The dilution water was aerated prior to tank preparation. Tanks were not aerated during the test. Fish were transferred to freshly prepared tanks. The test solutions were renewed after 48 hours.
Test parameters:	All test vessels were monitored for mortality and sub-lethal effects after 24, 48, 72 and 96 hours. Dissolved oxygen, temperature, conductivity and pH were measured in the control, solvent control, low, middle and high concentration containing surviving fish at beginning (day 0), on day 2 and on day 4. Temperature was measured daily using a mercury thermometer. The temperature was also measured hourly using data logger which was centrally located in a test chamber. Water hardness and alkalinity were measured were measured in the control, solvent control, low, middle and high concentration containing surviving fish (day 0) at the beginning and on the 4 day.
Analytical parameters:	The water samples were analysed with HPLC technique.
Statistic:	Binomial probability, moving average, angle, and probit. The slope of the toxicity curve was determined by linear regression.

Findings:

Analytical data:	The mean measured concentration of flufenacet during the test period ranged from 95-102% of the nominal concentration.
------------------	--

Biological effects: Behavioral/sublethal effects were observed at the 7.06, 4.25 and 2.39 and 1.53 mg a.s./L test concentrations. The effects observed were: abnormal position at the surface and at the bottom of the test chamber, loss of equilibrium, laboured respiration, quiescence, and vertical observation.

There were no behavioral/sublethal effects in the controls and 0.91 mg a.s./L test concentration.

**Table B. 9.2.1.2-1: Mortality and sub-lethal effects**

Mean measured concentration [mg a.s./L]	Mortality [%] (no. of dead fish / no. of treated fish)				
	0 h	24 h	48 h	72 h	96 h
Control	0 (0/20N)	0 (0/20N)	0 (0/20N)	0 (0/20N)	0 (0/20N)
Solvent control	0 (0/20N)	0 (0/20N)	0 (0/20N)	5(1/20)	5(1/20)
0.91	0 (0/20N)	0 (0/20N)	0 (0/20N)	5(1/20)	5(1/20)
1.53	0 (0/20N)	0 (0/20N)	10 (2/20) <sup>Q, OB</sup>	10(2/20) <sup>Q</sup>	10 (2/20) <sup>Q</sup>
2.39	0 (0/20N)	15(3/20) <sup>Q, LE, LR, OB</sup>	60(12/20) <sup>OB, Q</sup>	60 (12/20) <sup>Q, OB, LR</sup>	60 (12/20) <sup>Q, OB, LR</sup>
4.25	0 (0/20N)	35(7/20) <sup>LR, Q VO</sup>	100(20/20)	100(20/20)	100(20/20)
7.06	0 (0/20N)	75 (15/20) <sup>VO, LR, OB, Q</sup>	100 (20/20)	100 (20/20)	100 (20/20)

LE = Loss of equilibrium LR = Labored respiration, OB = On bottom, Q = Quiescent, D = Dead, N = Normal, VO = vertical orientation, N: normal

Conclusion: No mortalities or sublethal effects were recorded at the test concentration 0.91 mg a.s./L over 96 h exposure period. Therefore, the NOEC was determined to be 0.91 mg a.s./L and lowest-observed-effect-concentration (LOEC) was estimated to be 1.53 mg a.s./L. Based on the results the 96-hour LC<sub>50</sub> of 2.13 mg a.s./L (95% CI: 1.84 to 2.49 mg a.s./L) was determined.

**RMS comments:**

The study was conducted according to the US EPA/FIFRA 72-1 guideline, equivalent to OECD 203, (1984) test guideline.

Taking into account the validity criteria given in the test guidelines OECD 203 (1992) and US EPA ( OPPTS 850.1075 ,1996) the study is considered acceptable.

The mortality in the controls was <10 % (being 0 % in the control and 5% in the solvent control).  
 The dissolved oxygen throughout the test was >60% of the air saturation ( being 62-89%)  
 The loading rate of fish was 0.27 g fish/L.  
 The following deviations from OECD 203 (1992) test guideline were noted:  
 - The temperature decreased to 20.7°C by four hours during the test (21-25°C range is recommended in OECD 203(1992) guideline)  
 - The age of fish are not reported in the study protocol  
 Since all validity criteria were met, these deviations are considered as having no impact on results of the study.  
 The study is considered acceptable.  
**Agreed endpoints:**  
 96 h LC<sub>50</sub> = 2.13 mg a.s./L (95% CI:1.84-2.49 mg a.s./L), based on mean measured concentration  
 NOEC = 0.91 mg a.s./L, based on mean measured concentration

**B.9.2.1.3. Acute toxicity of FOE 5043 to the sheepshead minnow (*Cyprinodon variegatus*) under static renewal conditions.**

<b>Reference:</b>	Acute toxicity of FOE 5043 to the sheepshead minnow ( <i>Cyprinodon variegatus</i> ) under static renewal conditions.
<b>Author(s), year:</b>	██████████ 1994
<b>Report/Doc. number:</b>	Study No: 106421, Reference BCS No: M-002422-01-1
<b>Guideline(s):</b>	US EPA/FIFRA 72-3 (a) test guideline: Estuarine fish 96-h acute toxicity tests.
<b>GLP:</b>	Yes

Material and methods:

Test substance: FOE 5043 (Flufenacet technical), Batch No: FL036, Purity: 96.8% a.s.  
 Test species: Sheepshead minnow (*Cyprinodon variegatus*)

Holding of fish: Test medium: Synthetic saltwater (Hawaiian Marine Mix)  
 All test fish were held and observed prior to testing and were acclimatized to the actual test system for 48 hours prior to test commencement.  
 Feeding fish during the acclimatization period: Daily with newly hatched brine shrimp and/or a commercial fish food Tetramin® or trout chow).  
 No mortality occurred during this period.  
 During the 48-hours immediately prior to initiation of the 96 hour exposure period the fish were held under the test conditions, i.e. 22 ± 1°C and 16-hour day light photoperiod. The fish were not fed and no mortalities were observed

	during this period.
Number of organisms:	20 fishes per each test concentration and controls
Age, length, weight:	Age not given. Length: (19-37 mm, (mean, 23.7 mm $\pm$ 3.3 mm), weight: 0.15-0.74 g wet weight (mean , 0.39 $\pm$ 0.15 g wet weight)
Loading:	0.49 g fish /L
Type of test	Static- renewal, 96 hours. The renewal of test solution was made every 48 hours.
<u>Applied concentrations:</u>	
Nominal ( mean measured):	Control (0), Solvent Control (0), 0.63 (0.60), 1.25 (1.18), 2.5 (2.34), 5 (4.65), and 10 (9.62) mg/L
Solvent:	Acetone
<u>Test conditions:</u>	
Water quality:	Dilution water Synthetic saltwater (Hawaiian Marine Mix), Hardness: 52 mg/l as CaCO <sub>3</sub> , alkalinity: 48 mg/ l as CaCO <sub>3</sub>
Salinity:	12‰ (throughout the test)
Conductivity:	136 $\mu$ hmos/cm
Temperature:	Test start: 20.4°C – 22.1 °C Test end: 20.4°C-22.9 °C
pH:	Test start: 7.8 Test end: 7.5-7.9
O <sub>2</sub> content:	Test start: 6.8-6.9 mg /L Test end: 5.2-7.0 mg/L  The depressed (< 60 percent saturation) dissolved oxygen levels were observed on Day 2 in the old test solutions of 0.60 and 2.34 mg/L test concentration. Therefore, gentle aeration was added to all test chambers after they were renewed with fresh test solution. The gentle aeration did not affect the concentration of the test compound after 48 hours since the measured concentrations were 80 percent of the Day 0 measured concentrations.
Light regime:	16h light/8h dark. Light intensity 65-70 foot candles corresponding to 700-753 Lux.
Methods:	One replicate of twenty fish each was used at each test concentration. Test chambers were 22- liter glass aquaria contained 17 L of test solution.  Fish from the control and solvent control chambers were weighed and measured at test termination to determine the biomass loading factor.
Test parameters:	One replicates of test vessels (each with 20 fishes) were monitored for mortality and sub-lethal effects after 24, 48, 72 and 96 hours.

Temperature, salinity, dissolved oxygen, and pH were measured in the control, solvent control, low, middle and high test concentrations on Day 0 in the freshly prepared test solutions and on Days 2 and Day 4 in the old test solutions. Temperature was measured daily.

Statistic:

The water samples were analysed with HPLC technique.

LC<sub>50</sub> calculated by using the Probit analyses.

#### Findings:

Analytical data:

Measured concentration analysis was performed on new test solutions on Day 0 and Day 2, and on old test solutions on Day 4. All measured concentrations were between 79 and 106 percent of nominal throughout the study.

Biological effects:

The control, solvent control and 1.18, and 0.60 mg/L test concentrations exhibited no sublethal effects.

Behavioral/sublethal effects were observed at the 2.34, 4.65 and 9.65 mg a.s./L test concentrations.

The effects observed were: abnormal position at the surface and at the bottom of the test chamber, loss of equilibrium, labored respiration, quiescence, vertical observation, pale coloration, mottled coloration.

**Table B. 9.2.1.3-1: Mortality and sub-lethal effects.**

Nominal (mean measured) concentration [mg a.s./L]	mortality [%] (no. of dead fish / no. of treated fish)			
	24 h	48 h	72 h	96 h
Control	0(0/20N)	0(0/20N)	0(0/20N)	0(0/20N)
Solvent control	0(0/20N)	0(0/20N)	0(0/20N)	0(0/20N)
0.60 (0.60)	0(0/20N)	0(0/20N)	0(0/20N)	0(0/20N)
1.18(1.18)	0(0/20N)	0(0/20N)	0(0/20N)	0(0/20N)
2.34 (2.34)	0(0/20) <sup>LR,AS,LE,Q</sup>	0(0/20) <sup>LR,LE</sup>	5(1/20) <sup>Q,LR,P,LE,OB,VO</sup>	25(5/20) <sup>LR,OB,Q,LE,P,MC</sup>
4.65 (4.65)	0(0/20) <sup>AS,VO,LR,Q,LE,OB</sup>	20(4/20) <sup>LR,Q,LE,OB,VO,E</sup>	40(8/20) <sup>LR,LE,OB,Q,AS</sup>	75(15/20) <sup>LR,LE,OB,Q,E</sup>
9.62 (9.62)	20(10/20) <sup>LR,LE,OB,Q,AS,E,VO,AS</sup>	85(17/20) <sup>LR,LE,OB,Q</sup>	100(20/20)	100(20/20)

Observation: AS = At surface; E = erratic; LE = loss of equilibrium; LR = labored respiration; MC = mottled coloration  
N = normal; OB = on bottom; P = pale coloration; Q = quiescent; VO = vertical orientation,

**Conclusion:** The lowest, mean measured concentration tested, causing 100% mortality within the period of the test was 9.62 mg a.s./L.

Based on mortality and the mean measured concentration, 96 hour LC<sub>50</sub> was estimated to be 3.31 mg a.s./L (95%CI: 2.73 to 4.02 mg/L).

The no-observed-effect-concentration (NOEC) was 1.18 mg/L based upon the lack of mortality and sublethal effects at this concentration.

**Comments RMS:**

The study was conducted according to the US EPA 72-3 (a) guideline. Taking into account validity criteria given in the test guidelines OECD 203 (1992) and US EPA OPPTS 850.1075 (1996) the study is considered acceptable.

The mortality in the control was <10 % (being 0).

The loading rate of fish was 0.49 g fish/L.

The following deviations from OECD 203 (1992) and US EPA OPPTS 850.1075 (1996) test guidelines were noted:

- The depressed (<60 percent saturation) dissolved oxygen levels were observed on Day 2 in the old test solutions of 0.60 and 2.34 mg/L test concentrations. Therefore, gentle aeration was introduced to all test chambers after they were renewed with fresh test solution. The gentle aeration did not affect the concentration of the test compound after 48 hours since the measured concentrations were well above 80 % of the Day 0 measured concentrations.

- The measurements of test concentration were done on Day 0 and Day 2 in the new test solution and on Day 4 in old test solution (in static-renewal tests, the test substance should be measured in each replicate at the beginning and end of test and just before and after each renewal, as it is recommended by US EPA OPPTS 850.1075 (1996) ).

- The dissolved oxygen, pH and salinity were measured on day 0 (new test solution), Day 2 (old test solution) and Day 4 (old test solution, (the 24 hour interval for the quality measurements in semi-static system is recommended in the OECD 203 and US EPA OPPTS 850.1075 test guidelines is recommended).

- The temperature decreased to 20.5°C for the first hours of the test (range of 22.0 ± 1.0° is recommended in OECD 203 test guideline). It should be noticed that the temperature increased to acceptable range between 25 and 48 hour of the study.

Indicated deviation are, however, considered as having no impact on the study results, since all validity criteria were met. The study is considered acceptable.

**Agreed endpoints:**

LC<sub>50</sub> = 3.31 mg a.s./L ( 95% CI:1.84-2.49), based on mean measured concentration

NOEC = 1.18 mg a.s./L based on mean measured concentration

**B.9.2.1.4. Acute toxicity of flufenacet to (*Cyprinus carpio*) under static-renewal condition.**


---

Reference:	Acute toxicity of flufenacet (tech.) to fish ( <i>Cyprinus carpio</i> ) under static-renewal condition.
Author(s), year:	██████ 2010
Report/Doc. number:	Study No: E 280 3646-9, Reference BCS No: M-361666-03-1 Amendment 1 (2011-01-11), Amendment 2 (2012-04-05)
Guideline(s):	EPA-FIFRA § 72-1/SEP-EPA-540/9-85-006 (1982/1985) OPPTS 850.1075 (Public Draft, 1996) Directive 92/69/EEC, C.1 (1992) OECD No. 203 (rev. 1992) JMAFF, 11 Nousan No. 6283 (Oct. 1999)1
GLP:	Yes

---

Material and methods:

Test substance:	Flufenacet (tech), Batch No.: AE F133402-01-02, Purity: 97.5% (analysed)
Test species:	Common carp ( <i>Cyprinus carpio</i> )
Holding of fish:	Test medium: Reconstituted water All test fish were held and observed for 14 days prior to testing in the laboratory. Feeding of fish during acclimatization period: daily with commercial trout food. Photoperiod: 16 h light / 8 h dark. During the 48-hours immediately prior to initiation of the 96-hour exposure period the fish were held under the test conditions, 16-hour Light /8 h Dark. The fish were not fed and mortalities below 5% were observed during this period.
Number of organisms:	10 fish per test concentrations and controls, one replicate.
Age, length, weight:	Age: Not given. Mean length: 4.8 ±0.4 cm, mean body weight: 1.6 g ±0.3 g
Loading:	0.40 g/L fish loading per test vessel
Type of test: static-renewal:	Semi-renewal, 96 hours

Applied concentrations:

Nominal :	Control (0), Solvent control (0), 6.41, 12.8, 25.6, 51.3 and 103 mg a.s./L
Solvent:	DMF, dimethylformamide

Test conditions:

Water quality:	Reconstituted water prepared according to ISO
Conductivity:	< 0.2 mS/cm
Temperature:	Test start: 22.1-23°C Test end: 21.8-22.8°C
pH:	Test start: 7.0-7.3 Test end: 7.0-7.2
O <sub>2</sub> content:	Test start: 101% Test end: 83-96% Throughout the study the dissolved oxygen was > 60%
Light regime:	Photoperiod: 16 h Light /8 h Dark, Light intensity: not given
Feeding:	The fish were not fed throughout the duration of the tests.
Methods:	The test was carried out in glass aquaria (size: 38 h x 34 w x 36 d cm).The test volumes amounted to 40 L each. The aquaria were placed in the temperature controlled room. The reconstituted water was aerated prior to tank preparation. Tanks were not aerated during the test. Fish were transferred to freshly prepared tanks.
Test parameters:	<p>All test vessels were monitored for mortality and sub-lethal effects after 24, 48, 72 and 96 hours. At the termination of the definitive test, the length and weight of each fish in the control tank was recorded.</p> <p>Dissolved oxygen, water temperature and pH values were determined daily in each aquarium, water temperature was additionally measured in the control aquarium and recorded hourly with a data logger.</p> <p>Analytical determinations of measured substance concentrations were conducted in the test media at the beginning of the test, after 48h (aged and renewal water samples) and at test termination.</p> <p>Test solution samples were extracted on the same day as collected and were analyzed by HPLC technique.</p>
Statistic:	<p>Whenever possible, the LC<sub>50</sub> values and the 95%-confidence intervals were calculated every 24-hour using a computer program which estimated the LC<sub>50</sub> using one of three statistical techniques: moving average, logit analysis or probit analysis. The appropriate method was determined according to the data characteristics.</p> <p>All values calculated with Microsoft® Excel were shown as rounded values.</p>

Findings:

## Analytical data:

Accompanying chemical analysis of flufenacet revealed recoveries between 6.9 % and 94 % of nominal values. The observed recoveries demonstrate clear dose dependency. The used test concentrations exceeded the water solubility of the test item under exposure conditions (saturation concentration). In the two lowest concentrations mean recoveries were 94 and 86%. The analytical measurements revealed maximum concentrations of flufenacet in the test media of 15.7 mg/L. Based on the results of the accompanying chemical analysis it can be assumed that the solubility of the test item under exposure conditions is approximately 10 to 12 mg/L.

## Biological effects:

No lethal effects occurred up to the highest tested nominal concentration as up to the concentration with the highest analytically confirmed mean measured concentration (11.7 mg / L).

Based on these findings it can be stated that the LC<sub>50</sub> (96h) for the test item is greater than the saturation concentration.

In the controls no mortalities or sub-lethal findings were observed.

At the nominal test concentration of 6.41 mg test item / L behavioral changes were observed after 96 hours. The fish showed the following behavioural symptoms: remaining for unusually long periods on the bottom of the aquarium showed labored respiration were inactive or displayed abnormally low activity.

**Table B. 9.2.1.4-1: Mortality and sub-lethal effects.**

Nominal (mean measured) test concentration [mg a.s./L]	Mortality [%] (no. of dead fish / no. of treated fish)				
	4 h	24 h	48 h	72 h	496 h
Control	0 (0/10N)	0 (0/10 N)	0 (0/10N)	0 (0/10N)	0 (0/10N)
Solvent control	0 (0/10N)	0 (0/10 N)	0 (0/10N)	0 (0/10N)	0 (0/10N)
6.41 (5.88)	0 (0/10N)	0 (0/10 N)	0 (0/10N)	0 (0/10) <sup>BO,AP,</sup>	0(0/10) <sup>BO,AP,TF,</sup>
12.8 (10.7)	0 (0/10) <sup>BO,AT</sup>	0 (0/10 ) <sup>BO,AT</sup>	0 (0/10) <sup>BO,AT</sup>	0 (0/10) <sup>BO,AT</sup>	0 (0/10) <sup>BO,AT</sup>
25.6 (11.7)	0 (0/10) <sup>BO, AT</sup>	0 (0/10 ) <sup>BO, AT</sup>	0 (0/10) <sup>BO, AT</sup>	0 (0/10) <sup>BO,AT,DF</sup>	0 (0/10) <sup>BO,AT,DF</sup>

Nominal (mean measured) test concentration [mg a.s./L]	Mortality [%] (no. of dead fish / no. of treated fish)				
	4 h	24 h	48 h	72 h	496 h
51.3 (8.62)	0 (0/10) <sup>BO, AT</sup>	0 (0/10) <sup>BO, AT</sup>	0 (0/10) <sup>BO, AT</sup>	0 (0/10) <sup>BO,AT</sup>	0 (0/10) <sup>BO,AT</sup>
103 (6.91)	0 (0/10) <sup>BO, AT</sup>	0 (0/10) <sup>BO, AT</sup>	0 (0/10) <sup>BO, AT</sup>	0 (0/10) <sup>BO,AT,DF</sup>	0 (0/10) <sup>BO,AT,DF</sup>

Observation: AP inactive or displayed abnormally low activity, AT: labored respiration, BO: remained for unusually long periods on the bottom of the aquarium, DF: turned dark in coloration, N: did not show any abnormal signs

**Conclusion:** The highest concentration which did not result in any mortality within the exposure period (NOLEC) was the saturation concentration (10-12 mg /L). The no observed-effect-concentration (NOEC) was < 6.41 mg (nominal) test item /L. Based on these findings the LC<sub>50</sub> (96h) is greater than the saturation > 10-12 mg a.s./L.

#### RMS comments:

The study was conducted according to the US EPA, OPPTS 850.1075 (1996) test guideline.

Taking into account the validity criteria given in the test guidelines OECD (1992)

and US EPA OPPTS 850.1075(1996) the study is considered acceptable.

The mortality in the control was <10 % (being 0).

The dissolved oxygen throughout the test was >60% of the air saturation ( being 83-101% of saturation)

The loading rate of fish was 0.4 g fish/L.

The following deviations from OECD 203 (1992) and US EPA (1996) test guidelines were noted:

- No age of fish was given in the study report.

- Mean length of fish was 4.8 cm, (according to OECD the guideline preferred length is 3±1cm).

In opinion of RMS is not expected that slight deviations in length of tested organisms could significantly influence study results.

Indicated deviations are, however considered as having no impact on the study results, since all validity criteria were met. The study is considered acceptable.

#### Agreed endpoints:

96 h LC<sub>50</sub> > saturation concentration 10-12 mg /L mg a.s /L (based on nominal concentration)

NOEC < 6.41 mg /L (based on nominal concentration)

**Studies on metabolites****B.9.2.1.5. Acute toxicity of metabolite FOE5043-Sulfonic acid to Rainbow Trout (*Onchorynchus mykiss*) in a Static Test**

<b>Reference:</b>	FOE5043-Sulfonic acid. Acute toxicity (96 hours) to Rainbow Trout ( <i>Onchorynchus mykiss</i> ) in a Static Test.
<b>Author(s), year:</b>	██████████ 1995
<b>Report/Doc. number:</b>	Study No: 2800986-3, Report No: DOM 95031, Reference BCS No: M-004932-01-1
<b>Guideline(s):</b>	OECD 203 (updated on 1992) test guideline.
<b>GLP:</b>	Yes

**Material and methods:**

Test substance:	FOE-Sulfonic acid, Batch No: WAK 6222-3, Purity (as sodium salt): 93.6% corr. to free acid: 86.7%
Test species:	Rainbow Trout ( <i>Onchorynchus mykiss</i> )
Holding of fish:	Test medium: Reconstituted water Environmental condition: Photoperiod 16 h light/8 dark All test fish were held and observed at least 14 days prior to testing and were acclimatized to the actual test system for 48 hours prior to test commencement. Feeding fish during the acclimatization period: Daily with commercial trout diet. No significant mortality (<3%) occurred during this period. During the 48-hours immediately prior to initiation of the 96 hour exposure period the fish were not fed during this period.
Number of organisms:	10 fish per test concentrations and control
Age, length, weight:	Age not reported. Mean length: 4.1 cm ± 0.4 cm, mean weight: 0.6 g ± 0.3g
Loading:	0.15 g/L fish loading per test vessel
Type of test:	Static, 96 hours
<b><u>Applied concentrations:</u></b>	Nominal: 0 (control), 86.7 mg FOE 5043-Sulfonic acid /L
Solvent:	None

Test conditions:

Water quality:	Reconstituted water: Hardness 40-60 mg/L as CaCO <sub>3</sub> (mean)
Temperature:	12.7-15°C
Conductivity:	Not given
pH:	Test start: 7.0 Test end: 7.3-7.4
O <sub>2</sub> content:	Test start: 103% of air oxygen saturation Test end: 92% of air oxygen saturation Throughout the study the dissolved oxygen was > 90% of oxygen saturation
Light regime:	16 h Light /8 h Dark
Methods:	The test was carried out in glass aquaria with a size: 32 x 36 x 38 cm (length x width x height). The test volumes amounted to 40 L. The aquaria were placed in a temperature controlled room. Fish were transferred to freshly prepared tanks.
Feeding:	The fish were not fed throughout the duration of the tests.
Test parameters:	During the test fish were monitored for mortality and sub-lethal effects after 4 hour and then daily. Dissolved oxygen, and pH values were measured daily in each aquarium, water temperature was measured in the control and recorded hourly with a data logger. Analytical determinations of FOE sulfonic acid concentration were made in the test medium at the beginning of the test as well as at the end of the test. The water samples were analysed with HPLC technique.
Statistic:	Not statistical analysis were performed since no mortality were recorded.
<u>Findings:</u>	
Analytical data:	Under test conditions FOE sulfonic acid was stable resulting in measured values of 91 % of nominal at day 0 as well as at day 4. (based on analytical determination of sulfonic acid sodium salt). All results are related to nominal concentration of sulfonic acid.

Biological effects: In the tested concentration at 86.7 mg/L no fish showed any sublethal effects.

**Table B. 9.2.1.5-1: Mortality and sub-lethal effects.**

Nominal concentration [mg p.m./L]	Mortality [%] (no. of dead fish / no. of treated fish)				
	4 h	24 h	48 h	72 h	96 h
Control	0(0/10N)	0(0/10N)	0(0/10N)	0(0/10N)	0(0/10N)
86.7	0(0/10N)	0(0/10N)	0(0/10N)	0(0/10N)	0(0/10N)

Normal

Conclusion: No mortalities or sublethal effects were recorded at the test concentration 86.7 mg p.m./L over 96 h exposure period.  
Therefore, the NOEC was estimated to be > 86.7 mg p.m./L.  
Based on the mortality results and the nominal concentrations of sulfonic acid salt, the 96-hour  $LC_{50}$  > 86.7 mg p.m./L was determined.

**RMS comments:**

The study was conducted according to the OECD test guideline 203 (1992). Taking into account the valid criteria given in the OECD 203 (1992) test guideline, the acute fish study with rainbow trout is considered acceptable.

The mortality in the controls was <10 % (being 0).

The dissolved oxygen throughout the test was > 60% of the air saturation.

The loading rate of fish was 0.15 g fish/L.

Following deviations from OECD 203 (1992) test guideline were noted:

- The age of fish was not given.
- The measurements of conductivity, alkalinity during the study was missing from the study protocol.

Since all validity criteria were met, these deviations are considered as having no impact on results of the study.

The study is considered acceptable.

**Agreed endpoints:**

$LC_{50}$  > 86.7 mg FOE5043 sulfonic acid salt/L, based on nominal concentration

NOEC = 86.7 mg FOE 5043 sulfonic acid salt/L, based on nominal concentration

**B.9.2.1.6. The acute toxicity of Sodium Trifluoroacetate to the zebra fish (Brachydanio Rerio).**


---

<b>Reference:</b>	The acute toxicity of Sodium Trifluoroacetate to the zebra fish (Brachydanio Rerio).
<b>Author(s), year:</b>	████████████████████ 1992
<b>Report/Doc. number:</b>	Study No:56635/31/92, Reference BCS No:M-247889-01-1
<b>Guideline(s):</b>	OECD 203 (1984).
<b>GLP:</b>	Yes

---

Material and methods:

Test substance:	Sodium Trifluoroacetate, Batch No: ACA9135AB, Purity: 99%
Test species:	Zebra fish (Danio rerio, formerly Brachidanio Rerio)
Holding of fish:	Test medium: Reconstituted water Environmental condition: 21°C ± 1°C Daily fed with trout feed and water fleas.
Number of organisms:	10 fishes with two replicates per each test concentration and controls
Age length, weight:	Age young. Length 2.3-3.4 cm (22± cm), weight: 0.23 g
Loading:	Not given
Type of test:	Static, 96 hours

Applied concentrations:

Nominal (mean measured):	Control (0), 1200 (1210) mg metabolite Sodium Trifluoroacetate /L Due to that Trifluoroacetic acid is strong acid (pKa = 0.23) it was decided to test the sodium salt of trifluoroacetic acid following OECD Guideline 203 (OECD 1984). Based on the molecular weights 1.0 g trifluoroacetic acid corresponds to 1.2 g of its sodium salt.
--------------------------	---

Test conditions:

Water quality:	Reconstituted water
Temperature:	Test start: 22.1 °C Test end: 22.6-22.8 °C
O <sub>2</sub> :	Test start: 8.5 mg/L Test end: 8.3-8.5 mg/L Throughout the study the dissolved oxygen was > 60%, > 5 mg/L
pH:	Test start: 7.6-7.9 Test end: 7.8-7.9

---

Light regime:	Light/dark cycle of 16/8, Light intensity: not given
Feeding:	The fish were not fed during the test
Methods:	Two test aquaria were used per concentration and to each aquarium 10 fishes were added. Two other aquaria containing 4 L water only used as controls. The test solutions are aerated during the test.
Test parameters:	<p>Two replicates of test vessels (each with 10 fishes) were monitored for mortality and sub-lethal effects after 3, 24, 48, 72 and 96 hours. The fish were inspected for the following abnormalities: hyperactivity, hypoactivity, hyperventilation, uncontrolled movement, loss of equilibrium and discoloring.</p> <p>Temperature, dissolved oxygen, pH, were measured in all test solutions in the both replicates and the control were measured after 3, 24, 48, 72 and 96 hour.</p> <p>Samples of the solutions were taken 0 and 96 hours after test initiation and analyzed by ion chromatography. The test concentration of sodium trifluoroacetate remained constant during the test.</p>
Statistic:	<p>LC<sub>50</sub> calculated by using a probit analysis model (program Probit of SAS).</p> <p>NOEC calculated by using Fisher's exact test.</p>
<u>Findings:</u>	
Analytical data:	The mean measured concentration of during the test period was > 80% of the nominal concentration (99-108%). Therefore the conclusions are based on nominal values.
Biological effects:	<p>No mortality occurred when zebra fish were exposed to 1200 mg sodium trifluoroacetate/L. No mortality was observed in the control.</p> <p>No behavioral or sublethal effects were observed in the control and test level during the exposure period after. 3, 24, 48, 72, 96 hours.</p>

**Table B. 9.2.1.6-1: Mortality and sub-lethal effects.**

Nominal concentration Na-TFA [mg p.m./L]	Mortality [%] (no. of dead fish / no. of treated fish)				
	3h	24 h	48 h	72 h	96 h
Control	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/10N)
Control	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/10N)
1200	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/10N)
1200	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/10N)

Conclusion: Based upon mortality the lowest-observed effect-concentration (LOEC) was >1200 mg sodium trifluoroacetate/L and the no-observed effect-concentration (NOEC) was >1200 mg sodium trifluoroacetate /L .

The 96 hour LC<sub>50</sub> was >1200 mg sodium trifluoroacetate /L .

**Comments RMS:**

The study was conducted according to OECD 203 (1984). Taking into account the validity criteria given in the test guideline OECD 203 (1992) the study is considered acceptable.

The mortality in the controls was <10 % (being 0 %).

The dissolved oxygen throughout the test was >60% of the air saturation value.

The concentration of substance being tested was > 80 % of the nominal concentration during the test (being 99-108%).

The following deviations from OECD 203 (1992) test guidelines were noted:

-The information of loading fish/L, light intensity and duration of acclimatization period were missing from the study protocol.

-The measurement of hardness and alkalinity were not recorded during the study.

Since all validity criteria were met, these deviations are considered as having no impact on results of the study.

The study is considered acceptable.

**Agreed endpoints:**

The 96 hour LC<sub>50</sub> was >1200 mg sodium trifluoroacetate/L, based on mean nominal test concentration  
NOEC >1200 mg sodium trifluoroacetate/L, based on mean nominal test concentration.

**B.9.2.1.7. Acute toxicity of metabolite Thiadone to the Rainbow trout (*Oncorhynchus mykiss*) to under static condition.**

<b>Reference:</b>	Acute Toxicity of Thiadone to the Rainbow trout ( <i>Oncorhynchus mykiss</i> ) to under static condition.
<b>Author(s), year:</b>	██████████ 1998
<b>Report/Doc. number:</b>	Study No: 1 08738, Reference BCS No: M-005388-01-1
<b>Guideline(s):</b>	US EPA/FIFRA 72-1 test guideline: Acute toxicity test for freshwater fish.
<b>GLP:</b>	Yes

Material and methods:

	Test substance: Thiadone, Batch No.: K778, Purity: 99.4%
Test species:	Rainbow trout ( <i>Oncorhynchus mykiss</i> )
Holding of fish:	Test medium: Soft blended water
	All test fish were held and observed prior to testing in the laboratory.
	Fish were fed during acclimatization period daily with newly hatched brine shrimp and/or a commercial fish food (Tetramin™, Rangen™ Trout Chow).
	Photoperiod: 16 h daylight
	Temperature: 12± 1°C
	The mortality: <1%
	During the 48-hours immediately prior to initiation of the 96-hour exposure period the fish were held under the test conditions, i.e. 12 ± 1°C. Light regime: 16 h Light /8 h Dark.
	The fish were not fed this during this period.
Number of organisms:	10 fish with two replicates per test concentration and controls
Age, length, weight:	Age not reported. Length: 40-50 mm (mean, 48.9 mm ±1.6 mm), weight: 1-3.2 g wet weight (mean, 1.92 g ± 0.17 g)
Loading:	0.6 g/L fish loading per test vessel
Type of test:	Static, 96 hours

Applied concentrations:

Nominal (mean measured):	Control (0), Solvent Control (0), 2.5 (2.4), 5 (5.0), 10 (10.3), 20 (20.3) and 40 (41.7) mg metabolite/L
Solvent:	DMF, dimethylformamide 500µl/L

Test conditions:

Water quality:	Dilution water: Hardness 52 mg/L as CaCO <sub>3</sub> , alkalinity: 42-45 mg/L as CaCO <sub>3</sub> ,
Conductivity:	111-120 µmhos/cm
Temperature:	Test start: 12.2-12.9 Test end: 12-12.4
pH:	Test start: 7.0-7.8 Test end: 7.1-7.5
O <sub>2</sub> content:	Test start: 9-10 mg/L Test end: 8.3 mg/L  Due to that the oxygen concentration decreased on day 1 to 69%, gentle aeration was started after 24 hours. Throughout the study the dissolved oxygen was > 60%
Light regime:	16h light/8h dark. Light intensity ranged from 68 to 98 foot candles corresponding to 731-1054 Lux (mean, 867 Lux)
Feeding:	The fish were not fed throughout the duration of the tests.
Methods:	The test was carried out in glass aquaria of ca. 36 litter capacity. Test and control tanks were set up using 30 l final volumes of dilution water. The dilution water was aerated prior to tank preparation. The aquaria were placed in a temperature controlled room. Fish were transferred to freshly prepared tanks. Fish from the control and solvent control chambers were weighed and measured at test termination to determine the biomass loading factor.
Test parameters:	Two replicates of test vessels (each with 10 fishes) were monitored for mortality and sub-lethal effects after 24, 48, 72 and 96 hours. At the termination of the definitive test, the length and weight of each fish in the control tank was recorded.  Temperature, dissolved oxygen, conductivity, hardness, alkalinity and pH were measured in on replicate of all test levels with surviving fish on Day 0 and Day 4. The dissolved oxygen was measured in one replicate at all test levels on Day 1, in one replicate of the controls and the high, middle and low levels on Day 2 and 3, and in all replicates with surviving fish on Day 4.  The pH and conductivity were measured in one replicate of the controls, high, middle and low test solutions with surviving fish on Day 2. Temperature was

measured daily. In addition data logger documented hourly temperatures in a centrally located test chamber.

Dissolved oxygen concentrations were measured on Day 0, 2 and 4 and ranged from 6.5 to 10.0 mg/L representing 60 and 93 percent saturation, respectively. Additional measurements were taken on Day 1 and Day 3. Since the dissolved oxygen had declined to 69% of saturation in the static test on Day 1, gentle aeration was added to all test chambers. On the 3 day 63% to 85% saturation was noted.

Water samples were collected from each replicate of the control, solvent control and all test chambers on Day 0 and Day 4. The solutions were analyzed on Day 0 (new solutions) and Day 4 (old solutions) to measure actual exposure concentrations.

The water samples were analysed with HPLC-UV.

Statistic: The 96-hour LC<sub>50</sub> value and the 95 percent confidence limits were calculated using the following statistical methods: binomial probability, moving average angle, and probit.

#### Findings:

Analytical data: The mean measured concentration of thiadone during the test period ranged from 95 to 102% of the nominal concentration. All results based on mean measured concentration.

Biological effects: Sublethal effects were only observed at the 10.3 mg a.s./L test level. These effects included labored respiration and darkened coloration.

**Table B. 9.2.1.7-1: Mortality and sub-lethal effects.**

Mean measured concentration [mg p.m./L]	Mortality [%] (no. of dead fish / no. of treated fish)				
	0 h	24 h	48 h	72 h	96 h
Control	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/10N) <sup>J</sup>	0 (0/10N) <sup>J</sup>
Control	0 (0/10N)	0 (0/10N)	0 (0/10N)	0(0/10N)	0 (0/10N)
Solvent control	0 (0/10N)	0 (0/9N)	0 (0/9N) <sup>J</sup>	0 (0/9N) <sup>J</sup>	0 (0/9N) <sup>J</sup>
Solvent control	0 (0/10N)	0 (0/10N)	0 (0/10N)	0(0/10N)	0 (0/10N)

Mean measured concentration [mg p.m./L]	Mortality [%] (no. of dead fish / no. of treated fish)				
	0 h	24 h	48 h	72 h	96 h
2.4	0 (0/10N)	0 (0/10N)	0 (0/10N)	0(0/10N)	0 (0/10N)
2.4	0 (0/10N)	0 (0/10N)	0 (0/10N)	0(0/10N)	0 (0/10N)
5.0	0 (0/10N)	0 (0/10N)	0 (0/10N)	0(0/10N)	0 (0/10N)
5.0	0 (0/10N)	0 (0/10N)	0 (0/10N)	0(0/10N)	0 (0/10N)
10.3	40 (4/10) <sup>DC, LR</sup>	60(6/10) <sup>DC</sup>	60 (6/10) <sup>DC</sup>	60 (6/10) <sup>DC</sup>	60 (6/10) <sup>DC</sup>
10.3	50 (5/10) <sup>DC,LR</sup>	70(7/10) <sup>DC</sup>	70 (7/10) <sup>DC</sup>	70 (7/10) <sup>DC</sup>	70(7/10) <sup>DC</sup>
20.3	100(10/10)	100(10/10)	100(10/10)	100(10/10)	100(10/10)
20.3	100(10/10)	100(10/10)	100(10/10)	100(10/10)	100(10/10)
41.7	100(10/100)	100(10/10)	100(10/10)	100(10/10)	100(10/10)
41.7	100(10/10)	100(10/10)	100(10/10)	100(10/10)	100(10/10)

Observations: N = Normal; J = Fish jumped out of test chamber into surrounding water bath; LR = labored respiration; DC = darkened coloration

Conclusion: 96 h LC<sub>50</sub> = 9.1 mg metabolite/L (95% 5.0-10.3 mg a.s./L)  
 LOEC = 10.3 mg metabolite /L (based on mortality and sublethal effects)  
 NOEC = 5.0 mg metabolite /L (based on mortality and sublethal effects)

#### RMS comments:

The study was conducted according to the US EPA/FIFRA 72-1 guideline. Taking into account the validity criteria given in the test guidelines OECD 203 (1992) and US EPA OPPTS 850.1075 (1996) the study is considered acceptable.

The mortality in the controls was <10 % (being 0 %).

The dissolved oxygen throughout the test was >60% of the air saturation.

The loading rate of fish was 0.6 g fish/L.

The following deviations from OECD 203 (1992) and US EPA OPPTS 850.1075 (1996) test guidelines were noted:

- Gentle aeration after 24 hours of study initiation was added, because of decreasing of an oxygen concentration

on Day 1 to the level of 69% of air oxygen saturation. The addition of aeration did not impact the stability of the test material; metabolite concentrations were stable between Day 0 and Day 4.

- Test concentration was tested on day 0 and day 4 (in the static tests, the test substance concentration should be measured in each replicate minimally at the beginning (0 hour, before test organisms are added according to recommendation given in, at 48 h, and at the end of the test is recommended in US EPA OPPTS 850.1075 (1996) test guideline).

- The pH and conductivity parameter were measured on day 0, Day 2 and Day 4 (the 24 hour interval for the quality measurements in static system is recommended in the OECD 203 (1992) test guideline and US EPA OPPTS 850.1075 (1996) test guideline).

Since all validity criteria were met, these deviations are considered as having no impact on results of the study. The study is considered acceptable.

**Agreed endpoints:**

96 h LC<sub>50</sub> = 9.1 mg p.m./L (95% CI: 5.0-10.3), based on mean measured concentration

LOEC = 10.3 mg p.m./L, based on mean measured concentration

NOEC = 5.0 mg p.m./L, based on mean measured concentration

**B.9.2.1.8. Acute toxicity of metabolite Thiadone a metabolite of FOE5043 to the Bluegill (*Lepomis macrochirus*).**

<b>Reference:</b>	Acute Toxicity of Thiadone a metabolite of FOE5043 to the Bluegill ( <i>Lepomis macrochirus</i> ).
<b>Author(s), year:</b>	██████████ 1999
<b>Report/Doc. number:</b>	Study No: 108-455, Reference BCS No: M-016583-01-1.
<b>Guideline(s):</b>	US EPA/FIFRA 72-1 test guideline: Acute toxicity test for freshwater fish.
<b>GLP:</b>	Yes

Material and methods:

Test substance:	FOE 5043-Thiadone, Batch No: K778, Purity: 99.6%
Test species:	Bluegill ( <i>Lepomis macrochirus</i> )
Holding of fish:	Test medium: Soft blended water

---

	<p>All test fish were held and observed prior to testing in the laboratory.</p> <p>Fish were fed during acclimatization period daily with live food (D.magna, C.dubia, brine food) or commercial food.</p> <p>Photoperiod: 16 h daylight</p> <p>Temperature: 22°C</p> <p>The mortality: &lt; 3%</p> <p>During the 48-hours immediately prior to initiation of the 96-hour exposure Period the fish were held under the test conditions, i.e 22 ± 2°C, and light regime: 16 h Light /8 h Dark</p> <p>The fish were not fed this during this period.</p>
Number of organisms:	10 fishes with two replicates per test concentration and controls,
Age, length, weight:	Age: not given. Length: 21-39 mm, (mean, 30.6 ±4.5 mm ), weight 0.20-1.47 g wet weight (mean, 0.63 ± 0.30 g wet weight)
Loading:	0.21 g/L fish loading per test vessel
Type of test:	Static, 96 hours
<u>Applied concentrations:</u>	
Nominal (measured):	Control (0), Solvent Control (0), (0.62), 7.5 (6.61), 15 (14.9), 30 (28.0), 60 (58.6), and 120 (115) mg p.m./L
Solvent:	DMF, dimethylformamide 0.5 ml/L
<u>Test conditions:</u>	
Water quality:	Dilution water: mean hardness 60 mg/l as CaCO <sub>3</sub> , mean alkalinity: 51 mg/l as CaCO <sub>3</sub> ,
Conductivity:	106 µmhos/cm
Temperature:	Test start: 21.2-22.1 Test end: 21.6-22.1
pH:	Test start: 6.3-7.8 Test end: 7.3-7.6
O <sub>2</sub> content:	Test start: 9.1-9.3 mg/L Test end: 5.4-6.7 mg/L Dissolved oxygen concentrations ranged from 5.4 to 9.5 mg/L, representing 62 and 109 % of saturation at 22 °C, respectively.

On Day 2, dissolved oxygen levels were depressed, but they were not below 60% saturation. Slight aeration was added to the test system on Day 2 and aeration was slightly increased on Day 3 to maintain dissolved oxygen levels above 60% saturation throughout the study.

The addition of aeration did not impact the stability of the test material; thiadone concentrations were stable between Day 0 and Day 4.

Light regime: Light/dark cycle of 16h light /8h dark. Light intensity ranged from 44 to 65 foot candles corresponding to 470 to 695 Lux ( mean, 567 Lux).

Feeding: The fish were not fed throughout the duration of the tests.

Methods: The test was carried out in glass aquaria of ca. 38 litter capacity. Test and control tanks were set up using 30 L final volumes of dilution water. The dilution water was aerated prior to tank preparation. The aquaria were placed in a temperature controlled room. Fish were transferred to freshly prepared tanks. Fish from the control and solvent control chambers were weighed and measured at test termination to determine the biomass loading factor.

Test parameters: Two replicates of test vessels (each with 10 fishes) were monitored for mortality and sub-lethal effects after 24, 48, 72 and 96 hours. At the termination of the definitive test, the length and weight of each fish in the control tank was recorded.

Temperature, dissolved oxygen, pH, alkalinity, and hardness were measured in the both replicates of the control, solvent control and all treatment levels on Day 0 and Day 4. Dissolved oxygen was measured on Day 2 and Day 3.

Water samples for analytical determination of thiadone were collected from all replicates of the control, solvent control and all thiadone test levels on Day 0. On Day 4, water samples were collected from all vessels containing live fish; samples were not collected from those vessels in which fish died within 24 hours of test initiation. The water samples were analysed with HPLC technique.

Statistic: Since there were less than two concentrations with partial mortalities, the  $LC_{50}$  was calculated using nonlinear interpolation (the binomial probability method).

Findings:

Analytical data                      The mean measured concentration of thiadone during the test period ranged from 88.1-101% of the nominal concentration. All results based on mean measured concentration.

Biological effects:                      No behavioral or sublethal effects were observed in the control, solvent control and 6.61 mg/L test concentration during the exposure period. The sublethal or behavioral observations of darkened coloration, loss of equilibrium, labored respiration and fish on the bottom of the aquaria were noted in the 14.9 mg /L test concentration.

**Table B. 9.2.1.8-1: Mortality and sub-lethal effects.**

Mean measured concentration [mg p.m./L]	Cumulative mortality [%] (no. of dead fish / no. of treated fish)			
	24 h	48 h	72 h	96 h
Control	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/9N) <sub>j</sub>
Control	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/10N)
Solvent control	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/10N)
Solvent control	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/10N)
6.61	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/10N)
6.61	0 (0/10N)	0 (0/10N)	0 (0/10N)	0 (0/10N)
14.9	20 (2/10) <sup>DC, LE</sup>	20 (2/10) <sup>DC, LR</sup>	20 (2/10) <sup>DC, LR</sup>	20 (2/10) <sup>DC, LR</sup>
14.9	10 (1/10) <sup>LR</sup>	10 (1/10) <sup>LR</sup>	10 (1/10) <sup>LR</sup>	10 (1/10) <sup>DC, LR</sup>
28.0	100 (10/10)	100 (10/10)	100 (10/10)	100 (10/10)
28.0	100 (10/10)	100 (10/10)	100 (10/10)	100 (10/10)
58.6	100 (10/10)	100 (10/10)	100 (10/10)	100 (10/10)
58.6	100 (10/10)	100 (10/10)	100 (10/10)	100 (10/10)
115	100 (10/10)	100 (10/10)	100 (10/10)	100 (10/10)
115	100 (10/10)	100 (10/10)	100 (10/10)	100 (10/10)

N = Normal; DC = Darkened Coloration; LR = Labored Respiration; LE = Loss of Equilibrium; OB = On Bottom

Conclusion:                      The 96 hour LC<sub>50</sub> was estimated to be 18.6 mg p.m. /L (95% CI: 14.9 - 28.0).  
    The lowest observed effect concentration (LOEC) was determined to be 14.9 mg p.m./L.  
    No-observed effect-concentration (NOEC) was 6.61 mg p.m./L.

**Comments RMS:**

The study was conducted according to the US EPA/FIFRA 72-1 guideline.

Taking into the validity criteria given in the test guidelines OECD 203 (1992) and US EPA (1996) the study is considered acceptable.

The mortality in the controls was <10 % (being 0 %).

The dissolved oxygen throughout the test was > 60% of the air saturation during the study.

The loading rate of fish was 0.21g fish/L.

The following deviations from US EPA OPPTS 850.1075 (1996) test guideline were noted:

- The alkalinity ranged between 58 to 66 mg CaCO<sub>3</sub>/L, which is above the 40 to 60 hardness values designated in US EPA OPPTS 850.1075 (1996) test guideline. However, there was no apparent relationship between water hardness and thiadone concentration. These deviations had no impact on validity criteria of the test.

-The light intensity ranged between 44 to 65 foot candles (corresponding to app. 470 to 695 Lux).

which is below the 50 to 100 foot candle range given in US EPA OPPTS 850.1075 (1996) test guideline.

However, the mean measured light intensity was in acceptable range - 53 foot candles corresponding to 567 Lux, These deviations had no impact on validity criteria of the test.

-The environmental parameters, such as pH, were measured on Day 0 and Day 4 (the 24 hour interval for the quality measurements in static system is recommended in the OECD 203 (1992) and US EPA OPPTS 850.1075 (1996) test guidelines, respectively)

- Test concentration was tested on day 0 and day 4 (in the static tests, the test substance concentration should be measured in each replicate minimally at the beginning (0-hour, before test organisms are added according to recommendation given in, at 48 h, and at the end of the test is recommended in US EPA OPPTS 850.1075 (1996) test guideline).

- Slight aeration was added to the test system on Day 2 and aeration was slightly increased on Day 3 to maintain dissolved oxygen levels above 60% saturation throughout the study.

The addition of aeration did not impact the stability of the test material, FOE-Thiadone concentrations were stable between Day 0 and Day 4.

Since all validity criteria were met, these deviations are considered as having no impact on results of the study.

The study is considered acceptable.

**Agreed endpoints:**

96 h LC<sub>50</sub> = 18.6 mg p.m./L (95% CI: 14.9-28), based on mean measured concentration

LOEC = 14.9 mg p.m./L, based on mean measured concentration

NOEC = 6.61 mg p.m./L, based on mean measured concentration

---

**B.9.2.1.9. Acute toxicity of metabolite Thiadone to the sheepshead minnow (*Cyprinodon variegatus*) under static conditions.**

---

**Reference:** Acute toxicity of thiadone to the sheepshead minnow (*Cyprinodon variegatus*) under static conditions.

**Author(s), year:** [REDACTED] 1999

**Report/Doc. number:** Study No: 108809, Reference BCS No: M-009684-01-1

**Guideline(s):** US EPA/FIFRA 72-3 (a) test guideline. Estuarine fish 96-h acute toxicity tests.

**GLP:** Yes

---

Material and methods:

**Test substance:** Thiadone, Batch No: K778, Purity: 99.4%

**Test species:** Sheepshead minnow (*Cyprinodon variegatus*)

**Holding of fish:** Test medium: Aged salt water prepared from reverse osmosis water and Hawaiian Marine Mix® commercial sea salts.

All test fish were held and observed prior to testing in the laboratory.

The fish were fed daily with newly hatched brine shrimp and/or a commercial fish food (Tetramin®) or trout chow.

Photoperiod: 16 h daylight

Temperature: 22°±2 C

Salinity: 15-19 ‰

The mortality: No mortalities were recorded

During the 48-hours immediately prior to initiation of the 96-hour exposure period the fish were held under the test conditions: 22 ± 1°C, 16 h light /6 h dark.

Light intensity: 45-50 foot candles (app. 484-538 Lux). Salinity 15‰. The fish were not fed this during this period.

**Number of organisms:** 20 fishes per each test concentration and controls

**Age, length, weight:** Age: 3.5 to 4 months old. Length: 19-29 mm, (mean, 22.5 mm ±0.2), weight: 0.18-0.64 g wet weight

**Loading:** 0.4 g fish/L

**Type of test:** Static, 96 hours

**Applied concentrations:**

---

Nominal (mean measured):	Control (0), Solvent Control (0), 2.5 (2.48), 5.0 (5.20), 10 (9.97), 20 (20.5) and 40 (38.8) mg p.m./L
Solvent:	DMF, dimethylformamide 500 ul/L
<u>Test conditions:</u>	
Water quality:	Salt water prepared from reverse osmosis water and Hawaiian Marine Mix® commercial sea salts.
Salinity:	15 ‰
Conductivity:	Not given
Alkalinity:	Not given
Hardness	Not given
Conductivity:	Not given
Temperature:	Test start: 21.5°C - 22.9 °C Test end: 22.1°C - 22.4 °C
pH:	Test start: 6.9 -7.8 Test end: 7.2 - 7.5
O <sub>2</sub> content:	Test start: 92%-96% Test end: 80-87% Throughout the study the dissolved oxygen was > 60%
Light regime:	16h Light /8h Dark. Light intensity: 50-70 foot candles corresponding to app. 538-753 Lux (mean, 604 Lux)
Feeding:	The fish were not fed throughout the duration of the tests.
Methods:	One replicate of twenty fish each was used at each test concentration. Test chambers were 22- liter glass aquaria with a dimension of 21.5 x 34.5 x 29.5 cm. The aquaria contained 17 L of test solution, which was approximately 23 cm deep. Fish from the control and solvent control chambers were weighed and measured at test termination to determine the biomass loading factor.
Test parameters:	One replicates of test vessels (each with 20 fishes) were monitored for mortality and sub-lethal effects after 24, 48, 72 and 96 hours. Temperature, salinity, dissolved oxygen, and pH were measured in chambers which contained surviving organisms. The control, solvent control and all test concentrations were measured on Day 0 and 4. The dissolved oxygen and temperature were also measured in the controls and all test concentrations on Day 2. Temperature in the test system was measured daily.

In addition, an data logger documented hourly temperatures in a centrally located test chamber.

Water samples were collected for measured concentration analysis on Day 0 and Day 4.

Statistic: LC<sub>50</sub> calculated by using the following statistical methods: binomial probability, moving average angle, and probit. NOEC and LOEC calculated by using Fisher's exact test.

#### Findings:

Analytical data: The mean measured concentration of thiadone during the test period was >80% of the nominal concentration ( ranged between 97-104%).

Biological effects: Behavioral and sublethal effects were observed at 5.20, 9.97 and 20.5 mg metabolite/L test concentrations. These effects included erratic behavior, abnormal position at the surface, abnormal position at the bottom of the water column, quiescence, loss of equilibrium, vertical orientation and labored respiration. All fish in the control, solvent control and 2.48 mg p.m./L were normal throughout the test period.

There was no mortality in the control, solvent control, 2.48 and 5.20 mg p.m../L test concentrations.

**Table B. 9.2.1.9-1: Mortality and sub-lethal effects.**

Mean measured concentration [mg p.m./L]	mortality [%] (no. of dead fish / no. of treated fish)			
	24 h	48 h	72 h	96 h
Control	0 (0/20N)	0 (0/20N)	0 (0/20N)	0 (0/20N)
Solvent control	0 (0/20N)	0 (0/20N)	0 (0/20N)	0 (0/20N)
2.48	0 (0/20N)	0 (0/20N)	0 (0/20N)	0 (0/20N)
5.20	0 (0/20) <sup>Q</sup>	0 (0/20N)	0 (0/20) <sup>Q</sup>	0 (0/20) <sup>Q,E</sup>
9.97	5 (1/20) <sup>Q,AS,E</sup>	5 (1/20) <sup>Q,LE</sup>	5(1/20) <sup>Q,OB,LE</sup>	15 (3/20) <sup>LE,VO,E</sup>
20.5	3 0(6/20) <sup>E,Q,AS</sup>	65 (13/20) <sup>E,Q</sup>	70(14/20) <sup>Q,VO,LR</sup>	75 (15/20) <sup>E,VO,LR</sup>
38.8	100(20/20)	100(20/20)	100(20/20)	100(20/20)

N = normal; Q = quiescent; E = erratic; AS = at surface; LE = loss of equilibrium; OB = on bottom; VO = vertical orientation; LR = labored respiration

Conclusion: Based on mortality and the mean measured concentration, the thiadone 96 hour  $LC_{50}$  was 15.3 mg p.m./L (95% 12.7-18.3 mg p.m./L).  
The no-observed effect-concentration (NOEC) for sublethal effects of 5.20 mg p.m./L was determined.

**RMS comments:**

The study was conducted according to the US EPA/FIFRA 72-3 (a) guideline.

Taking into account the validity criteria given in the test guidelines OECD 203 (1992) and US EPA OPPTS 850.1075 (1996) the study is considered acceptable.

The mortality in the controls was <10 % (being 0 %).

The dissolved oxygen throughout the test was >60% of the air saturation during the study.

The loading rate of fish was 0.4g fish/L.

The following deviations from the test guidelines US EPA OPPTS 850.1075 (1996) and OECD 203 (1992) were noted:

- The environmental parameter such as pH was measured on Day 0 and Day 4 (the 24 hour interval for the quality measurements in static system is recommended in the test guidelines OECD 203 (1992) and US EPA OPPTS 850.1075 (1996))

- The measurements of alkalinity, hardness and conductivity were missing from the study protocol.

Since all validity criteria were met, these deviations are considered as having no impact on results of the study.

The study is considered acceptable.

**Agreed endpoints:**

96 h  $LC_{50}$  = 15.3 mg p.m./L (95% CI:12.7-18.3 mg p.m./L), based on mean measured concentration

NOEC = 5.20 mg p.m./L, based on mean measured concentration

**B.9.2.2. Long-term and chronic toxicity to fish**

For the first EU peer-review of the active substance flufenacet a fish early life stage toxicity study ([REDACTED] 1995) with *Oncorhynchus mykiss* was submitted addressing the chronic risk to fish.

The study is still valid and appropriate for the re-evaluation of flufenacet. The NOEC of 0.334 mg a.s./L was determined from this study.

For the renewal the new early life stage 35 day study has been conducted with *Cyprinodon variegatus* (Sheepshead Minnow). Based on mean measured concentration of flufenacet in this study and the most sensitive parameters- standard length and dry weight, the NOEC value was determined to be 0.049 mg a.s./L.

In addition, a new fish full life cycle study has been performed with Fathead Minnow (*Pimephales promelas*). The long-term (chronic) effects of flufenacet were investigated at mean measured concentrations of 0, 0.075 0.138, 0.274, 0.600 and 0.1211 mg/L over 279 days.

Based on mean measured concentrations of flufenacet and all of the biological endpoints evaluated, the NOEC and LOEC values for this study were determined to be 0.138 and 0.274 mg a.s./L, respectively.

The most sensitive species to flufenacet was *Cyprinodon variegatus* (Sheepshead minnow) with the NOEC value of 0.049 mg a.s./L.

**B.9.2.2-1: Long term and chronic toxicity to fish.**

Organism	Test substance	Endpoint (type of the test)	Value (mg a.s./L)	Reference
<i>Oncorhynchus mykiss</i> (Rainbow trout)	Flufenacet	97-day NOEC (flow-trough, ELS study )	0.334 mm	[REDACTED] (1995) M-002357-01-1
<i>Cyprinodon variegatus</i> (Sheepshead Minnow)	Flufenacet	35 –day NOEC (flow-through, ELS study, growth)	0.049 mm	[REDACTED] (2013) M-464909-01-1
<i>Pimephales promelas</i> (Fathead minnow)	Flufenacet	279 day NOEC (flow-through, FFLC study)	0.138 mm	[REDACTED] (2002) M-082934-01-1

**Study on flufenacet****B.9.2.2.1. Early life stage toxicity of FOE 5043 Technical to the Rainbow Trout (*Oncorhynchus mykiss*) under flow-through conditions.**

<b>Reference:</b>	Early life stage toxicity of FOE 5043 Technical to the Rainbow Trout ( <i>Oncorhynchus mykiss</i> ) under flow-through conditions.
<b>Author(s), year:</b>	██████████ 1995
<b>Report/Doc. number:</b>	Study No: 106978, Reference BCS No: M-002357-01-1
<b>Guideline(s):</b>	US EPA/FIFRA 72-4 test guideline. Early life stage.
<b>GLP:</b>	Yes

**Material and methods:**

Test substance:	FOE 5043 technical, Batch No: 303-0057 purity: 98.8%
Test species:	Rainbow Trout ( <i>Oncorhynchus mykiss</i> )
Holding of fish:	Eggs were incubated in glass incubation cups, constructed from 8 cm diameter glass jars with 10 mesh stainless steel screen replacing the bottom. These incubation cups were suspended in each of replicate test chamber. The glass aquaria used for egg hatching and as growth chambers measured approximately 22 x 34 cm with a water depth of 25 cm, yielding an approximate chamber volume of 17 liters. All chamber drains were covered with stainless steel screen to prevent fry escape. Test chambers were placed randomly in a temperature controlled water bath.
Environmental conditions:	Dilution water, hardness 55 mg/l as CaCO <sub>3</sub> alkalinity: 50 mg/L CaCO <sub>3</sub> , pH 7.4-8.3. Temperature: 9.2°C
Feeding of adult fish:	Feeding began on Day 50 (post-hatch Day 17). Twice daily, except on weekends when food was added once daily.  Live brine shrimp ( <i>Artemia salina</i> ) nauplii were fed to the fry until post hatch Day 45 at which time the fry were fed with ground trout/salmon starter until 18 hours prior to study termination.
Number of organisms:	Four replicates per test concentration, 35 embryos per replicate.

Additional 50 eggs in separate incubation cups were randomly placed in each of four control chambers for viability determination. The extra eggs were used to ensure that at least 15 fry per replicate (60 per treatment level) would be available for the early life-stage growth portion of the study.

Age: Embryos < 48 hours old at test initiation

Loading: The biomass loading factor for the study was determined using the wet weights of the control and solvent control fish at study termination. The mean wet weight was 0.58 g/fish. The maximum loading factor based upon 15 fish in the 17 liter volume of the growth chambers was 0.51 g/L. The loading factor based upon a flow of 187 liters per day through each test chamber, was 0.05 g/L.

Type of test: Flow-through test, 97 days

#### Applied concentrations:

Nominal (mean measured): Control (0), Solvent Control (0), 50 (44.5), 100 (87.5), 200 (179), 400 (334), and 800 (735) µg a.s./L

Solvent: Acetone

Test conditions:

Water quality: Dilution water, hardness: 50-64 mg/l as CaCO<sub>3</sub>, alkalinity: 40-56 mg/L as CaCO<sub>3</sub>

Conductivity: 122-147 µmhos/cm

Temperature: 9.3-10.8°C

pH: Test start: 7.1

Test end: 7.9

O<sub>2</sub> content: 9.0 to 11.6 mg/L corresponding to 82-103 % of oxygen saturation

Light regime: Light/dark cycle of 16/8. Light intensity 53 foot candles (574 Lux).

A 30 min transition period to stimulate the dawn and dusk was used.

Methods: 35 embryos were placed in each of four replicate test chambers (total of 140 embryos per test group). An additional 50 eggs in separate incubation cups were randomly placed in each of four control chambers for viability determination. The eggs set aside for viability determination (fertilization success) were removed from the test system on Day 13. Observations of embryonic stage (as evidenced by spinal development) were made and the eggs discarded. Those eggs exhibiting no indication of embryonic development were deemed non-viable. The mean viability was 83%. The number of eggs hatched in each incubation cup was recorded daily until 3 days post-hatch. The post hatch period

began after 90 percent of all eggs in the controls had hatched (study Day 36, day post hatch). Alevin were impartially reduced to 15 individuals per replicate on study Day 36 (post hatch Day 3). This was accomplished by removing all but 15 impartially selected fry and removing any remaining eggs from each incubation cup. On Day 41 (post-hatch Day 8), alevin were released from incubation cups into the aquaria.

Observations of abnormal behavior, swim-up behavior, abnormal physical changes and mortality were recorded daily by visually inspecting each growth chamber. Dead fry were removed and discarded.

Test parameters:

Temperature, conductivity, total alkalinity, total hardness and pH were measured in alternating replicates of the control, solvent control, low, middle and high concentrations on days 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91, and 97. Dissolved oxygen was measured in all aquaria on the listed study days. Temperature was measured manually twice daily (except weekends).

Samples of test solutions, including controls, were taken from two alternating replicate test chambers on days: -1, 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91, and 97 to measure actual exposure concentrations. Samples were extracted for HPLC analysis on the same day sampled.

Statistic:

Data for the replicate chambers for each concentration were grouped together for analysis. Replicate means were used for statistical analysis since each chamber was the experimental unit based on the design of the test system. For each parameter analyzed the following statistical tests were conducted:

- 1) t-test to determine if control and solvent control data could be pooled;
- 2) chi-square test to test for normality and Levene's test for homogeneity of variance;
- 3) ANOVA followed by Dunnett's multiple comparison test.

Control data were pooled if the t-test criteria was met, otherwise only solvent control data were used for further analyses.

All data were analyzed without transformations when the chi-square test and homogeneity of variance test indicated normality and homogeneity of variance ( $\alpha = 0.05$ ). If the raw data did not pass the tests for normality and/or the homogeneity of variance, the data were transformed using one of several algorithms until the assumptions were met. Growth, percent hatch, survival and swim up data were analyzed by a one-way analysis of variance (ANOVA) to

determine if there was a significant difference between the treatment groups and control groups. If the results of the ANOVA showed a significant difference ( $\alpha = 0.05$ ) then a Dunnett's two-ailed multiple means comparison test was conducted to identify which treatment group(s) were significantly different from the control groups. The results of the Dunnett's test were used to determine the no observed effect concentration (NOEC). Statistical analyses were conducted using a PC based computer program developed by WEST Inc. and Gulley (1994) with conclusions of statistical significance based on a 95 percent confidence level ( $\alpha = 0.05$ ).

All biological data (survival at test termination, time to hatch, percent hatch, time to swim up, and growth) met the assumptions of normal distribution. These data also met the assumptions for homogeneity of variance, except for survival, percent hatch and swim-up. The variances for survival and swim-up could not be tested because there were replicates with a variance of zero. Therefore, untransformed, raw data was used for statistical analysis of these endpoints. The percent hatch data was transformed with various standard statistical algorithms until a transformation was found that allowed the data to meet the assumption for homogeneity of variance. In this case, the inverse algorithm was used ( $1/Y$ ) to transform the percent hatch data prior to analysis of variance and multiple comparisons testing. In addition, based on the results of the t-test, there was no significant difference in the control and solvent control data for any of these biological endpoints (survival at test termination, time to hatch, percent hatch, time to swim up, and growth).

Therefore, all control data were pooled for comparison to treatment data.

#### Findings:

##### Analytical data:

Mean measured concentration ranged between 87 and 92 % of nominal test concentration. Based on analytical measurement of test concentration the all results are presented on mean measured concentration.

##### Biological effects:

The following parameters were observed and recorded: survival at test termination, time to hatch, percent hatch, time to swim up, and growth. Growth as length was determined by standard length (mm), fry survival on post hatch Day 33 and post hatch day 64. Fish dry weights were measured at post hatch day 64. Percent hatchability was evaluated on study Day 36 ( Day 3 post hatch). Newly hatched fry began swimming up from the bottom of the test chambers on

Day 43 (day 7 post hatch). Swim-up was observed for a 13 day period between study day 43 and 56. Fry growth, as length, was measured using a photographic technique on study Day 66 (post-hatch Day 33). Fry growth, as length, was again measured at the end of the study (post-hatch Day 64). Fry growth, as dry weight, was measured after study termination (post-hatch Day 64).

The summary of the results of the study are presented in the Table B.9.2.2.1-1 below:

**Table B.9.2.2.1-1: Summary of effects following exposure of Rainbow trout to Flufenacet for 97 days.**

Mean measured [µg s.a./L]	Egg hatchability <sup>1</sup> [%]	Fry survival [%]		Fry length [mm]		Fry weight [g]	
		Post hatch day 33	Post hatch day 64	Post hatch day 33	Post hatch day 64	wet weight	dry weight
						Post hatch day 64	
Control	94	100	100	31	32.1	0.5760	0.0951
Solvent control	100	100	98	31.2	33.7	0.5771	0.0997
Pooled control	-	-	-	31.1	32.9	0.05765	0.0997
44.5	100	95	95	31.3	33.8	0.5771	0.1037
87.5	90	100	100	31	31.7	-	0.0987
179	97	100	98	30.7	33.9	-	0.0982
334	96	97	95	<b>28.7*</b>	32	-	0.0966
735	94	93	92	26.6*	31.1	-	0.0840*
NOEC (µg a.s./L)	>735	>735		179		-	334
LOEC (µg a.s./L)	>735	>735		334		-	735

<sup>1</sup>Percent hatched of live embryos

\*Statistically significant different compared to pooled control (Dunnet 's test , α=0.05)

**Table B.9.2.2.1-2. Percent Swim-up of Newly Hatched Rainbow Trout Fry during exposure to flufenacet .**

Mean measured test concentration [µg s.a./L]	Percent Swim-up by study day														
	Study Days	43d	44d	4d5	46d	47d	48d	49d	50d	51d	52d	53d	54d	55d	56d
Control	0	0	0	5	17	53	100								
Solvent control	2	2	3	3	10	17	100								
44.5	2	0	3	5	10	19	90	98	98						
87.5	0	0	2	5	8	15	80* <sup>1</sup>	100							
179	0	0	3	3	10	18	87	100							
334	0	0	2	3	7	10	78* <sup>1</sup>	98							
735	0	0	0	0	0*	2	13*	40*	57*	63*	63*	63*	93*	100	
NOEC = 334 µg a.s/L based on % swim up, LOEC =735 µg a.s./L based on % swim up															

\* Percent swim-up statistically significantly different compared to solvent control or pooled controls (Wilima's test,  $\alpha = 0.05$ ) depending on the day (solvent control used for day 48, pooled controls used on all other days).

<sup>1</sup> Statistically significant difference from the pooled controls. However, the statistically significant difference found at that treatments group was not considered significant since the percent swim up in this treatment concentrations on the following day (50 day) achieved the level comparable to controls.

#### Results:

For controls and all tested concentration the egg hatchability was >90%.

Percent hatch on Day 36 corrected for viability resulted in a mean percent hatch ranging from 90 percent to 100 percent. There was no statistical difference in percent hatchability in any test treatment compared to the pooled control data.

Fry survival was analyzed on Day 66 (post-hatch Day 33) and on Day 97 (post-hatch Day 64; study termination). There was no significant difference between the pooled controls and any test levels.

During the post hatch period, morphological and behavioral effects observed included darkened coloration, position at the bottom of the chamber, quiescence, and lethargy. These anomalies were confined to the 334 and 735 µg/L test levels. Newly hatched fry began swimming up from the bottom of the test chambers on Day 43 (day 7 post hatch). Swim-up was observed for a 13 day period between

study day 43 and 56. Swim-up of >90% was achieved on Day 49 in the control, solvent control and 44.5 µg/L test level. All other test levels had >90% swim-up fry on Day 50, except for the highest test level, which achieved >90% swim-up on Day 55. There was a significant difference in percent swim-up between the pooled controls and the 735 µg/L test level on day 47 and days 49-55 (8 of 9 days examined). Swim-up at this concentration lagged behind controls by about a week, and so this was considered a potentially biologically significant treatment effect. Statistically significant differences between the controls and the 87.5 and 334 µg/L concentrations were found on day 49. All of the test concentrations, with the exception of 735 µg/L, achieved virtually 100% swim-up by day 50 (within 1 day of controls). For this endpoint, the 334 µg/L test concentration was identified as the NOEC and the 735 µg/L test level was identified as the LOEC.

Fry growth, as length, was measured using a photographic technique on study Day 66 (post-hatch Day 33). Analysis of the data showed a statistically significant difference between the pooled controls and the 334 and 735 µg/L test concentrations. Fry growth, as length, was again measured at the end of the study (post-hatch Day 64). There was no significant difference in any test concentrations compared to the pooled controls Fry growth, as dry weight, was measured after study termination (post-hatch Day 64).

There was a significant difference from the pooled controls at the 735 µg/L test concentration.

Conclusion:

Based on the statistical analysis of survival, hatchability, and growth the no-observed-effect-concentrations (NOECs) and the lowest-observed-effect-concentrations (LOECs) were determined as follows:

NOEC > 735 µg a.s./L and LOEC >735 µg a.s./L (Survival at post-hatch Day 33 (66 day study) and post hatch day 64 (97 day study)).

NOEC > 735 µg a.s./L and LOEC > 735 µg s.s./L (Egg hatchability)

NOEC = 334 µg a.s./L and LOEC = 735 µg a.s./L (Percent swim-up )

NOEC = 179 µg a.s./L and LOEC = 334 µg a.s./L (Growth, as length, on post-hatch Day 33 ( 66 day study))

NOEC = 334 µg a.s./L and LOEC = 735 µg a.s./L (Growth, as dry weight on post-hatch Day 64, 97 day study)

NOEC > 735 µg a.s./L and LOEC > 735 µg a.s./L (Growth, as length, on post-hatch Day 64, 97 day study)

Growth, determined as mean total length, was the most sensitive endpoints of the study. The 97-day NOEC and LOEC in rainbow trout, based on mean, measured concentrations of Flufenacet and growth were 179 and 334 µg a.s./L, respectively. Toxicant Concentration (MATC) was based on the swim-up and dry weight endpoint is 495 µg/L.

In accordance with the new data requirement (Commission Regulation EU No 283/2013), the EC<sub>10</sub>, EC<sub>20</sub>, values should be calculated. Where they can not be estimated, an explanation shall be provided.

**RMS comments:**

The study was conducted according to the US EPA/FIFRA 72-4 guideline.

The study is in line with the current OECD 210 (1992 and 2013) test guideline regarding the early life stage test with fish.

The following criteria should be met in the study according to the test guideline OECD 210 (2013)

- The dissolved oxygen concentration should be >60% of the air saturation value throughout the test.

In this study the dissolved oxygen concentration ranged between 82-103 % of oxygen saturation.

- The water temperature should not differ by more than + 1.5°C between test chambers or between successive days at any time during the test, and should be within the temperature ranges specified for the test species (range 10±1.5°C is recommended in the test guideline).

In this study temperature not differ more than + 1.5°C and was in acceptable ranges (being 9.3-10.8°C)

- The analytical measurements of the test concentrations is compulsory.

The analytical measurements of test concentrations were provided weekly.

- Overall survival of fertilized eggs and post-hatch success in the controls and, where relevant, in the solvent controls should be greater than or equal to 75% (being 94-100% ( survival in the control, hatching success) and 98-100 % (survival in the control at post hatching success)).

The following deviations from OECD 210 (2013) test guideline were noted:

- The feeding began on 17<sup>th</sup> post-hatch day not on 19<sup>th</sup> day post hatch or swim up.
- The hardness on Day 42 was measured to be 62 to 64 mg/L as CaCO<sub>3</sub> (while range between 40 to 60 mg/L as CaCO<sub>3</sub> is recommended in the OPPTS 850.1400 (1996) test guideline).

Indicated deviations are, however, considered as having no impact on the study results, since all validity criteria were met. The study is considered acceptable.

**Agreed endpoints:**

NOEC > 735 µg a.s./L and LOEC >735 µg a.s./L (Survival at post-hatch Day 33 (66 day study) and

post hatch day 64 (97 day study).

NOEC > 735 µg a.s./L and LOEC > 735 µg s.a./L (Egg hatchability)

NOEC = 334 µg a.s./L and LOEC = 735 µg a.s./L (Percent swim-up )

NOEC = 179 µg a.s./L and LOEC = 334 µg a.s./L (Growth, as length, on post-hatch Day 33 (66 day study)

NOEC = 334 µg a.s./L and LOEC = 735 µg a.s./L (Growth, as dry weight on post-hatch Day 64, 97 day study)

NOEC > 735 µg a.s./L and LOEC > 735 µg a.s./L (Growth, as length, on post-hatch Day 64, 97 day study)

Growth, measured as fish length, was statistically different from controls on post-hatch day 33 (study-day 66). This proved to be biologically not relevant on post-hatch day 62 (study-day 97), where no effects were observed for length. The NOEC for growth (as length) at the end of the study is given as 0.8 mg/L.

(measured 0.735 mg/L). In RMS opinion due to the fact that the effects for growth as length on post hatch Day 33 at 334 µg a.s./L was moderate and only transistant the overall NOEC of 334 µg a.s./L for the whole study should be based on the parameters "percent swim-up" and "97d-dry weight".

**Therefore, overall NOEC of 334 µg a.s./L was estimated from the study.**

#### **B.9.2.2.2. Early life stage toxicity of flufenacet technical to the Sheepshead minnow (Cyprinodon variegatus) under flow-through conditions.**

<b>Reference:</b>	Early life stage toxicity of flufenacet technical to the Sheepshead minnow (Cyprinodon variegatus) under flow-through conditions.
<b>Author(s), year:</b>	██████████ 2013
<b>Report/Doc. number:</b>	Study No: EBF OL 244, Reference BCS No: M-464909-01-1
<b>Guideline(s):</b>	FIFRA Guideline 72-4 (1982) OPPTS Guideline 850.1400 (1996 draft) OECD Guideline 210 (1992)
<b>GLP:</b>	Yes

#### Material and methods:

Test substance:	FOE 5043 (flufenacet tech.), Batch No: NK61CX0617, Purity: 98.8%
Test species:	Sheepshead minnow (Cyprinodon variegatus)
Age/life stage at dosing:	24 to 48 hours old eggs in the neurula stage
Initial population:	35 eggs at initiation, thinned to 20 alevin after hatching phase.

---

	Four replicates per test vessel.
Diet:	Brine shrimp nauplii ( <i>Artemia salina</i> ) starting on Day 5.
Feeding:	Twice daily on weekends and two to three times daily on weekdays until approximately 24 hours prior to study termination.
Test chamber:	9-L glass aquaria containing 7 L of test solution (25.5-cm test solution depth) One oscillating egg cup per replicate placed in each replicate through the egg hatching phase.
Loading:	The biomass loading factor for the study was determined using the wet weights of the control fish at study termination. The mean wet weight was 0.23 g/fish and 0.096 g/L/day (mean wet weight based on controls).
Type of test:	Flow-through test
In -life duration	35 days

Applied concentrations:

Nominal (mean measured): Control (0), 50 (49), 100 (95), 200 (174), 400 (339), and 00 (667) mg a.s./L

Test conditions:

Water quality: Dilution water: Synthetic sea water.

Salinity 18 to 22 ‰

Temperature: 24.7-25.5°C

pH: 8.1-8.2

O<sub>2</sub> content: 5.3 to 7.5 mg/L corresponding to 70-99 % saturation

Light regime: 16 hours light /8 hours dark. Light intensity: 736-805 Lux (mean, 778 Lux) with 30 min the dawn and dusk transition period.

Methods: Thirty five eggs per replicate were used at initiation, thinned to 20 alevin per replicate after hatching phase (Day 6, thinning day). Day 6 is the day when 90 % of the viable eggs completed hatching and was the most representative day for the time to hatch data analysis. Post hatch phase began after thinning.

Test parameters: Water quality parameters including pH, salinity, and dissolved oxygen were measured at the test start and the least weekly thereafter including experimental finish (in all test concentrations).

The temperature of the test system was represented by a centrally located test

vessel was recorded on hourly basis. Daily temperature measurements were also recorded manually.

The following biological parameters were observed and recorded during the study: sublethal effects, percent hatch (time to hatch), alevin and fry survival and growth as standard length and dry weight for all surviving fish on Day 35. Observations for sublethal effects and survival made daily, hatching observations made daily during hatching phase, growth determinations made at the end of the exposure.

Samples of test solutions, including controls, were taken from two alternating replicates at each interval at experimental start, at least one a week (+ 2 days) thereafter including experimental finish.

The analysis was performed using a Liquid Chromatograph/ Tandem Mass spectrometry system (HPLC-MS/MS).

Findings:

Analytical data:

Mean measured test concentration of flufenacet ranged from 85 to 98 % of nominal. All results of the study are based on mean measured concentration.

Statistic:

For survivorship, hatchability, and growth data (length and dry weight measurements), the aquaria were considered to be the smallest experimental unit for a replicate based on the design of the test system, and hence replicate means were used for statistical analysis. For each parameter analyzed, the following statistical tests were conducted:

- 1) Chi-square test to test for normality and Bartlett's test for homogeneity of variance.
- 2) One way Analysis of Variance (ANOVA) was used to determine if there was a significant difference between the treatment groups and the control.

If the results of the ANOVA showed significant differences ( $p=0.05$ ) then the Dunnett's test and the William's test (if appropriate) were conducted to identify which treatment group(s) were significantly different from the control groups. The results were used to determine the No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC). Statistical analyses were conducted using a PC based computer program (TOXSTAT ver. 3.4).

Biological effects:

Fish throughout all test levels, excluding the 677  $\mu\text{g a.s./L}$ , appeared normal during the course of the study, with the exception of a few fish small in size

being noted throughout various test levels. Fish in the 677 µg a.s./L test level were observed to be swimming at the bottom of the test vessel, except when being fed, beginning on study Day 32. All fish appeared normal in the 677 µg a.s./L test level on study Day 35. Additionally, one fish in the 174 µg a.s./L test level was observed to have a blunt snout at termination, which appears to be incidental and not biologically significant.

**Table B.9.2.2.2-1: Summary of effects following exposure of flufenacet for 35 days to Sheepshead minnow (*Cyprinodon variegatus*).**

Mean measured concentration [µg a.s./L]	Mean % hatch <sup>a</sup>	Mean % hatch <sup>b</sup>	Mean % Survival		Growth	
	Day 5	Day 6	Alevin <sup>c</sup>	Fry <sup>d</sup>	Mean standard length [mm]	Dry weight [mg]
Control	37.9	85.7	90.7	98.8	19.4	62.7
49	36.4	87.1	87.9	96.3	19.3	61.9
95	39.3	84.3	90.0	98.8	19*	58.1*
174	35	86.4	90.0	98.8	18.8*	58.8*
339	38.6	87.1	90.7	97.5	18.6*	56.9*
677	29.3	89.3	89.3	95.0	18.1*	50.8*
NOEC (µg a.s./L)	677	677	677		49	49
LOEC (µg a.s./L)	>677	>677	>677		95	95

- a The mean percent of embryos that hatched by day 5. % Hatch on Day 5 = (alevin/total eggs on Day 0) x 100. There was not statistical significantly difference from control in any test concentrations (William's test, p=0.05).
- b The mean percent of embryos that hatched by day 6. % Hatch on Day 6 = (alevin/total eggs on Day 0) x 100. There was not statistical significantly different from controls in any test concentrations (William's test, p=0.05).
- c The mean percent alevin survival on Day 6. Survivorship on Day 6 = (live alevin and eggs/eggs on Day 0) x 100%. There was not statistical significantly different from controls in any test concentrations (William's test, p=0.05).
- d The mean percent of survivorship on Day 35. % Fry survival on day 35 = (fish on Day 35/ fish at thinning) x100. There was not statistical significantly different from controls in any test level. p=0.05, William's test).
- \* Statistically significant difference from control (William's test p=0.05).

#### Conclusion:

Procent Hatch NOEC = 677 µg a.s./L

LOEC >677 µg a.s./L

Time to Hatch NOEC = 677 µg a.s./L

LOEC >677 µg a.s./L

Alevin survival: NOEC = 677 µg a.s./L

LOEC >677 µg a.s./L

Fry survival: NOEC = 677 µg a.s./L

LOEC >677 µg a.s./L

Growth (Length) NOEC = 49 µg a.s./L

LOEC = 95 µg a.s./L

Growth (Dry weight) NOEC = 49 µg a.s./L LOEC = 95 µg a.s./L

The most sensitive parameter was growth, determined as mean standard length and dry weight,

resulting in NOEC<sub>growth</sub> = 49 µg a.s./L

In accordance with the new data requirement (Commission Regulation EU No 283/2013), the EC<sub>10</sub>, EC<sub>20</sub>, values should be calculated. Where they can not be estimated, an explanation shall be provided.

#### **RMS comments:**

The study was conducted according to OECD 210 (2013) guideline.

The following validity criteria should be met in the study:

- The dissolved oxygen concentration should be > 60% of the air saturation value throughout the test.

In this study the dissolved oxygen concentration ranged between 70 and 99 % saturation.

- The water temperature should not differ by more than + 1.5°C between test chambers or between successive days at any time during the test, and should be within the temperature ranges specified for the test species (range 25.5°C ± 1.5 is recommended in the test guideline).

In this study temperature not differ more than + 1.5°C and was in acceptable range between 24.7 and 25.5°C.

- The analytical measure of the test concentrations is compulsory.

The analytical measurements of test concentrations were provided weekly.

- Overall survival of fertilized eggs and post-hatch success in the controls and, where relevant, in the solvent controls should be greater than or equal to 75% and 80%, respectively (being: 90.7% survival in the control, (hatching success) and 98.8% survival in the control (post hatching success)).

The following deviation from OECD 210 test guidelines was noted:

- Length of fish in the control group was 19.4 mm at the end of study and therefore was slight above the value recommended in the 210 OECD test guideline (should be 17 mm).

This slight deviation in length of tested in organisms in opinion of RMS could not significantly influence of the study results.

Based on the evaluation of the study the ELS fish toxicity test is considered acceptable.

#### **Agreed endpoints:**

Procent Hatch NOEC = 677 µg a.s./L LOEC > 677 µg a.s./L

Time to Hatch NOEC = 677 µg a.s./L LOEC > 677 µg a.s./L

Alevin survival: NOEC = 677 µg a.s./L LOEC > 677 µg a.s./L

Fry survival: NOEC = 677 µg a.s./L LOEC > 677 µg a.s./L

Growth (Length) NOEC = 49 µg a.s./L LOEC = 95 µg a.s./L

Growth (Dry weight) NOEC = 49 µg a.s./L LOEC = 95 µg a.s./L

**B.9.2.2.3. Fathead minnow (*Pimephales promelas*) Fish Life Cycle Test with Flufenacet (FOE 5043 Technical).**

<b>Reference:</b>	Fathead minnow ( <i>Pimephales promelas</i> ) Fish Life Cycle Test with Flufenacet (FOE 5043 Technical).
<b>Author(s), year:</b>	██████████ 2002
<b>Report/Doc. number:</b>	Study No: 109767, Reference BCS No: M-082934-01-1
<b>Guideline(s):</b>	FIFRA 72-4 A, US EPA (1975, 1982, 1986a and b), APHA (1989) and Benoit (1981).
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet, Batch No. 803-1087, Purity: 95.6 %
Test species:	Fathead minnow ( <i>Pimephales promelas</i> )
Holding:	<p>The fathead minnow eggs (at the gastrulation stage or earlier) used to initiate the test were obtained from an in-house fathead minnow breeding culture. The breeding system consisted of a series of 21-liter aquaria, each of which contained at least two male and four female fathead minnows, and two to three breeding substrates.</p> <p>Eggs from at least five breeding tanks were collected on the morning of test initiation and placed in a crystallizing dish that contained dilution water. Microscopic observations on the eggs were made to determine the stage of development, and any eggs that were noticeably nonviable or were at a later stage than gastrulation were removed from the egg pool prior to distribution into treatment tanks.</p> <p>The holding area for the breeding fish was maintained on 16-hour daylight, 8-hour dark photoperiod.</p> <p>Environmental conditions: dilution water: hardness 51 mg/L as CaCO<sub>3</sub>, alkalinity 3 mg/L CaCO<sub>3</sub>, pH: 7.4-8.1, conductivity: 115 µhos/cm.</p>
Number of organisms:	<p>At start 50 eggs per egg cup, four egg cups per treatment.</p> <p>After hatching (the hatch Day 6, F0 generation,) the number of alevin was reduced to 25 viable individuals per replicate per growth chamber, two growth chamber per aquarium in total 200 individual in four replicates.</p> <p>After 36 day the fish were impartially thinned to 15 fish per growth chamber, and were transferred to extended growth chamber resulting in 30 fish per extended</p>

	growth chamber.
Loading:	The biomass loading factor did not exceed 1 gram of fish tissue per liter of test solution passing through the aquaria in 24 hours, and instantaneous loading did not exceed 5 g/L at any one time.
Type of test:	Flow-through test, 279 days
<u>Applied concentrations:</u>	
Nominal (mean measured)	Control (0), solvent control (0), 0.087 (0.075), 0.175 (0.138), 0.35 (0.274), 0.70 (0.600) and 1.4 (1.211) mg a.s./L
Solvent:	Aceton, 0.1 ml acetone/L
Test conditions:	<p>The mean and range of the alkalinity were 44 mg/L as CaCO<sub>3</sub> and 26 to 50 mg/L as CaCO<sub>3</sub>, respectively.</p> <p>The mean and range of hardness were 52 mg/L as CaCO<sub>3</sub> and 36 to 66 mg/L as CaCO<sub>3</sub>, respectively.</p> <p>Conductivity: The mean and range of conductivity were 115 µhos/cm and 94 to 131 µhos/cm, respectively.</p>
Temperature:	<p>The daily test temperature ranged from 23.5 to 26.7 °C as measured hourly by the datalogger, and the mean temperature was 25.0 °C.</p> <p>The temperature fluctuated outside of the 25 ± 1.5° C test parameter on five different occasions during the 279 day study.</p> <p>On Day 9 the temperature reached 26.1 °C for 3 hours.</p> <p>On Day 34 the temperature with a maximum of 26.7 C was out of range during 6 hours. On Day 110 the temperature fell out of range at 23.9 °C for 1 hour.</p> <p>On Day 167, the temperature fell to 23.9 C for 2 hours.</p> <p>On Day 227, the temperature fell out of range for 4 hours, with a minimum of 23.5 C.</p> <p>The temperature range of the study did not vary by more than 25 ± 2 °C.</p> <p>Since these temperature fluctuations were short (&lt;48 h), minor in degree and would have occurred in all test vessels, they would have no adverse impact on the results of the study.</p>
pH:	The pH ranged from 7.3 to 7.9
O <sub>2</sub> content:	The mean dissolved oxygen (DO) (mean for all replicate for all test concentration) concentrations ranged from 6.2 to 7.2 mg/L (75 to 87% of air saturation, respectively, at 25° C throughout the study. It should be, however, noted at particular time points dissolved oxygen dropped below the

recommended concentration and usually ranged from 4.2 to 8.2 mg/L (51 to 99%, respectively) at 25°C. The dissolved oxygen dropped below 60% of air saturation on Days 104, 111, 150, 153, 174, 185, 197, 213, 248, 251 and 249 in the extended growth chamber/spawning (replicates A and B). On day 150 oxygen concentrations was 2.2 mg/L (27%). This significant deviation was in a single test chamber (replicate 3) that was experiencing restricted water flow (0.600 mg a.s./L). On the previous day and later on that same day (after flow was restored), dissolved oxygen values equaled 6.0 mg/L in that replicate.

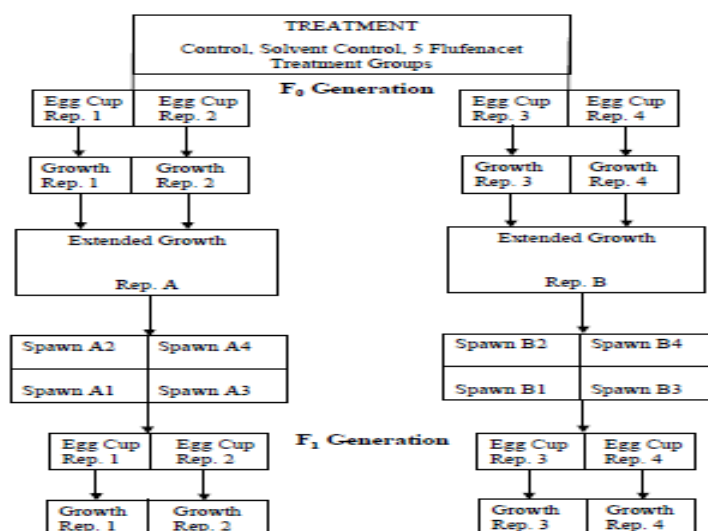
According to the study director due to microbial growth and potential to decreased dissolved oxygen it was necessary to clean the diluter (once per week) and test vessels (3 to 5 times per week). Due to extensive cleaning on day 92 the aeration was added to help maintain dissolved oxygen level. In most cases DO concentration recovered > 60% on the same day or the following days (1-3). For one vessel on Day 248 the dissolved oxygen were not documented until 5 days later. Taking into account that oxygen concentrations below the recommended values were transient, it is concluded that they had no impact on the integrity of the study and obtained results, especially all validity criteria were met.

Light regime:

Photoperiod for the test followed the Indiana light cycle, with a 15 to 30 minute transition period to simulate dawn and dusk conditions. The day length was depended from the study day and ranged from approx. 11 hours on November-December, 12 hours on October, 15 hours on May-August. Average light intensity was 69.7 foot-candles (750 Lux).

Test chambers:

Each treatment, control, and solvent control group had two exposure chamber units. Each unit consisted of two egg cups, immersed in two growth chambers, and one chamber that acts as either an extended growth chamber or a spawning chamber, depending on the stage of the study Figure B.9.2.2.2-1.



**Figure B.9.2.2.2-1. Experimental design of the flufenacet fish life cycle.**

Parental generation: F<sub>0</sub>:

#### Early life stage:

Embryo exposure for the F<sub>0</sub> generation began at test initiation on Day 0 with a target of app. 50 eggs per egg cup, four egg cups per treatment (total 200 eggs). Incubation cups, constructed of glass jars (7 cm diameter, 11.5 cm height, 440 mL volume) with the bottom removed and replaced with 20 mesh nytex screen, were suspended in each growth chamber.

The egg cups were gently oscillated in the test solution. One egg cup was placed into each growth chamber.

Three additional egg cups containing 50 eggs were placed in the control extended growth tanks to act as viability checks for the test. Egg viability was determined on Day 2, when the embryos had visible eye spots.

The mean egg viability was 92% ± 2SD.

Hatching of the eggs was monitored until all control replicates reached at least 90% hatch. Hatch began from Day 2 and on Day 6 - the 90% of all control replicate achieved > 90% hatch (Hatch Day 6). The percent of eggs hatched and egg and alevin survivorship was monitored from Day 0 (egg) through Day 6 (alevin). The % survivorship equaled the number of live organism divided by the numbers of eggs on the test initiation day.

% Hatch on Day 6 as well as % survivorship of eggs and alevin was corrected by viability 92%±2.

Larvae/Juvenile Exposure

After hatching (Day 6) and counting the number of alevin was reduced to 25 viability per replicate. These 25 alevin were transformed from egg cups into growth chamber in which the egg cup had been oscillating. Observation on larva/juvenile fish were made daily. The number of individuals. Fish were reared in the growth chamber for approximately 30 days after post hatch.

Observations of larvae/juvenile fish were made daily. The number of live individuals was counted at least once per week.

On Day 36, the fish were impartially thinned to 15 fish per growth chamber, and were transferred 39.7 liter glass extended growth chamber (width x length x height equal to 30.5 x 52 x 30.5 cm), resulting in 30 fish per extended growth chamber. The fish remained until the initiation spawning phase. The remaining fish were weighed for wet weight and measured for standard length.

On approximately Days 34 and 63, the standard length of the fish was measured using digital photographic techniques.

The survivorship of individuals was monitored from Day 6 (transference from egg cup to growth chamber) and from Day 36 (transference from growth chamber to extended growth chamber) and from Day 36 (transference to extended growth chamber) through Day 146 (transference from extended growth chamber to spawning chambers).

Percent survivorship equaled the number of live organism on Day 36 divided by number of fish on Day 7 and the number of organism present on Day 146 divided by number of fish on Day 36.

Adult Reproductive Exposure:

On Day 146, the spawning phase of the study was initiated.

Perforated stainless steel dividers were placed into the extended growth chambers to provide 4 separate spawning areas per tank.

One male and two females were placed into each spawning area, for a total of 4 males and 8 females per spawning tank. A spawning substrate of 4 inch PVC pipe cut in half was placed in each spawning area.

Observations were made daily for adult survivorship and reproductive output of each spawning area (number of eggs present on the spawning substrate). When eggs were present on a substrate, the eggs were removed and counted, and a general observation of their condition was made (number that appear viable, number that appear nonviable and the number that are popped).

On Days 238 and 239, white spots were noted on a few fish in the adult

spawning tanks. The spots were across all treatment groups and warranted treatment for disease Ichthyophthiriasis, also known as Ick.

On Day 240, the adult spawning tanks were treated with Ick Guard (active ingredients (0.20%) triethylene glycol, victoria green, nitromersol, acriflavine; Jungle Laboratories Corporation). Since the observation of and treatment for the disease Ick occurred late in the study, it had minimal impact on the validity of the results. The adult reproductive period ended on Day 254. At the end of the adult reproductive period, all adults were sexed, sacrificed, and measured for standard length and wet weight.

#### Second Generation, F1:

##### Embryo exposure

##### Early life stage

Multiple groups of F1 generation eggs/embryos from spawning tanks were selected and incubated in the embryo incubation cups for either the calculation of the hatching success (referred to as hatch tests) or for conducting F1 early life stage exposures (referred to as F1 early life stage tests). Each group of eggs for either type of trial originated from a single spawning substrate. When possible, the target number of eggs with which to start a test was 35 for the hatch tests and 50 for the early life stage tests.

Embryo viability (percentage of live embryos) and hatchability were determined. When at least 90% of the eggs had hatched or reached the F1 age of 7 days, the hatch tests were terminated (remaining eggs and larvae were discarded), and the early life stage tests entered the growth period.

For the early life stage tests, the number of alevin was reduced to 25 viable individuals per replicate. Alevin were transferred from the egg cups to a growth chamber. Observations of larvae/juvenile fish were made daily.

The number of live individuals was counted at least once per week.

At approximately 28 days after release from the egg cup, the juvenile fish were sacrificed and measured for standard length and wet weight.

##### Feeding:

Feeding began on the day hatching was first observed in the test, control or solvent control chambers. Food was added at least twice per day, except on weekends or holidays when they were fed at least once. Fry were fed brine shrimp nauplii, but ground trout chow or Tetra-Min was supplemented after 7 days. All tanks were fed equal amounts of food depending on the life stage of the organism. The amount of food was increased during the course of the study to compensate for growth. All approximate feeding volumes/masses were recorded.

	<p>Fish were not fed for 24 hours prior to test termination.</p>
Test parameters:	<p>On Day 0, pH, hardness, alkalinity and conductivity were measured in the growth chambers of the control, solvent control, <u>low</u>, middle and high concentrations. The pH, hardness, alkalinity and conductivity were measured in the control, solvent control, low, middle and high concentrations (with eggs or fish present) approximately weekly thereafter.</p> <p>All pH, hardness, alkalinity and conductivity measurements were made with a composite sample from the respective treatment or control group.</p> <p>A data logger with thermocouple was used to monitor temperature hourly in one centrally located test chamber.</p>
Statistic:	<p>The data from each test chamber was used for the statistical analysis since the experimental unit for this study was the replicate test chambers.</p> <p>For each test parameter, a T-test was used to determine if the control and solvent control were statistically different. If no statistical difference was detected (<math>p &gt; 0.05</math>) then the two control groups were pooled. However if statistical difference was detected between the control and solvent control (<math>p &lt; 0.05</math>), then the solvent control group was used in the subsequent analyses.</p> <p>The chi-square test was used to test the assumption of normality in the data, and Bartlett's test was used to test for homogeneity of variance.</p> <p>To determine if the flufenacet treatment groups were significantly different from the control, the data were analyzed by a one-way analysis of variance (ANOVA) followed by the Dunnett's (control groups not pooled) or the Bonferoni test (control groups pooled), or a nonparametric analysis if the assumptions of normality and/or homogeneity of variance were not met.</p> <p>Statistical analyses were conducted using PC based computer programs (TOXSTAT) developed by West, Inc. and Gulley (1994) with conclusions of statistical significance based on a 95 percent confidence level (<math>\alpha = 0.05</math>).</p>

#### Findings:

Analytical data:	<p>Samples of test solutions, including control and solvent control, were taken from alternating replicate test chambers on Day-3, Day 0 and weekly thereafter to measure actual exposure concentrations of flufenacet. On occasion, additional samples were taken to verify flufenacet concentrations if a diluter malfunction occurred. Samples were analyzed on the same day as sampled. The mean measured concentration of flufenacet during the test period ranged from 78-87%</p>
------------------	---

of the nominal concentration. Periodically a diluter malfunction occurred during the conduct of the test.

#### Results:

The results of % hatch and survival of egg/alevin/juvenile are presented in the Table B 2.2.3-1 below.

**Table B. 2.2.3-1: F0. Hatch, survival, growth. Egg/Alevin/Juvenile exposure.**

Mean measured concentration (mg a.s./L)	% Hatch <sup>a</sup>	% Survival <sup>b</sup>	%Survival		Length (mm)	
	Day 6	Day6	Day 36 <sup>c</sup> (Day 7-36)	Day 146 <sup>d</sup> (Day 36-146)	Day 34 <sup>e</sup>	Day 63
Control	96.1	91.3	90±2.31	100	19.6 ±0.74	37.2±0.87
Solvent control	94.7	92.5	86±8.33	98±2.36	20.7 ±0.30	35.7±0.95
Pooled control	-	-	88±6.05	99±1.67	-	36.4±1.15
0.075	97.9	93.1	91±6.00	93±4.71	19.7 ±0.58	37.2±0.55
0.138	98.9	95.6	81±10.0	98±2.36	19.9±1.42	38.2±1.97
0.274	95.6	94.6	84±5.66	95±7.07	19.9±0.69	35.8±0.11
0.600	93.9	90.6	93±2.0	98±2.36	19.5±0.44	35.7±0.33
1.211	94.3	93.8	81 ±3.83	98±2.36	19.6±0.19	31.6±1.36 <sup>f</sup>
NOEC <sub>hatch</sub> = 1.211 mg a.s./L, NOEC <sub>survival</sub> = 1.211 mg a.s./L, NOEC <sub>length</sub> =0.600 mg a.s./L						

±SD Standard deviation

a Mean % hatch on 6 day with viability correction. The % of eggs hatched in the parental generation (F0) was monitored from Day 0 through Day 6. Not statistically significant difference between flufenacet and pooled control were detected on Day 6

Hatching began on Day 2, with some variability across the treatment groups. No dose response relationship was observed on this day.

On Day 3 and Day 4 the highest treatment group (1.211 mg a.s./L) had lower % hatch compared to the other test concentrations, control and solvent control.

By Day 5, this difference was no longer evident. On Day 6 no statistically significant difference compared to pooled control was recorded.

b Mean % survivorship eggs and alevin with viability correction. Not statistically significant difference between flufenacet and pooled control were detected on Day 6

c Mean % survivorship on Day 36-equalled the number of live organism on Day 36 divided by the number of fish on Day 7. No statistically significant differences compared to pooled control was noted.

d % survivorship on Day 146-equalled the number of live organism on Day 146 divided by the number of fish on Day 36. No statistically significant differences compared to pooled control was detected.

e No statistically significant differences compared to solvent control was recorded.

f Statistically significant differences compared to pooled control (p<0.05)

Parental generation, F0Early life stage:

The hatching success was not affected and was > 90 % in all treatments at the end of the hatching period (Day 6).

The mean % survivorship eggs and alevin was for all test concentration above also above 90%.

In general, the majority of the alevin in the control groups and in the flufenacet treatment groups appeared normal throughout the test. The sublethal effect such as lethargic, lordosis, scoliosis were observed in the controls and tested concentrations but these effects were in low proportion to the number of organism in the test and none of these observations appear to be related to the flufenacet treatment.

Juvenile stage

No effects on survival of the juvenile stage were observed between 6-36 days and between 36 and 46 day.

The post hatch survival larvae/juvenile on 36<sup>th</sup> and on 146<sup>th</sup> day for none of the concentrations tested was found to be statistically significant when compared to the pooled control.

Growth in terms of length performed on Day 34 and Day 63 was found to be statistical significantly reduced at the highest test concentration rate at 1.211 mg a.s/L on 63 day.

Adult stage:

Adult survivorship was monitored from the day spawning was initiated (Day 146) through the termination of the study (Day 254).

Due to the fact that on the Day 238 the white spot were noted on some adult fish and on Day 240 the adult spawning tanks was treated with medicine the statistical analysis for survival was performed for two time period:

from 146 to 237 day (the day prior disease) and from Day 146 to 254 Day

No test item related effects on the survival of the adult fish were observed for both time periods.

With regard to growth, a decrease of weight was detected for male at 0.274 mg a.s./L, 0.600 mg a.s./L, 1.211 mg s.a./L and for female at 1.211 mg s.a./L.

No statistical differences between the flufenacet treatments and the pooled control were detected for male and female length.

The results of survival and growth of adults for F0 generation are presented in the Table B 2.2.3-2 below:

**Table B.2.2.3-2: F0-generation – Survival and growth. Adult exposure.**

Test Flufenacet concentration (mg a.s./L)	Survival %		Length at test termination (mm)		Weight (g)	
	Day 237 <sup>b*</sup> (Day 146 <sup>a</sup> -237)	254 <sup>c*</sup> (146-254)	Male*	Female*	Male	Female
Control	100	92±12	57.8±2.03	44.6±0.79	4.89±0.19	2.18±0.08
Solvent control	92±12	79±18	57.8±1.06	46.4±0.62	5.81±0.03	2.42±0.17
Pooled control	96± 6	85±9	57.8±1.32	45.8±1.47	-	-
0.075	96± 6	83±12	58.6±1.50	46.3±1.00	5.07±0.67	2.08±0.06
0.138	75±0	71± 6	57.2±2.36	47.8±1.12	4.67±0.49	2.35±0.09
0.274	96± 6	96.6± 6	57.0±1.06	45.9±0.22	4.24±0.68 <sup>d</sup>	2.02±0.13
0.600	83±12	83±12	52.6±0.88	45.9±0.44	3.93±0.00 <sup>d</sup>	2.00±0.24
1.211	92 ± 0	83±12	54±3.18	45.3±0.35	3.60±0.02 <sup>d</sup>	1.81±0.19 <sup>d</sup>
NOEC <sub>survival</sub> =1.211 mg a.s./L, NOEC <sub>growth</sub> = 0.138 mg a.s./L						

\* No statistically significant differences between the flufenacet and the pooled control

a Day 146 is the day the spawning phase was initiated

b Day 237 is the day prior to the first observation of the signs of disease

c Day 254 is the day the spawning phase was terminated

d Statistically significant different compared to solvent control (p<0.05)

#### Reproduction of adults

(F0-generation)

##### Egg production:

Due to the onset of the Ick disease, egg production data analysis was performed for eggs laid between the Days 148 and 237 (the duration of time between the onset of spawning and the prior to the first observing the symptoms of the Ick) and for eggs laid between Days 148 and 254 (the total duration of the spawning phase). No significant difference in egg production for Days 148 through 237 between the pooled control and the flufenacet treatment groups was detected. However, RMS does not agree with the latter statement. The mean number of eggs for the highest treatment dose was twice as low as in the pooled control. It is then unclear why, at the same time, the egg production for Days 148 through 254 for the highest treatment group (1.211 mg a.s./L) was estimated as significantly

different from the pooled control, at similar differences between the tested concentration and pooled control.

The RMS notes a large difference in egg numbers between replicates A and B, thus it seems that the results obtained for the highest treatment dose may be questionable.

Since the reproductive output trends were similar for the two time periods, egg viability and other reproductive endpoints were examined only for the Days 148 to 237 (i.e., the time period prior to the onset of disease).

Statistically significant differences between the flufenacet treatments and the pooled control were detected for the percent of viable eggs for 1.211 mg a.s./L for Days 148 to 327.

Mean egg production (total eggs) and % of viable eggs are presented in the Table B. 9.2.2.3- below:

**Table B.2.2.3-3: F0 generation. Total Eggs production ( total eggs) and percent of viable eggs.**

Mean measured concentration [mg s.a./L]	Days 148-237	Days 148-254	% viable eggs * 148-237
Control	4205±886	4919±1142	88.2±0
Solvent Control	4606±114	5431±254	87.5±2.8
Pooled control	4405±284	5175±362	87.8±0.8
0.075	4714±1445	5862±1353	87.9±4.3
0.138	4661±354	5812±494	87.8±4.3
0.274	4186±165	4926±93	89.5±1.9
0.600	3773±66	4507±186	83.3±.8
1.211	2084±2446 <sup>b</sup>	2550±2625 <sup>a</sup>	77.2 ±9.0 <sup>a</sup>
NOEC <sub>reproduction</sub> = 0.600 mg a.s./L			

±SD Standard deviation

\* The percent of viable eggs was calculated as the number viable eggs (i.e., those eggs with signs of embryonic development as observed under the microscope) divided by the total number of eggs times 100).

<sup>a</sup> Statistically significant different from the pooled control (p<0.05)

<sup>b</sup> No statistically significant different from the pooled control was indicated in the study.

However in opinion of RMS, due to that fact that there is a high variability between replicates for the concentration of 1.121 (355 of total eggs in replicate A vs 3814 in replicate B), the NOEC reproduction for that days should be 0.600 mg a.s./L.

Egg production was divided into following reproductive parameters: the number eggs laid per spawn, the number of eggs laid per female and the number of spawns per female. No significant difference between the flufenacet treatment groups and the pooled control was observed for the number of eggs laid per female, the number of eggs laid per spawn and the number of spawns per female.

The results of number of eggs per spawn, eggs per female and spawns per female are presented in the Table below:

**Table B.2.2.3-4: Number of Eggs per Spawn, Eggs per Female and Spawns per Female <sup>a</sup>**

Mean measured concentration (mg a.s./L)	Eggs/Spawn	Eggs/Female	Spawns/d/Female
Control	129±4	2102±443	16±3
Solvent control	179±25	2303±57	13±3
Pooled control	154±35	2203±142	14±2
0.075	143±27	2455±584	17±0
0.138	181±20	2671±221	13±2
0.274	170±2	2134±141	12±0
0.600	132±8	2096±20	14±3
1.211	108±53	1056±1243 <sup>b</sup>	8±7 <sup>c</sup>
NOEC= 600 mg a.s./L			

±SD standard deviation

<sup>a</sup> The number of eggs laid per spawn equaled the total number of eggs divided by the number of spawns (the number of days eggs were present on the tiles). The number of eggs per female equaled the sum of the total number of eggs each day divided by the number of females present on that day. The number of spawns per female equaled the sum of number of spawns divided by the number of females present on each day. No statistically significant differences between the flufenacet treatments and the pooled control were detected.

<sup>b</sup> In the opinion of RMS, the NOEC is 0.600 mg a.s./L, different than that proposed by the Applicant (1.211 mg a.s./L) due to that fact that there is a great discrepancy between replicates concerning the parameter eggs/female for the concentration of 1.211.

<sup>c</sup> In the opinion of RMS the NOEC should be 0.600 mg /L instead of 1.211 mg a.s./L proposed by the Applicant. The spawns/female in the highest concentration clearly decreased. However, this effect was not confirmed by the statistical analysis. A high variability between replicates was observed.

F1 generation

The % hatch of the F1 larvae was relatively high and was >90%.

On incubation Day 7, the highest test level of 1.211 mg a.s./L had significantly reduced hatching success compared to the pooled control.

The post hatch survival on Days 7 and Day 35 was not negatively affected.

The growth in terms of lengths and weight at the highest test level

(1.211 mg a.s./L) was significantly different from the pooled control.

**Table B. 2.2.3-5. F1 – generation, Hatch/ Survival eggs and alevin and growth.**

Mean measured concentration (mg a.s./L)	% Hatch <sup>b</sup>	% Survival <sup>c</sup>	% Survival <sup>c</sup>	Length <sup>f</sup> (mm)	Dry weight <sup>f</sup> (g)
Incubation Day <sup>a</sup>	Day 7	Day 7	Day 35	Day 35	Day 35
Control	98.1±3.3	95±7.5	93±7	22.8±1.4	0.22±0.026
Solvent control	94.0±9.3	89.3±10.3	81±17	22.1±1.3	0.21±0.028
Pooled control	96.0±4.1	92.2±2.0	87±8	22.5±0.5	0.217±0.0034
0.075	91.0±18.5	86.1±18.6	90±14	22.3±1.3	0.215±0.0386
0.138	93.1±9.8	91.7±9.5	93±7	22.7±1.3	0.214±0.0297
0.274	97.5±3.2	94.9±6.5	9±73	22.1±0.9	0.20±0.0233
0.600	91.4±13.3	89.5±13	91±7	21.9±0.7	0.20±0.0247
1.211	77.4±24.1 *	75.1±24.7 <sup>d</sup>	87±11	20.3 ±0.7*	0.16 ±0.02*
NOEC <sub>post hatch survival F1</sub> =0.600 mg a.s./L				NOEC <sub>growth F1</sub> =0.600 mg a.s./L	

\* Statistically significantly different from the pooled control (p<0.05).

a The incubation day is equal to the number of days after which the eggs were placed in the incubation cup.

b Mean % hatch for incubation Day 7. The % of hatch for F1 generation was determined between Day 2 and Day 7. The hatch began on incubation Day 2 and monitored when at least 90% of the eggs had hatched ( Day7) or reached the F1 age of 7 days. Statistically significant reduction was observed only for the highest treatment group 1.211 mg a.s./L.

c Mean % survivorship eggs and alevin on incubation Day 7. The survivorship was monitored from incubation Day 0 (egg) through incubation Day 7 (alevin). The % survivorship equaled the number of live organism divided by the number of eggs placed in incubation cup.

d On incubation Day7 even though that not statistically significant different from the pooled control were indicated, the decrease in survivorship may be biologically relevant.

e Mean % survivorship of F1 generation in early life stage. % survivorship id the number present on incubation day 35/number present on day transferred from egg cup times 100.

f Length and dry weight of F1 individuals were taken 35 days of incubation in the early life stage tests.

Summary of results are presented in the Table B. 2.2.3-6.

**Table B. 2.2.3-6. Flufenacet Fish Life Cycle Test Endpoints**

Parameter	NOEC (mg a.s./L)	LOEC (mg a.s./L)	MATC (mg a.s./L)
FO Percent Hatch	1.211	>1.211	>1.211
FO Egg and Alevin Survivorship	1.211	>1.211	>1.211
FO Day 36 Survivorship	1.211	1.211	>1.211
FO Day 146 Survivorship	1.211	1.211	>1.211
FO Adult Survivorship on Day 237	1.211	1.211	>1.211
FO Adult Survivorship on Day 254	1.211	>1.211	>1.211
FO Day 36 Length	1.211	>1.211	>1.211
FO Day 63 Length	0.600	1.211	0.852
FO Adult Female Length	1.211	>1.211	>1.211
FO Adult Female Weight	0.600	1.211	0.852
FO Adult Male Length	1.211	>1.211	>1.211
FO Adult Male Weight	0.138	0.274	0.194
Egg Production between Days 148 and 237	1.211	>1.211	>1.211
Egg Production between Days 148 and 254	0.600	1.211	0.852
Egg Viability between Days 148 and 237	0.600	1.211	0.852
Number of Eggs per Female	1.211	>1.211	>1.211
Number of Eggs per Spawn	0.600	1.211	0.852
Number of Spawns per Female	0.600	1.211	0.852
F1 Percent Hatch	0.600	1.211	0.852
F1 Egg and Alevin Survivorship	0.600	1.211	0.852
F1 Incubation Day 35 Survivorship	1.211	>1.211	>1.211
F1 Length	0.600	1.211	0.852
F1 Weight	0.600	1.211	0.852

Conclusion:

Based on the data derived from the study, the growth in terms of length of parental fish larvae (F0-parental generation), the growth in terms of weight of the parental adult fish (F0 - parental generation) and for F1 (filiis-generation) was found to be the most sensitive endpoints.

The hatching success and post hatch survival of the fish was not affected in the F0-generation.

The hatching success in the F1-generation and post hatch survival of the fish was affected but these parameters were less sensitive. The parameter reproduction as egg production was affected by the exposure to the test item at level 0.600 mg s.a./L.

Based on the most sensitive parameter growth in terms of weight of parental adult fish (male, F0) the overall NOEC was 0.138 mg a.s./L and LOEC=0.274 mg a.s./L based on mean measured concentrations.

In accordance with the new data requirement (Commission Regulation EU No 283/2013), the EC<sub>10</sub>, EC<sub>20</sub>, values should be calculated. Where they can not be estimated, an explanation shall be provided.

**Comments RMS:**

The study was conducted according to US EPA/FIFRA guideline 72-4 A.

No validity criteria were performed in the US/FIFRA guideline 72-4 for full life stage test. The RMS verified the validity of this study taken into consideration the OECD test guideline 210 (1992, 2013) for early life stage test. According to recommendation given the test guideline OECD 210 (2013) the following validity criteria should be met:

- Dissolved oxygen concentration must be between 60 and 100% of the air saturation value throughout the test.

The mean dissolved oxygen (DO) concentration ranged from 75% to 87% during the all study.

However, the dissolved oxygen concentration in some occasion was below 60% of air saturation but recovered either the same day the following days. No effects on fish due to low oxygen levels were observed throughout the test.

- The water temperature must not differ by more than  $\pm 1.5^{\circ}\text{C}$  between test chambers or between successive days at any time during the test, and should be within the temperature ranges specified for the test species ( temperature should be at range  $25 \pm 2^{\circ}\text{C}$  according to OECD 210, 1992 and at range  $25 \pm 1.5^{\circ}\text{C}$  according to OECD 2010, 2013).

The temperature range of the study did not vary by more than  $25 \pm 2^{\circ}\text{C}$ . The max temperature was  $26.7^{\circ}\text{C}$ .

- Evidence must be available to demonstrate that the concentrations of the test substance in solution have been satisfactorily maintained within  $\pm 20\%$  of the mean measured values.

The mean measured concentration of flufenacet during the test period ranged from 78-87% of the nominal concentration. Some deviations of the concentration of flufenacet were caused by a diluter malfunction occurred during the conduct of the test and has no impact of the validity criteria.

- Overall survival of fertilized eggs in the controls must be greater than or equal to the given limits (hatching success at least 70% and post hatch success 75%)

For ELS (F0 and F1 generation) hatching success was  $> 90\%$  in the controls.

The following deviation was noted from the study protocol:

- There was no information of the sex ratio in the study protocol, however it was declared by the study director that the sexes of male and female was done.

In opinion of RMS the study is acceptable.

**Agreed endpoints:**

Parameter	NOEC (mg a.s./L)	LOEC (mg a.s./L)	MATC (mg a.s./L)
FO Percent Hatch	1.211	>1.211	>1.211
FO Egg and Alevin Survivorship	1.211	>1.211	>1.211
FO Day 36 Survivorship	1.211	1.211	>1.211
FO Day 146 Survivorship	1.211	1.211	>1.211
FO Adult Survivorship on Day 237	1.211	1.211	>1.211
FO Adult Survivorship on Day 254	1.211	>1.211	>1.211
FO Day 36 Length	1.211	>1.211	>1.211
FO Day 63 Length	0.600	1.211	0.852
FO Adult Female Length	1.211	>1.211	>1.211
FO Adult Female Weight	0.600	1.211	0.852
FO Adult Male Length	1.211	>1.211	>1.211
FO Adult Male Weight	0.138	0.274	0.194
Egg Production between Days 148 and 237	1.211	>1.211	>1.211
Egg Production between Days 148 and 254	0.600	1.211	0.852
Egg Viability between Days 148 and 237	0.600	1.211	0.852
Number of Eggs per Female	0.600	1.211	0.852
Number of Eggs per Spawn	0.600	1.211	0.852
Number of Spawns per Female	0.600	0.600	0.600
FI Percent Hatch	0.600	1.211	0.852
FI Egg and Alevin Survivorship	0.600	1.211	0.852
FI Incubation Day 35 Survivorship	1.211	>1.211	>1.211
FI Length	0.600	1.211	0.852
FI Weight	0.600	1.211	0.852

Based on the most sensitive parameter growth in terms of weight of parental adult fish (male, F0) the overall NOEC was 0.138 mg a.s./L and LOEC was 0.274 mg a.s./L based on mean measured concentrations.

**B.9.2.2.4. Potential for endocrine disruption**

Population relevant effects of Flufenacet on fish were studied in an early life-stage test (ELS) with rainbow trout and Sheepshead minnow. The fish full life cycle test (FFLC) with fathead minnow (*P. promelas*) was also conducted.

The lowest NOEC of 49 µg a.s./L based on the effects length and dry weight was estimated for Sheepshead minnow. In the ELS test for rainbow trout the NOEC of 334 µg/L was estimated based on swim up and dry weight on 97 d.

In the FFLC after 279 days flow-through exposure, a NOEC of 138 µg a.s./L was obtained for effects on F0 adult weight (but not on male length, nor on female weight or length). For all other endpoints, such as survival, reproduction and growth (other than male weight) higher NOECs of either 600 or 1211 µg a.s./L were established.

Member states should note that there are currently no defined criteria for indentifining endocrine disruptors or interpreting the significance of any effects in ecotoxicology studies under the Commission Regulation (EU) No. 2009/1107. Therefore, it is today not possible to conclude that endocrine disruptive effects are/are not taking place.

**B.9.2.3. Bioconcentration in fish**

As the log P<sub>OW</sub> of the active substance is above the trigger (log Pow > 3), the determination of the bioconcentration potential in fish is needed. The bioaccumulation study submitted for the first EU approval of the active substance was re-evaluated and summarized by the RMS in the RAR.

The study is divided in two sections and reported in two reports. The first report of [REDACTED] (1994) describes the in-life phase and the determination of the steady-state BCF on basis of radioactivity measurements. The second report of [REDACTED] (1994) describes the nature of residues (metabolite's identification) in the fish following uptake from of radiolabelled flufenacet from the fish water.

**B.9.2.3.1. Uptake, depuration and bioconcentration of 14C-FOE 5043 Technical by Bluegill (*Lepomis macrochirus*) under flow-through condition.**

<b>Reference:</b>	Uptake, depuration and bioconcentration of 14C-FOE 5043 Technical by Bluegill ( <i>Lepomis macrochirus</i> ) under flow-through condition.
<b>Author(s), year:</b>	██████████ 1994
<b>Report/Doc. number:</b>	Study No: 106760, Reference No: BCS No: M-003803-01-1
<b>Guideline(s):</b>	US-EPA/FIFRA 72-6 test guideline.
<b>GLP:</b>	Yes

<u>Test substance:</u>	<sup>4</sup> CFOE 5043 (radiolabelled flufenacet) , Batch No: No. C-584; Purity: 98.9% s.a., Activity = 66.5 m Ci/mmol FOE 5043 (tech.), Batch No: FL -036: Purity: 96.8%
Solvent:	Acetone
<i>Test species:</i>	Bluegill ( <i>Lepomis macrochirus</i> )
Size of fish:	The bioconcentration study and the first metabolism phase Fish length: 16-21 mm (mean, 19.2 ± 1.5 mm), wet weight: 0.084-0.233 g wet weight (mean, 0.17 ± 0.05 g) The second metabolism phase (larger fish) Fish length: 100 - 150 mm
Holding of fish:	<u>Environmental condition:</u> Water quality (soft blended water): Hardness 44 mg/l as CaCO <sub>3</sub> , alkalinity: 37 mg/l as CaCO <sub>3</sub> , total carbon <1 mg/l, pH = 7.9 Temperature: 22 ± 2 C Conductivity: 129 µmhos/cm Light regime: 16-hour daylight photoperiod <u>Acclimatization of fish</u> : 1 month prior to testing (the bioconcentration study and the first metabolism phase) and acclimatization to the actual test system for 48 hours prior to test commencement. The antibiotic treatment ended 10 days prior to the study. During the 48-hour period immediately prior to initiation of the 28-day exposure less than 1% mortality was noted. The second metabolism phase (with larger fish): 4 days prior to the initiation of the second metabolism phase. During the 4-day period, immediately prior to initiation of the second metabolism phase no fish died during this period.

---

Feeding of fish:	Newly hatched brine shrimp and/or a commercial fish food.
Loading:	Biomass loading in the treatment chambers ranged from 0.057 grams fish per liter of test solution passing through the test chambers every 24 hours (g/L/d) during the uptake phase to 0.024 g/L/d during the depuration phase. Control biomass loading was 0.06 g/L/d.
Type of test:	Flow-through
<u>Applied concentrations:</u>	
Nominal:	100 µg <sup>14</sup> C FOE 504/ L Control (0) and Solvent control (0)
<u>Test conditions:</u>	
Water quality:	Dilution water (spring blended water)
Temperature:	20.8 to 22.8°C  Due to a data logger malfunction, hourly temperatures were not recorded for 9 hours on Day 11, 24 hours on Day 12, 23 hours on Day 13, 20 hours on Day 17 and 4 hours on Day 18 for a total of 104 out of 1043 study hours. All of the recorded 939 hourly temperatures, as well as weekly manual temperatures were within the specified range of 22±C.
pH:	7.1-7.5
O <sub>2</sub> content:	6.3-9.0 mg/l corresponding to 72 and 103% saturation. The test chamber was not aerated during the study.  Throughout the study the dissolved oxygen was > 60%.
Light regime:	16 hours light /8 hours dark. Light intensity ranged 50-80 foot-candles corresponding to 538 and 862 Lux.
Feeding:	Fish were fed a commercial fish food (trout chow and/or Tetra-Min™) during the test.
<u>Methods:</u>	Bluegill ( <i>Lepomis macrochirus</i> ) were exposed to <sup>14</sup> C FOE 5043 under one test concentration of 100 µg/L (nominal) conducted under flow-through conditions.  The bioconcentration study consisted of the following phases:  The first phase—uptake phase was designed to determine the bioconcentration potential of <sup>14</sup> C-FOE 5043 by bluegill.  The second phase—deputation phase was designed to determine the depuration potential of the test compound by bluegill.  In addition, a group of fish was exposed to <sup>14</sup> C-FOE 5043 for use in a metabolite characterization study.

The metabolites identification was performed in the separate report (Leimkuehler and Moore, 1994).

The metabolism exposure was divided into two phases.

The first metabolism phase was conducted using smaller fish (length: 19-21 mm) on the same size as fish same in bioconcentration study.

The second metabolism phase was conducting using larger fish (length 100-150 mm).

The uptake portion of bioconcentration and first metabolism phase (with smaller fish) of study was 28 days in duration.

Following exposure, the fish from bioconcentration chamber were transferred to clean flowing water for 15-day elimination periods.

The second metabolism phase of the study (with larger fish) consisted of an uptake only phase and was 14 days in duration.

Study design:

A total of five aquaria (100 l) were used in the study.

One aquarium holding 150 fish was used for the bioconcentration portion of the study and one aquarium with 150 fish was used for the solvent control.

Three aquaria holding 150 fish each were used for the first metabolism phase of the study.

Two aquaria holding 6 fish each were used for the second metabolism phase of the study.

The uptake phase of the bioconcentration and first metabolism phase of the study was initiated after the test solutions had been flowing through the aquaria for approximately 67 hours. Bluegill in an amount 150 was distributed to each test chambers. On each sampling date, six fish each from the solvent control and one exposure chamber were sampled during the uptake period for tissue analysis.

Fish were sampled on Day 0 approximately 3 hours after study initiation, and on Day 1, 3, 7, 14, and 28. Three fish were analyzed on a whole body basis and three fish were dissected into edible and non-edible portions for analysis. Fillet (edible) and viscera (non-edible) portions were sectioned as follows: fillet portions consisted of muscle tissue with skin, scales and associated skeletal structures; viscera portions consisted of head, all fins, and all internal organs.

The depuration phase of the study was initiated with the fish remaining in the bioconcentration chamber after the Day 28 uptake phase.

Fish and water samples were collected. During the depuration phase six fish each from the bioconcentration chamber and solvent control chamber were sampled

on Day 1, 3, 7, 10 and 14.

Due to the loss of Day 14 sample data caused by a malfunction of the oxidation instrument on Day 15 fish were sampled.

Fish tissues were dissected and analyzed in the same manner as during the uptake phase. On Day 21, part of fish from the metabolism exposure chambers were sampled and the remaining metabolism fish were sampled on Day 28. The fish exposed for the metabolite study were dissected, processed and analyzed for metabolite characterization by LSC and HPLC analysis

The results of identification of residues in bluegill sunfish exposed to  $^{14}\text{C}$ -FOE5043 from first metabolism phases were presented in separate report (Leimkuehler and Moore, 1994).

The second metabolism phase of the study was initiated in the same test chambers immediately after the first metabolism phase was completed,

The first metabolism phase was completed after all remaining fish were removed from the test chambers on Day 28 of the uptake period.

Bluegills were impartially distributed to two of the metabolism test chambers until each chamber contained 6 fish. These fish were exposed to the test compound for a 14-day period: half of the fish were sampled after 7 days and the other half after 14 days. At the end of the exposure period the fish were collected for metabolite analysis by LSC and HPLC methods.

Test parameters:

Temperature, dissolved oxygen and pH were measured in all of the test chambers and control chamber on Day 0, 7, 14, 21 and 28 during the uptake phase and on Day 1, 3, 7, 10 and 14 during the depuration phase. Temperature was measured on these days using a mercury thermometer while an ECD Model 50 data logger documented hourly temperatures from a centrally located test chamber.

Analytical data:

Approximately 24 hours prior to test initiation, duplicate water samples were collected from all test chambers, including the solvent control and radioanalyzed for FOE 5043 to verify proper diluter system function. Triplicate water samples were collected from all test chambers, including the solvent control, on Day 0, 1, 3, 7, 14, 21 and 28 during the uptake phase for determination of actual concentration of FOE 5043. Single water samples were collected from all test chambers, including the control, on Day 1, 3, 7, 10 and 14 during the depuration phase and during the 2 and metabolism study for determination of actual concentration of FOE 5043. On each sampling day, water samples were collected for radio analysis from each test chamber. Water samples for percent parent

determination of  $^{14}\text{C}$ -FOE 5043 were collected from all test chambers, except the control, on Day 0, 7, 14, 16, 21 and 28 of the uptake phase. Samples were analysed quantitatively for the content of radioactivity, by LSC. The further analysis at the nature of radioactivity in water – identification and quantitation of the individual compounds, was performed by means of chromatographic analysis-TLC or HPLC techniques. The concentration of  $^{14}\text{C}$  residues in fish tissues for bioconcentration analysis was determined by combustion to  $^{14}\text{CO}_2$  and followed by quantification by LSC.

**Biological data analysis:**

The steady-state BCF was determined by averaging the BCFs calculated during the plateau (steady-state) period. The plateau period was defined as the period when the BCFs from each sampling period were not significantly ( $\alpha = 0.05$ ) different when tested by analysis of variance. The uptake rate constant ( $K_1$ ) and depuration rate constant ( $K_2$ ) were determined by the Dow BIOFAC computer program. BIOFAC, a nonlinear kinetic modeling program, provided optimal parameter estimates of the rate constants by utilizing the actual bioconcentration study data.

The bioconcentration factor at steady-state, the time to reach 90 percent of the steady-state body burden, and the time to reach 50 percent of test material depuration were also calculated from the estimated rate constants. A measure of the variability of the estimated parameters was provided by the standard deviation of each estimate.

**Results:**

Mortality during 28 day exposure was 1.3% in the solvent control and less than 1 percent in all chambers. No behavior or sublethal effects were observed. During the uptake phase in the bioconcentration and in the first phase of metabolism test chambers the mean measured concentration of total  $\text{C}^{14}$ -FOE5043 ranged from 98% to 106% of nominal. During the second metabolism phase the mean measured concentration of  $\text{C}^{14}$ -FOE5043 ranged from 76 to 113 % of nominal. The solvent control water samples analyzed during the exposure and depuration phases were all no-detectable. In the depuration phase in the bioconcentration chamber the range of measured concentration of  $\text{C}^{14}$ -FOE5043 was from  $<0.006$  to  $0.90 \mu\text{g/l}$ . Results of total  $^{14}\text{C}$  radioactivity measured in the bioconcentration test chamber test water and fish tissue during 28 uptake phase and 15 day depuration phase with Bluegill was presented in the B.9.2.3.1-1.

**Table B.9.2.3.1-1: Total <sup>14</sup>C radioactivity measured in the bioconcentration test chamber test water and fish tissue during 28 uptake phase and 15 day depuration phase with Bluegil.**

Day	Water	Filet		Viscera		Whole body	
	Mean measured µg/L*/**	µg/kg	BCF	µg/kg	BCF	µg/kg	BCF
Uptake phase							
0	99.6	833	8.4	5899	59.2	3315	33.3
1	99.5	1269	12.8	11078	111	4827	48.5
3	101	1199	11.9	10846	107	9900	98.0
7	101	1871	18.5	9629	95.3	6905	68.4
14	100	2213	22.1	9531	95.3	7057	71.3
21	101	1708	16.9	9345	92.5	7455	73.8
28	100	1600	16.0	10301	103	6173	61.7
Depuration phase							
1	0.90	349	-	665	-	427	-
3	0.30	270	-	397	-	289	-
7	<0.006	169	-	311	-	235	-
10	0.05	156	-	225	-	157	-
15	0.03	84	-	227	-	147	-

\* Mean measured water concentration up to and including the respective sampling day for uptake phase

\*\* Mean measured water actual concentration for depuration phase

Depuration of total <sup>14</sup>C as <sup>14</sup>C-FOE 5043 during the 15-day clearance period is summarized in Table B.9.2.3.1-2.

**Table B.9.2.3.1-2: Depuration<sup>1</sup> of total <sup>14</sup>C as <sup>14</sup>C-FOE 5043 during the 15-day clearance period in bioconcentration test chamber.**

Depuration day	Fillet		Viscera		Whole fish	
	Depuration concentration µg/kg	% depuration	Depuration concentration µg/kg	% depuration	Depuration concentration µg/kg	% depuration
1	349	79	665	94	427	94
3	270	84	397	97	289	96
7	169	90	311	97	235	97
10	156	91	225	98	157	98
15	84	95	227	98	147	98

<sup>1</sup> Depuration concentrations are expressed as a percentage of the day 28 <sup>14</sup>C-FOE5043 concentrations of 1600 µg/l for fillet, 10301 µg/l for whole fish and 6173 µg/l for viscera.

The bioconcentration of total  $^{14}\text{C}$  residues after 28 exposures in tissue types were 6173  $\mu\text{g/kg}$  3811  $\mu\text{g/kg}$ , 10301  $\mu\text{g/kg}$  for whole fish, filet and viscera, respectively.

The bioconcentration factor associated with these body burdens were 61.7, 38.1 and 103 for whole body, fillet and viscera, respectively.

Mean daily bioconcentration factors during the uptake phase range from 33.3 to 98 for whole body, 8.4-38.1 for fillet and 59.2 to 111 for viscera.

Based on the analysis of variance for the data the whole body tissue residues appeared to reach a plateau after 7 days of exposure to FOE5043.

The steady-state bioconcentration factor defined as the average of the BCFs calculated for whole body during the plateau period was 68.

Tissue residues decreased steadily during depuration phase. By day 15 of the depuration period the tissue residues showed 95% elimination in filet and 98% in viscera and whole body.

The study steady-state BCKF value, estimated time to reach 90% steady state of bioconcentration factor, and time to reach 50% clearance were calculated by BIOFAC model and are shown in the Table B.9.2.3.1-3.

**Table B.9.2.3.1-3. Summary of bioconcentration potential for flufenacet to bluegill using non linear regression BIOFAC.**

Tissue	$K_1$	$K_2$	Kinetic Steady state BCFK	Estimated time to reach 90% steady state (days)	Time to reach 50% clearance (days)
Whole body	$2.3 \pm 0.3$	$165 \pm 23$	$71.4 \pm 1$	$0.99 \pm 0.13$	$0.30 \pm 0.04$

Conclusion:

The FOE 5043 concentrations (based on radioactivity) increased rapidly with a calculated time of 0.99 days to reach 90% of the steady-state level. The steady-state bioconcentration factor was calculated as 71.4 for the exposure level. During the depuration phase, residues in fish declined with a half-time for clearance of 0.3 days.

**RMS comments:**

The study is in general agreement with the current valid test guidelines, e.g. US EPA (OPPTS 850.1730, April 1996) and OECD 305 (July 2012).

The following validity criteria given in the test guideline OECD 305 (July 2012) should be met:

- The concentration of dissolved oxygen should not fall below 60% saturation

In this study the dissolved oxygen ranged between 72 and 103% of saturation in the bioconcentration study and metabolism test.

- The concentration of the test substance in the chambers is maintained within 20 percent of the mean of measured values during the uptake phase

During the uptake phase in the bioconcentration and in the first phase of metabolism test chambers the mean measured concentration of total C<sup>14</sup>-FOE 5043 ranged from 98% to 106% of nominal.

During the second metabolism phase the mean measured concentration of C<sup>14</sup>-FOE 5043 ranged from 76 to 113 % of nominal.

- The concentration of the test substance should be below its limit of solubility in water, taking into account the effect that the test water may have on effective solubility.

The concentration tested (0.1 mg a.s./L ) was below the limit of solubility in the water the active substance (56 mg a.s./L water solubility is estimated for flufenacet)

- The mortality or other adverse effects/disease in both control and treated fish is less than 10% at the end of the test; where the test is extended over several weeks or months, death or other adverse effects in both sets of fish should be less than 5% per month and not exceed 30% in all. Significant differences in average growth between the test and the control groups of sampled fish could be an indication of a toxic effect of the test chemical.

Mortality during 28 day exposure was <10% (being 1.3% in the solvent control and less than 1 percent in all chambers) and no behavior or sub lethal effects were observed.

-The water temperature variation should be than  $\pm 2^{\circ}\text{C}$ , because large deviations can affect biological parameters relevant for uptake and depuration as well as cause stress to animals.

The water temperature ranged between 20.8 and 22.8°C.

It should be noted that, the temperature was not recorded hourly on Days 11, 12, 13, 16, 17, and 18 (104 hours total) due to a data logger malfunction. Since, the temperature records from the remaining 939 hours of the study were within the specified range of  $22 \pm 2^{\circ}\text{C}$  according to the guideline OECD 305 (1996) this deviation did not appear to adversely affect of the study.

In addition the following deviations were noted :

The protocol stated that fish tissue should be sampled on Day 1, 3, 7, 10 and 14 during the depuration phase.

Fish were sampled on Day 15 of the depuration phase due to the loss of Day 14 sample data caused by a malfunction of the oxidation instrument.

-Water quality parameters (dissolved oxygen, pH, and temperature) were not measured on Day 1 and Day 3 of the uptake phase. Since the water quality throughout the study (Day 0, 7, 14, 21) was consistent, it did not appear that this deviation affected results of the study.

-No lipid content performed

- Only one concentration was tested (two concentrations are recommended by OECD 305 test guideline).

- The mean length of fish was 1.92 cm ranged between 1.6 and 2 cm, (preferred length of  $3.0 \pm 1$  cm is recommended by US EPA test guideline and preferred length of  $5 \pm 1$  cm is recommended in OECD 305 test guideline).

It should be pointed out that no lipid content measurement and smaller fish used than was recommended by the current guideline OECD 305 may increase the uncertainty associated with the estimation of the BCF.

However, taking into account that TERs are **comfortably** above the triggers for fish-eating birds and mammals and not close to the trigger for B in the PBT assessment, RMS would recommend not repeating the study.

Therefore, the study is considered acceptable.

**Agreed endpoints:**

Steady-state has been achieved after 7 days of exposure.

whole fish BCF (for uptake and depuration rate constant) = 71.4

CT<sub>50</sub> (whole fish) = 0.3 days

CT<sub>90</sub> (whole fish) = 0.99 days

**B.9.2.3.2. Identification of radioactive residues of phenyl-14 C FOE5043 in Bluegil**

<b>Reference:</b>	Indentification of radioactive residues of phenyl- ( <sup>14</sup> C) FOE 5043 in Bluegil Sunfish ( <i>Lepomis macrochirus</i> ).
<b>Author(s), year:</b>	██████████ 1994
<b>Report/Doc. Number:</b>	Study No:106577, Reference no: M-003804-01-1,
<b>Guideline(s):</b>	US EPA/FIFRA 165-4 test guideline.
<b>GLP:</b>	Yes

The description of the test chemical, test system and procedures are described in the main study (██████████ 1994):

Executive summary of the study (██████████, 1994):

For the first metabolism phase three 100-L glass aquaria holding initially 150 smaller fish each (body length approx. 19 mm, body weight approx. 0.17 g) were used. The fish were kept in flow-through condition for a total uptake period of 28 days and a subsequent depuration period of 14 days (two aquaria with the test substance, one control aquarium without test substance). During the uptake period radiolabelled flufenacet was added to the inflowing water to reach a concentration in the fish water of approx. 100 µg/L. During the depuration period pure water with no test substance was introduced.

Following complete removal of the smaller fish six larger fish (body length approx. 10 – 15 cm) were inserted in each of the two aquaria and exposed to radiolabelled flufenacet in the same way as done with the smaller fish.

The smaller fish of the BCF trial were sampled after different exposure periods, i.e. 0, 1, 3, 7, 14, 21, and 28 days. They were directly radioassayed (following cutting in suitable pieces) or first dissected into fillet (edible) and viscera (non-edible tissue). The fillet and viscera samples were also radioassayed to determine the total radioactive residues (TRR) in the whole body, fillet and viscera.

Fillet and viscera samples of collection days 21 and 28 were extracted with methanol and a mixture of methanol and 0.1N hydrochloric acid at room temperature. The methanol extract was partitioned against hexane. The hexane solution was discarded. The methanol fraction was concentrated, centrifuged and analyzed by radio-HPLC. The larger fish used in second metabolism phase, were collected after a 7 (first aquarium) and 14-day exposure (second aquarium). These fish were dissected and their bladders were carefully removed, punctured and drained. The removed urine was centrifuged and analyzed by radio-HPLC.

The total radioactive residue (TRR) in fish tissue amounted to 833 - 2213 µg equ/kg in edible fillet, to 5899 - 10846 µg equ/kg in non-edible viscera and to 3315 - 9900 µg equ/kg in whole fish. Comparing the residue levels in fish tissue and fish water resulted in daily bioconcentration factors (BCF values) of 8.4 – 22.1 for fillet, 59.2 – 111 for viscera and 33.3 – 98.0 for the whole body. The plateau levels (steady-state levels) were already reached after approximately 7 days of exposure. The mean steady-state BCF for the whole body was determined to 68 (mean BCF

of the last four sampling dates 7, 14, 21 and 28 days of uptake) or 71.4 when calculated using the BIOFAC model operating on the basis of an uptake and depuration rate constant.

The composition of the radioactive residues obtained from the first and the second metabolism phase performed by [REDACTED] 1994.

The composition of the radioactive residues in viscera and fillet of bluegill sunfish following 21 and 28-day exposure of radiolabelled flufenacet ( including the metabolite from the first and second metabolism phase) are presented in Table 9.2.3-1 and Table 6.2.3- 2.

The structures of the metabolites were derived from their mass spectra and by comparison of the retention behavior in reversed phase HPLC. The composition of residues in viscera and fillet was almost identical during the 21 and 28-day exposure indicating a steady state metabolism. The major metabolites in non-edible viscera were identified as FOE cysteine conjugate (FACS, M23) amounting to approximately 50% of TRR and its acetylated derivative FOE acetyl cysteine (FANACS, “mercapturic acid”, M10) amounting to approximately 24% of TRR. Other four minor metabolites(<10% of TRR) were also identified. The parent substance flufenacet was observed at a low level of approximately 5% of TRR.

The major metabolites in edible fillet proved to be also FOE cysteine conjugate (FACS, M23) amounting to approximately 37% of TRR and FOE acetyl cysteine (FANACS, M10) amounting to approximately 16% of TRR. Eight unknown minor metabolites could be characterized according to their polarity (retention behavior in reversed phase liquid chromatography). The parent compound flufenacet contributed significantly to pattern of residues accounting for 18% of TRR.

**Table B 2.3.2-1: Radioactive residues in viscera of bluegill sunfish following 21 and 28-day exposure of [fluorophenyl-UL-14C]flufenacet at a concentration of 100 µg/L fish water in a flow-through study.**

Exposure period	21 Days		28 Days	
TRR [mg equ/kg] (after combustion)	10.99		10.22	
Metabolite detected by radio-HPLC	% of TRR	[mg equ/kg]	[% of TRR] [mg equ/kg]	[mg equ/kg]
FOE isopropyl hydroxy cysteine (FAIOCS) and FOE oxalate (FOE OX, M1)	2.6	0.258	1.8	0.191
FOE cysteine sulfoxide conjugate (FACSO, M39)	3.3	0.327	1.1	0.114
FOE S-oxo-acetylcysteine (FANACSO, M12)	4.4	0.444	0.9	0.097

Exposure period	21 Days		28 Days	
FOE glutathionate (FOE GSH, M22)	3.5	0.335	2.3	0.239
FOE cysteine conjugate (FACS, M23)	46.9	4.694	54.8	5.719
FOE acetyl cysteine (FANACS, M10)	24.0	2.408	23.3	2.431
FOE isopropanol glucuronide (FOE GLU)	5.2	0.520	4.5	0.467
Flufenacet (FOE 5043, parent substance)	3.8	0.381	5.7	0.599
Unextracted	6.3	0.631	5.6	0.586
Total	100	10.018	100	10.443

**Table B. 2.3.2-2: Radioactive residues in the fillet of bluegill sunfish following 21 and 28-day exposure of [fluorophenyl-UL-14C]flufenacet at a concentration of 100 µg/L fish water in a flow-through study.**

Exposure period	21 Days		28 Days	
TRR [mg equ/kg] (after combustion)	10.99		10.22	
Metabolite detected by radio-HPLC	% of TRR	[mg equ/kg]	[% of TRR] [mg equ/kg]	[mg equ/kg]
Unknown 1	1.3	0.026	1.5	0.026
Unknown 2	1.4	0.027	2.1	0.036
Unknown 3	1.2	0.022	2.4	0.041
Unknown 4	2.1	0.039	3.0	0.052
FOE cysteine conjugate (FACS, M23)	36.2	0.692	37.3	0.631
FOE acetyl cysteine (FANACS, M10)	17.1	0.326	15.3	0.260
Unknown 5	2.0	0.038	1.9	0.032
Unknown 6	1.4	0.027	1.6	0.027
Unknown 7	1.6	0.030	0.7	0.001
Unknown 8	1.6	0.030	0.8	0.014
Flufenacet (FOE 5043, parent substance)	18.1	0.345	17.6	0.297
	16.0	0.308	15.8	0.268

Exposure period	21 Days		28 Days	
Unextracted				
Total identified	69.3	1.326	68.4	1.159
	100	1.910	100	1.696

**Conclusion:**

The metabolism of [fluorophenyl-UL-14C]flufenacet was investigated in bluegill sunfish after 21 and 28-day exposure in the fish water a concentration of approx.100 µg/L. The TRR levels in the fillet and viscera were essentially the same for both exposure periods amounting to approx. 1.7 (fillet) and 11 (viscera) mg equ/kg. The pattern of metabolites was also nearly identical at both periods. This indicates that residues and the metabolism had reached a steady state.

A total of nine metabolites were identified, four of these were greater than **5%** of TRR in the respective tissue.

The data indicate that the primary metabolic pathway starts with a glutathionate conjugation of the isopropyl acetanilide moiety (M22) of the parent molecule followed by subsequent formation of FOE cysteine (M23) and its acetylated derivative, the mercapturic acid or FOE acetyl cysteine (M10). A minor metabolic pathway in fish is the hydroxylation of the isopropyl group followed by conjugation with glucuronic acid.

**RMS comments:**

The study was accepted by RMS-FRANCE during the first approval of flufenacet.

The aim of this study was indicated the composition of the radioactive residues in viscera and fillet of bluegill sunfish following 21 and 28-day exposure of radiolabelled flufenacet (including the metabolite from the first and second metabolism phase). None of the main aquatic metabolites were identified in the viscera or fillet at the time of evaluation.

During the current assessment RMS repeated the analysis of the results. It was noticed that in fillet, in addition to two major degradates of Flufenacet – FOE cysteine conjugate and FOE acetyl cysteine, several minor fractions were identified, named Unknown 1 – Unknown 8. Each of these fractions was low, not surpassing 2.5% of TRR, therefore there was no need for their further characterization.

In case of viscera the identified metabolites were mainly conjugates with cysteine and glutathione, or their derivatives. Therefore it may be stated that the conclusion drawn by the former RMS for Flufenacet – France, with regard to the nature of the metabolites, remains valid.

**B.9.2.4. Acute toxicity to aquatic invertebrates****B.9.2.4.1. Acute toxicity to *Daphnia magna***

One acute study with *Daphnia magna* have been re-evaluated during the first EU assessment for Annex I listing of Flufenacet. The study is still valid and appropriate for the re-evaluation of flufenacet.

An EC<sub>50</sub> of 30.9 mg a.s./L from this study was obtained after 48 hours of exposure in static conditions.

The acute toxicity studies to *Daphnia magna* for two metabolites – FOE-Thiadone and FOE-Sulfonic acid with 48 hour EC<sub>50</sub> values of 31.7 mg met./L and >87.3 mg met./L have been also evaluated during the first EU assessment for Annex I listing of Flufenacet.

In addition, the effects of trifluoroacetate acid (TFA) metabolite on *Daphnia magna* has been investigated according to OECD 202 test guideline. Results of this study indicated that exposure of TFA metabolite mortality and sublethal effects occurred up tested rate of 1200 mg met. /L.

**Table B.9.2.4.1-1: Acute toxicity data of flufenacet to *Daphnia magna***

Organism	Test substance	Endpoint (type of the test)	Value (mg /L)	Reference
<b>Aquatic invertebrates</b>				
<i>Daphnia magna</i> (Waterflea)	Flufenacet	48 h EC <sub>50</sub> (static, moratlity )	30.9 mm	Bowers L.M (1994) M-003805-01-1
<i>Daphnia magna</i> (Waterflea)	FOE-Thiadone	48 h EC <sub>50</sub> (static, moratlity )	31.7 mm	Bowers & Lam C.V. (1998) M-005390-01-1
<i>Daphnia magna</i> (Waterflea)	TFA	48 h LC <sub>50</sub> (Static, mortality)	>1200 nom	Groeneveld et al. (1992) M-247890-01-1
<i>Daphnia magna</i> (Waterflea)	FOE sulfonic acid	48 h EC <sub>50</sub> (static, moratlity )	>87.3 nom	Heimbach F., (1995) M-004930-01-1

**Active substance:****B.9.2.4.1.1. Acute toxicity of FOE5043 technical to waterflea (*Daphnia magna*) under static condition,**

<b>Reference:</b>	Acute of FOE 5043 Technical to the waterflea ( <i>Daphnia magna</i> ) under static conditions.
<b>Author(s), year:</b>	Bowers L.M., 1994
<b>Report/Doc. number:</b>	Study No: 106-597, Reference BCS No: M-003805-01-1
<b>Guideline(s):</b>	US EPA/FIFRA 72-2 test guideline. Acute toxicity test for freshwater invertebrates.
<b>GLP:</b>	Yes

**Material and methods:**

Test substance:	FOE 5043 ,(flufenacet tech.), Batch No: FL-036: Purity 96.8% <sup>14</sup> C-FOE 5043; Batch No: C-584, Purity 96.9%
Test species:	Water flea ( <i>Daphnia magna</i> )
Number of organism:	2 replicates each with 10 daphnids per treatment, control and solvent control
Age:	< 24 hour old neonates
Type of test, duration:	Static, 48 hour
<b><u>Applied concentrations:</u></b>	
Nominal (measured):	Control (0), Solvent control (0), 6.6 (6.38), 11 (10.8), 18 (17.7), 30 (29.0), 50 (47.9) mg a.s./L
Solvent:	Acetone, 0.5 mL/L

**Test conditions:**

Water quality:	Dilution water (blended spring water), Hardness 177 mg/L as CaCO <sub>3</sub> , alkalinity: 123 mg/L as CaCO <sub>3</sub>
Conductivity:	396 mg/L
Temperature:	19.9 - 20.2 °C
pH:	8.0-8.3
O <sub>2</sub> content:	8.2- 8.8 mg/L corresponding to 90-95% saturation The test solution was not aerated during the study
Light regime:	16 hours light /8 hours darkness. Light intensity: 40-60 foot-candles, corresponding to 430-645 Lux
Feeding:	Daphnia were not fed throughout the duration of the tests.

Test parameters:	<p>All test vessels were monitored for mortality and sub-lethal effects after 0, 24, 48, hours. Dissolved oxygen, temperature, pH were measured in the control, solvent control, low, middle and high concentration containing surviving daphnia, at the test start and at the end of the test. Temperature was monitored continuously.</p> <p>Water samples were collected from all test concentrations, including controls, on Day 0 and on Day 2 to measure actual exposure concentrations. At test termination the analytical samples were collected after the two replicates of each test concentration were combined into one vessel. All samples were processed for Liquid Scintillation Counts and Radio-TLC Analysis on the same day the samples were collected.</p>
Statistic:	<p>Mortality data was analyzed using Toxcal, a multi-method program which determines the LC<sub>50</sub> and 95% confidence interval using the Binomial, Moving Average, and Probit methods.</p>
Findings:	
Analytical data:	<p>The analytical data indicated that measured concentration of flufenacet on day 0 ranged between 95 and 98.2% of nominal on Day 0 and between 96.2 and 98.3 % on Day 4 of nominal. The mean measured concentration of flufenacet during the test period ranged from 96-98 % of the nominal concentration. The results were based on mean measured concentration.</p>
Biological effects:	<p>Sublethal effects including daphnia positioned at the bottom of test vessels and exhibiting very little movement was observed at 29 and 47.9 mg a.s./L. The 48-hour EC<sub>50</sub> value was calculated using the number of dead and sublethally effected organisms at each test level: 6.38, 10.8, and 17.7 mg/L (0); 29.0 mg/L (8); 47.9 mg/L (20). Based upon combined endpoints of mortality and sublethal effects 48 hour EC<sub>50</sub> was 30.9 mg a.s./L with 95% confidence limits of 29 to 47.9 mg a.s./L. The 48 h NOEC was 17.7 mg a.s./L.</p>

**Table B. 9.2.4.1.1-1: Cumulative mortality and sub-lethal effects on *Daphnia magna* exposed to technical flufenacet.**

Mean measured concentration (mg a.s./L)	Cumulative Mortality [%] (no. of dead / no. of treated )	
	24 hours	48 hours
Control	0 (0/20N)	0 (0/20N)
Solvent control	0 (0/20N)	0 (0/20N)
6.38	0 (0/20N)	0 (0/20N)
10.8	0 (0/20N)	0 (0/20N)
17.7	0 (0/20N)	0 (0/20N)
29.0	0 (0/20N)	0 (0/20) <sup>OB, VLM</sup>
47.9	0 (0/20) <sup>OB</sup>	90 (18/20) <sup>OB, VLM</sup>

Observation: OB- on the bottom, VLM -every little movement

**Conclusion:**

The 48 h LC<sub>50</sub> of flufenacet to *Daphnia magna* based on mortality under static test conditions of the study was determined to be 39.4 mg a.s./L (95% C.I: 29-47.9 mg a.s./L) based on mean measured concentration.

The 48 EC<sub>50</sub> of flufenacet based on mortality and sublethal effects of flufenacet was determined to be 30.9 mg a.s./L (95% C.I: 29-47.9 mg a.s./L) and 48 h NOEC is 17.7 mg a.s./L based on mean measured concentration.

**RMS comments:**

The study was conducted according to US EPA/FIFRA 72-2 guideline. The validity criteria given in the test guidelines OECD 202 (2004) and US EPA, OPPTS 850.1010 (1996) are met.

The mortality in the controls was <10 % (being 0).

The dissolved oxygen throughout the test was >3 mg a.s./L in control and all test vessels. The measured dissolved oxygen was in ranged from 8 to 8.8 mg a.s./L corresponding to 90-95% saturation.

The following deviations from OECD 202 (2004) and US EPA OPPTS 850.1010 (1996) test guidelines were noted.

- The water quality parameters were measured on low, middle and high test concentrations (in the all test chamber is recommended by the test guideline).

Indicated deviations are, however, considered as having no impact on the study results, since all validity criteria were met. Therefore the study is considered to be acceptable.

**Agreed endpoints:**

48 h EC<sub>50</sub> = 30.9 mg a.s./L (95% C.I: 29-47.9 mg a.s./L), based on mean measured concentration

48 h NOEC = 17.7 mg a.s./L, based on mean measured concentration

**Metabolites:****B.9.2.4.1.2. Acute toxicity of FOE 5043-sulfonic acid to *Daphnia magna***

<b>Reference:</b>	Acute toxicity of FOE 5043-sulfonic acid to the Water fleas ( <i>Daphnia magna</i> )
<b>Author(s), year:</b>	Heimbach F. 1995
<b>Report/Doc. number:</b>	Study No: E320 097-8, Report No: HBF/Dm 145, Reference BCS No: M-004930-01-1
<b>Guideline(s),:</b>	OECD 202, 1984.
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Sodium salt of the Flufenacet-sulfonic acid (FOE 5043-sulfonic acid). Batch No: WAK 6222-3, Purity: 93.6% (corresponding to 86.7% free acid)
Test species:	<i>Daphnia magna</i>
Number of organism:	3 replicate each with 10 daphnids per treatment, control water
Age:	The first instars < 24 hours old
Type of test, duration:	Static, 48 hours

Applied concentrations:

Nominal:	Control (0), 87.3 mg Flufenacet-sulfonic acid /L
Solvent:	None

Test conditions:

Water quality:	Dilution water: Total hardness 213 mg/L as CaCO <sub>3</sub> , alkalinity 50 mg /L as CaCO <sub>3</sub>
Conductivity:	580 µS/cm
Temperature:	Test end: 20 ± 1 °C
pH:	Test start: 8-8.08 Test end: 7.97-7.99
O <sub>2</sub> content:	Test start: 8.1-8.5 mg/L Test end: 8.8-8.9 mg/L
Light regime:	16 hours light /8 hours darkness. Light intensity: 41 foot-candles corresponding to 437 Lux
Feeding:	Daphnia were not fed throughout the duration of the tests

Test parameters:	<p>All test vessels were monitored for number of live <i>Daphnia</i> and sub-lethal effects after 0, 24, 48, hours.</p> <p>Dissolved oxygen, pH were measured in the control and test concentration at the test start and at the end of the test. Temperature was monitored continuously in the climatic chamber and verified in vessels of the control at the termination of the study.</p> <p>Total hardness, alkalinity and conductivity were measured at the beginning of the test in the dilution water. Water samples were collected from test level including controls on Day 0 and 2 (day 2 without control) to measure actual test concentration. At test termination the analytical samples were collected after the three replicates of the test concentration were combined into one vessel. All samples were processed for Liquid Scintillation Counts and Radio-TLC.</p>
Statistic:	A calculation and statistical evaluation was not applicable (limit test).
<u>Findings:</u>	
Analytical data:	<p>The analytical results indicated that measured concentration on Day 0 was 82 % of nominal and on Day 4 was 91% of the nominal.</p> <p>The mean measured concentration of flufenacet during the test period was 86.8% of the nominal concentration.</p>
Biological effects:	No sublethal effects were observed for control and test concentration.

**Table B. 9.2.4.1.2-1: % Mortality/Immobility of *Daphnia magna* exposed to Flufenacet-sulfonic acid.**

Nominal concentration (mg p.m./L)	Number of living / no. of treated		% immobilization	
	24 hours	48 hours	24 hours	48 hours
Control	10/10 10/10	9/10 10/10	0	3 ±6
87.3	10/10 10/10	10/10 10/10	0	0

Conclusion:

The 48 EC<sub>50</sub> of flufenacet based on mortality and sublethal effects was determined to be > 87.3 mg p.m./L and 48 h NOEC was estimated to be >87.3 mg p.m./L.

**RMS comments:**

The study was conducted according to OECD 202 (1984) test guideline. The validity criteria given in the former (OECD 202, 1984) and current test guidelines (OECD 202, 2004 and US EPA, OPPTS 850.1010) are met.

The mortality in the control was <10 %.

The dissolved oxygen throughout the test was >3 mg a.s./L in control and all test vessels being at range between 8.2 to 8.9 mg /L in the study corresponding to 96 to 105% of air saturation.

The study is considered acceptable.

**Agreed endpoints:**

48 h EC<sub>50</sub> > 87.3 mg Flufenacet sulfonic acid /L based on nominal concentration

48 h NOEC > 87.3 mg /Flufenacet sulfonic acid /L, based on based on nominal concentration

**B.9.2.4.1.3. Acute toxicity of Thiadone to *Daphnia magna* (static test)**

<b>Reference:</b>	Acute toxicity of Thiadone ( a metabolite of FOE 5043) to the Waterflea <i>Daphnia magna</i> under static condition.
<b>Author(s), year:</b>	Bowers L.M., Lam C.V., 1998
<b>Report/Doc. number:</b>	Study No: 108464, Reference BCS No: M-005390-01-1
<b>Guideline(s):</b>	US EPA/FIFRA 72-2 guideline
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Thiadone. Batch No: l K778 6222-3, Purity 94.4%
Test species:	<i>Daphnia magna</i>
Number of organism:	Two replicates each with 10 daphnids per treatment, control water, solvent control
Age:	< 2 4 hour old neonates
Type of test, duration:	Static, 48 hour

Applied concentrations:

Nominal (mean measured):	Control (0), Solvent control (0), 7.5 (7.84), 15 (16), 30 (30.1), 60 (60.9) 120 (119.7) mg metablite/L
Solvent control	DMF, dimethylformamide 500 µl/L

Test conditions:

Water quality:	Dilution water (hard blended water): hardness 176 mg/L as CaCO <sub>3</sub> , alkalinity 119 mg as CaCO <sub>3</sub>
Temperature:	19.9-20.3 °C (mean, 19.9°C)
pH:	Test start : 7.2-8.0 Test end: 8.2-8.4
O <sub>2</sub> content:	Test start: 8.6-8.8 mg/L Test end: 8.0-8.8 mg/L
Light regime:	16 h light/8 hours dark. Light intensity: 41 foot-candles corresponding to 437 Lux.
Feeding:	Daphnia were not fed throughout the duration of the tests.
Test parameters:	All test vessels were monitored for number of live Daphnia and sub-lethal effects after 0, 24, 48 hours. Dissolved oxygen, temperature, pH were measured in the controls and all test concentrations at the test start and at the end of the test. Temperature was monitored continuously. Hardness, alkalinity were measured at the beginning of the test in the controls and high concentration. Water samples were collected from test concentrations including controls on Day 0 and Day 2. The mean measured thiadone concentrations were analyzed using (HPLC) technique.
Statistic:	The 48-hour EC <sub>50</sub> value and the 95 percent confidence limits were calculated by a EC <sub>50</sub> computer program (CT-TOX, 1990) using the following statistical methods: Spearman-Kärber, binomial probability, moving average angle, and probit.

Findings:

Analytical data:	The mean measured concentration of thiadone during the test period range from 100-116 % of the nominal concentration.
Biological effects:	The sublethal effects including floating at surface or abnormal position at the bottom of vessel were observed after 24 hours and 48 hours at test concentrations of 16.0, 30.1, 60.9 and 119 mg p.m/L.

**Table B. 9.2.4.1.3-1: Cumulative mortality and behavioral observation of *Daphnia magna* exposed to Thiadone.**

Mean measured concentration (mg p.m. /L)	% mortality (Number of dead/ no. of treated )	
	24 hours	48 hours
Control	0 (0/20 N)	0 (0/20N)
8.7	0 (0/20 N)	0 (0/20N)
16.0	0 (0/20N)	10 (/2/20 <sup>F</sup>
30.1	0 (0/20) <sup>F</sup>	30 (6/20) <sup>F</sup>
60.9	95 (19 /20) <sup>OB</sup>	100 (20)
119.7	100 (20)	100 (20)

\*Observation: N-normal, F-floating at surface, OB-abnormal position at the bottom

**Conclusion:**

The 48 EC<sub>50</sub> of Thiadone based on mortality and sublethal effects was determined to be 31.7 mg p.m./L (CI: 95% 26.5-38.2 mg met./L) and 48 h NOEC was estimated to be 8.7 mg p.m. /L.

**Comments RMS:**

The study was conducted according to US EPA-FIFRA 72-2 guideline.

The validity criteria given in the test guidelines OECD 202, (2004) and US EPA, OPPTS 850.101 are met.

The mortality in the control were <10 % (being 0%). The dissolved oxygen throughout the test was >3 mg/L in controls and all test vessels. The measured dissolved oxygen was in range from 8.0-8.8 mg metabolite/L corresponding to 88-97% saturation.

The study is considered acceptable.

**Agreed endpoints:**

48-h EC<sub>50</sub> = 31.7 mg Thiadone/L, based on mean measured concentration

NOEC = 8.7 mg Thiadone/L, based on mean measured concentration

**B.9.2.4.1.4. The acute toxicity of sodium trifluoroacetate to *Daphnia magna* (static test).**


---

<b>Reference:</b>	The acute toxicity of sodium trifluoroacetate to <i>Daphnia magna</i>
<b>Author(s), year:</b>	Groeneveld A.H.C., de Kok H.A.M., Berg G., 1992
<b>Report/Doc. number:</b>	Study No: C 047203, Reference BCS No: M-247890-01-1
<b>Guideline(s):</b>	OECD 202 (1984)
<b>GLP:</b>	Yes

---

Material and methods:

Test substance:	Sodium trifluoroacetate (sodium-TFA), Batch No: ACA9135AB, Purity 99%
Test species:	<i>Daphnia magna</i>
Number of organism:	Three replicates each with 10 daphnids per treatment, control water
Age:	<24 hour old neonates
Type of test, duration:	Static, 48 hour
<u>Applied concentrations:</u>	
Nominal (measured):	Control (0), 1200 (1215) mg sodium-TFA salt /L

Solvent: None

Test condition:

Water quality:	ISO reconstituted dilution water: Hardness: 250 mg/L as CaCO <sub>3</sub> , pH: 7.8
Temperature:	Test start: 19.4-19.9°C Test end: 20.4-20.5°C
pH:	Test start: 7.8-8.0 Test end: 7.7-7.8
O <sub>2</sub> content:	Test start: 8.6- 8.6 mg/L Test end: 8.4-8.5 mg/L
Light regime:	16 hours light /8 hours dark
Feeding:	Daphnia were not fed throughout the duration of the tests.
Test parameters:	All test vessels were monitored for immobility of Daphnia and sub-lethal effects after 24, 48, hours. Dissolved oxygen, temperature, pH were measured in the control and test concentration at the test start and at the end of the test. Temperature was monitored continuously. At test initiation a sample of 50 ml was taken from the test solution. At the end of the test samples of 15 ml were taken from each vessel and pooled. This was done in duplicate of which only one

sample was analysed. The samples were stored cool and dark until analysis by means of ion chromatography.

**Statistic:** Due to the absence of effects no statistical analysis could be done. The 24-hour, 48-hour EC<sub>50</sub>-values and the NOEC and LOEC values were determined to be at least or higher than 1200 mg sodium-TFA/L. These values were determined directly from the raw data.

**Findings:**

**Analytical data:** The mean measured concentration of sodium-TFA salt metabolite during the test period 101 % of the nominal concentration.

**Biological effects:** No mortality and sublethal effects occurred in the control and 1200 mg metabolite/L test level after 24 and 48 hours exposure.

**Table B.9.2.4.1.4-1: Influence of sodium-TFA salt on the mobility of *Daphnia magna***

Nominal concentration (mg p.m./L)	% of immobilized dapniads after	
	24 hours	48 hours
Control	0	0
1200	0	0

**Conclusion:** The 48-hour EC<sub>50</sub> value was calculated as 1200 mg sodium TFA salt /L based on nominal concentration. Based on the molecular weights, a concentration of 1200 mg sodium TFA-Na/L  
NOEC was estimated to be >1200 mg TFA-Na/L

**RMS comments:**

The study was conducted according to test guidelines OECD 202 (1984). The validity criteria given in the former (OECD 202, 1984) and current test guidelines (OECD 202, 2004 and US EPA, OPPTS 850.1010) are met.

The mortality in the control were <10 % (being 0%).

The dissolved oxygen throughout the test was >3 mg/L in controls and all test vessels. The measured dissolved oxygen was in range from 8.4 to 8.6 mg /L corresponding to 100-103% air saturation.

The study is considered acceptable.

**Agreed endpoints:**

48 h EC<sub>50</sub> >1200 mg TFA-Na/L, based on nominal concentration

NOEC = 1200 mg TFA-Na/L, based on nominal concentration

**B.9.2.4.2. Acute toxicity to an additional aquatic invertebrate species**

For flufenacet three toxicity studies on three additional aquatic invertebrates' species were performed.

The study with the most sensitive species *Hyaella azteca* was evaluated within the process of Annex I inclusion and was considered acceptable by RMS-France. However, the toxicity endpoint  $LC_{50} = 2.45$  mg a.s./L was not included in the LoEP.

*Hyaella azteca* is epibenthic detritivore that burrows into the sediment surface. The behavior and feeding habits of *H. azteca* make them an excellent test organisms for sediment assessments. It is a widespread and abundant species of amphipod crustacean in North America. It lives among vegetation in permanent bodies of freshwater, including lakes and rivers. RMS, included this species in the risk assessment even though it is not a representative species for the EU. However, due to the fact that it is the most sensitive organism, the RMS is of the opinion that it covers the sensitivity of the other not tested species.

For metabolite FOE-Thiadone two studies with two different species were conducted.

For *Mysidiopsis bahia* and *Crassostrea virginica* no mortality occurred at the highest tested dose, resulting in an  $LC_{50} > 15.1$  mg met./L and  $> 22$  mg met./L, respectively.

**Table B.9.2.4.2-1: Acute toxicity data of flufenacet**

Organism	Test substance	Endpoint (type of the test)	Value (mg /L)	Reference
<b>Aquatic invertebrates</b>				
<i>Americamysis bahia</i> Mysid shrimp	Flufenacet	96 h $LC_{50}$ (flow-through, mortality)	5.6 mm	Claude M.B., et al (2013) M-452205-01-1
<i>Crassostrea virginica</i> Eastern oyster	Flufenacet	96 h $LC_{50}$ (mortality), 96 h $LC_{50}$ (shell growth)	>13.9 mm 12.6 mm	Wheat & Evans (1993) M-002427-01-1
<i>Hyaella azteca</i>	Flufenacet	96 h $LC_{50}$ (acute, static)	2.45 mm	Bowers L.M. (1995) M-002374-01-1
<i>Mysidiopsis bahia</i> Mysid shrimp	FOE-Thiadone	96 h $LC_{50}$ (Flow-through, mortality)	>15.1 mm	Bowers & Lam (1998) M-005110-01-1
<i>Crassostrea virginica</i> Eastern oyster	FOE-Thiadone	96 h $LC_{50}$ (Flow-through, mortality mortality)	22 mm	Palmer S.J. & Krueger H. (1998) M-005108-01-1

**B.9.2.4.2.1. Acute toxicity of FOE 5043 (Technical) to *Hyalella azteca* under static condition.**

<b>Reference:</b>	Acute toxicity of FOE 5043 (Technical) to <i>Hyalella azteca</i> under static condition.
<b>Author(s), year:</b>	Bowers L.M., 1995
<b>Report/Doc. number:</b>	Study No: 106908, Reference BCS No: M-002374-01-1
<b>Guideline(s):</b>	US EPA/FIFRA 72-2 guideline: Acute toxicity test for freshwater invertebrates.
<b>GLP:</b>	Yes

Material and methods:

Test substance:	FOE 5043 (flufenacet tech.), Batch No: 3030057, Purity 98.8%
Test species:	<i>Hyalella azteca</i>
Number of organism:	Two replicates each with 10 per treatment and controls
Age:	7-10 days old
Type of test, duration:	Static, 96 hours

Applied concentrations:

Nominal (mean measured)	Control (0), Solvent Control (0), 1.25 (0.83), 2.5 (2.28), 5 (3.69), 10 (8.51), 20 (15.2) mg a.s./L
Solvent:	Acetone, 500 µl/L

Test condition:

Water quality:	Dilution water: Hardness 166 mg/L as CaCO <sub>3</sub> , alkalinity: 112 mg/L as CaCO <sub>3</sub> , conductivity: 369 µhos/cm
Temperature:	Test start: 19.9-20.4°C Test end: 20.2- 20.6°C
pH:	Test start: 8.1-8.2 Test end: 8.1-8.2
O <sub>2</sub> content:	Test start: 9.0- 9.1 mg/L Test end: 8.8-9.2 mg/L
Conductivity:	Test start: 375-377 µmhos/cm Test end: 359-364 µmhos/cm
Hardness:	Test start: 162-172 as CaCO <sub>3</sub>

---

	Test end: 160-166 as CaCO <sub>3</sub>
Alkalinity:	Test start: 114-117 as CaCO <sub>3</sub>
	Test end: 109-111 as CaCO <sub>3</sub>
Light regime:	16 hours light/8 hours darkness. Light intensity: 775 Lux
Feeding:	<i>Hyalella azteca</i> were not fed and test solutions were not aerated throughout the duration of the tests.
Test parameters:	<p>Dissolved oxygen, temperature, pH, conductivity, hardness, alkalinity were measured in the controls and in the low, middle and high test concentrations containing surviving <i>Hyalella azteca</i> at the test start and at the end of the test. Temperature was monitored continuously.</p> <p>All test vessels were monitored for mortality and sub-lethal effects after 24, 48, 72, 96 hours.</p> <p>The body lengths of the controls <i>Hyalella azteca</i> were measured at the end of test. Test solution samples were collected from all test concentrations, including controls, on Day 0 and Day 4 to measure actual exposure concentrations using HPLC analysis.</p>
Statistic:	The LC <sub>50</sub> value was estimated by a LC <sub>50</sub> computer program TOXCAL using the following statistical methods: binomial probability, moving average angle, and probit. The slope of the toxicity curve was determined by the probit method.
<u>Findings:</u>	
Analytical data	<p>The measured concentration of flufenacet during the test period was ranged between 66 to 91% of the nominal concentration.</p> <p>The mean measured concentrations of FOE 5043 (technical) during the test period were 0.83, 2.28, 3.69, 8.51 and 15.2 mg a.s./L which correspond to 66, 91, 74, 85, and 76% of the nominal concentration .</p> <p>The lowest concentration tested was 1.25 mg/L (nominal).</p> <p>The fact that mean measured flufenacet concentration was less than 70% of nominal in the lowest test concentration (1.25 mg/L nominal) according to the study director, should not invalid the study due to a good dose response obtained and the variability of the measured concentration within the lowest test level was well within acceptable limits 1.5. All study results are based in mean measured concentration. The compound was stable in the test system for the duration of the 96-hour exposure. No undissolved test substance was observed in the test chambers.</p>

Biological effects: Behavioral/sublethal effects were observed at the 15.2, 8.51 and 3.69 mg a.s./L test concentrations. These effects observed were: abnormal position at the bottom of the test chamber and quiescence. There were no behavioral or sublethal effects in the control, solvent control, 2.28 and 0.83 mg a.s./L test concentrations.

**Table B.9.2.4.2.1-1: Effects on *Hyalella azteca* exposed to technical flufenacet.**

Mean measured concentration (mg a.s./L)	Mortality [%] (no. of dead / no. of treated )			
	24 h	48 h	72 h	96 h
Control	5 (1/20)	10 (2/20)	10 (2/20)	10 (2/20)
Solvent control	0 (0/20)	0 (0/20)	0 (0/20)	0 (0/20)
0.83	5 (1/20)	5 (1/20)	10 (2/20)	10 (2/20)
2.28	5 (1/20)	15 (3/20)	30 (6/20)	35 (7/20)
3.69	20 (4/20) <sup>VLM</sup>	40(8/20) <sup>VLM</sup>	65(13/20)	65 (13/20)
8.51	65 (13/20) <sup>OB.,VLM</sup>	80 (16/20) <sup>VLM</sup>	90 (18/20)	100 (20/20)
15.2	95 (19/20) <sup>VLM</sup>	100 (20/20)	100 (20/20)	100 (20/20)

Observation: N: Normal, VLM: very little movment, OB: abnormal position on the bottom

#### Conclusion:

The 96 hour LC<sub>50</sub> of flufenacet technical to *Hyalella azteca* was determined under the static test conditions of this study, is 2.45 mg a.s./L (95% CI: 1.80-3.15 mg a.s./L) based on mean measured concentrations.

#### **RMS comments:**

The study was conducted according to US EPA/FIFRA 72-2 test guideline.

The study was accepted during the first inclusion of flufenacet to the Annex I.

RMS to verify the validity criteria of the study took into consideration the validity criteria given in test guidelines OECD (202), 2004. However, it should be noted, that in OECD 202 (2004) test guideline *H.azteca* species was not listed as species tested.

During the test no mortality in the solvent control was observed. However, the 10% mortality was noted in water control.

The dissolved oxygen throughout the test was >3 mg/L in the controls and all test vessels (measured dissolved oxygen was in range between 8.8 and 9.2 mg/L, representing 97 - 101% of air saturation).

The following deviation from the OECD 203 was noted:

-The mean measured concentration was between 66-98 % during the study. The lowest mean measured

concentration was observed at the lowest tested rate of 1.25 mg a.s./L.

Indicated deviation is however, considered as having no impact on the study results, since all validity criteria were met. The study is considered acceptable.

**Agreed endpoints:**

96 h LC<sub>50</sub> = 2.45 mg a.s./L (95% CI: 1.80-3.15 mg a.s./L), based on mean measured concentration

NOEC = 0.83 mg a.s./L, based on mean measured concentration

**B.9.2.4.2.2. Acute toxicity of flufenacet to *Americamysis bahia* (static test).**

<b>Reference:</b>	Flufenacet: A 96-Hour. A 96 static acute toxicity test with the saltwater mysid ( <i>Americamysis bahia</i> ).
<b>Author(s), year:</b>	Claude M.B., Martin K.H., Gallagher S.P., Krueger H.O., 2013
<b>Report/Doc. number:</b>	Study No: EBFOL242, Reference BCS No: M-452205-01-1
<b>Guideline(s):</b>	US EPA/FIFRA 72-3 guideline
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet technical, Batch No: AE F133402-01-17, Purity 97.49%
Test species:	<i>Americamysis bahia</i> , saltwater mysid
Number of organism:	2 replicates each with 10 per treatment, control and solvent control
Age:	<24 hours old
Type of test:	96 hours, flow-trough

Applied concentrations:

Nominal:	0 (control), 0.31, 0.63, 1.3, 2.5, 5.0 and 10 mg a.s./L
Mean Measured:	0 (control), 0.29, 0.59, 1.2, 2.3, 4.7, 9.5 and 9.5 mg a.s./L

Test condition:

Water quality:	Dilution water (Natural seawater, salinity: 20‰)
Temperature:	Test start: 23.1-24.1 °C Test end: 25.2- 26.5°C
pH:	Test start: 9-8.2 Test end: 7.9-8.0
O <sub>2</sub> content:	Ranged between 5.2 and 7.6 mg/L corresponding to 80 - 103 % of air saturation.
Light regime:	16 hours light /8 hours darkness. Light intensity: 272 Lux.

Feeding:	The juvenile mysids were fed live brine shrimp ( <i>Artemia</i> sp.) nauplii ad libitum daily during the test.
Test parameters:	<p>Dissolved oxygen and pH measurements were made at approximately 24-hour intervals. Salinity was measured in the dilution water at test initiation and termination.</p> <p>Temperature was measured in each chamber at the beginning and the end of test. Temperature also was monitored continuously in a container of water placed adjacent to the test chambers using a Fulscope ER/C Recorder.</p> <p>Test vessels were monitored for mortality and sub-lethal effects after 5, 24, 48, 72 and 96 hours after test initiation.</p>
Statistic:	The Probit analysis was used to calculate the 48, 72 and 96-hour LC <sub>50</sub> values and the 95% confidence intervals. Since there was <50% mortality at 24 hours, the 24-hour LC <sub>50</sub> value, as well as the no-mortality concentration and NOEC, were determined by visual interpretation of the mortality and observation data.
Analytical data:	<p>Samples were collected from the batch solution of each concentration at the beginning of the test, and from each replicate test chamber of each treatment and control group at 48 and 96 hours (<math>\pm 1</math> hour) to measure concentrations of the test substance.</p> <p>High-performance liquid chromatography HPLC-UV was used as analytical method.</p>
Findings:	
Analytical results:	The mean measured concentration of flufenacet during the test period ranged from 92 to 95% of the nominal concentration.
Biological effects:	Behavioral/sublethal effects were observed at the 4.7 and 9.5 mg a.s./L test concentrations. These effects observed were lethargy.

**B.9.2.4.2.2-1. Observed mortality and sublethal effects of saltwater mysids (*Americamysis bahia*) exposed to flufenacet for during 96 hours.**

Mean measured concentration (mg a.s./L)	Replicate	(No. of dead / no. of treated organism)					% Cumulative mortality
		5 h	24 h	48 h	72 h	96 h	
Control	A	(0/10)	(0/10)	(0/10)	(0/10)	(0/10)	0
	B	(0/10)	(0/10)	(0/10)	(0/10)	(0/10)	0
Solvent control	A	(0/10)	(0/10)	(0/10)	(0/10)	(0/10)	0

Mean measured concentration (mg a.s./L)	Replicate	(No. of dead / no. of treated organism)					% Cumulative mortality
		5 h	24 h	48 h	72 h	96 h	
	B	(0/10)	(0/10)	(0/10)	(0/10)	(0/10)	0
0.29	A	(0/10)	(0/10)	(0/10)	(0/10)	(0/10)	0
	B	(0/10)	(0/10)	(0/10)	(0/10)	(0/10)	0
0.59	A	(0/10)	(0/10)	(0/10)	(0/10)	(0/10)	0
	B	(0/10)	(0/10)	(0/10)	(0/10)	(0/10)	0
1.2	A	(0/10)	(0/10)	(0/10)	(0/10)	(0/10)	0
	B	(0/10)	(0/10)	(0/10)	(0/10)	(0/10)	0
2.3	A	(0/10)	(0/10)	(0/10)	(0/10)	(0/10)	0
	B	(0/10)	(0/10)	(0/10)	(0/10)	(0/10)	0
4.7	A	(0/10)	(0/10)	(1/10)	(1/10)	(1/10)	30
	B	(0/10)	(0/10)	(1/10)	(3/10)	(5/10) <sup>C,IM</sup>	
9.5	A	(0/10)	(0/10)	(3/10)	(6/10)	(9/10) <sup>C,IM</sup>	95
	B	(0/10)	(0/10)	(7/10)	(8/10)	(10/10)	

<sup>1</sup> Observation: c-lethargy, M-missing and assumed dead

**Conclusion:**

Based on mortality the 96 hour LC<sub>50</sub> is estimated to be 5.6 mg a.s./L, based on mean measured concentration of flufenacet. NOEC based on mortality and sublethal effects is determined to be 2.3 mg a.s./L.

**RMS comments:**

The study was conducted according to US EPA/FIFRA 72-3 guideline.

The validity criteria given in the test guideline US EPA, OPPTS 850.1035 are met.

Mortality in the control was <10% (measured: 0%). The dissolved oxygen throughout the test ranged from 80 to 103 % of air saturation. The test conditions parameters such as: a temperature: (measured: 23.1-26.5°C), salinity (measured: 20‰) were in line with those recommended by the relevant test guideline.

The following deviation from guideline was noted:

-The photoperiod in this study was 16 hours light /8h darkness, instead of 14 hours light/10 h darkness.

Indicated deviation is however, considered as having no impact on the study results, since all validity criteria were

met. The study is considered acceptable.

**Agreed endpoints:**

96h LC<sub>50</sub> = 5.6 mg a.s./L, based on mean measured test concentration

NOEC = 2.3 mg a.s./L, based on mean measured test concentration

**B.9.2.4.2.3. Acute of FOE 5043 ( technical) on new shell growth of the eastern oyster (*Crassostrea virginica*)**

<b>Reference:</b>	Acute of FOE 5043 (technical) on new shell growth of the eastern oyster ( <i>Crassostrea virginica</i> ).
<b>Author(s), year:</b>	Wheat, J., Evans, J.; 1993
<b>Report/Doc. number:</b>	Study No: J 9201017b, Reference BCS No:M-002427-01-1
<b>Guideline(s):</b>	US EPA/FIFRA 72-3 test guideline. Oyster shell growth toxicity test.
<b>GLP:</b>	Yes

Material and methods:

Test substance:	FOE5043 technical, Batch 2030019, Sec. ref.no. 898113006, purity 98.2%
Test species:	<i>Crassostrea virginica</i>
Number of organism:	20 organisms per treatment and control groups
Length at initiation:	Length of 20.0 to 37.7 mm (mean 28.0 ± 4.6 mm) Wet tissue weight of 0.19 to 1.68 g (mean 0.74 ± 0.40 g).

Type of test, duration: Flow-trough, 96 hours

Applied concentrations:

Nominal:	Control (0), Solvent (control 0), 1.2, 1.9, 3.2, 5.4, 9.0 and 15 mg a.s./L
Measured:	Control (0), Solvent (control 0), 1.2, 1.7, 3.0, .4.9, 8.4 and 13.9 mg a.s./L
Solvent:	DMF (100 µL)

Test conditions:

Water quality:	Dilution water (Natural unfiltered saltwater)
Temperature:	19.7-21.4 °C (mean, 20.3±0.4 °C)
pH:	Test start: 8.4-8.5 Test end: 7.7-8.0 pH=7.5 (Day 3)
O <sub>2</sub> content:	Test start: 6.3-7.2 Test end: 2.6-3.1 On day 2, the lowest dissolved oxygen concentration was 1.9 mg/L at 3 mg a.s./L

---

	<p>test concentration, representing 26% saturation. The dissolved oxygen concentration remained &gt; 26% saturation until test termination and ranged from 2.6 to 3.1 mg/L representing 36-42% saturation in test concentration. In the controls ranged from 3.7 to 5.5 mg/L representing 51-76% saturation at terminations.</p>
Salinity:	29-32 ‰
Light regime:	16 hours Light /8 hours Darkness. Light intensity: 275-367 Lux
Feeding:	<p>The oysters in each test treatment were supplementally fed by the addition of one liter of <i>Isochrysis galbana</i> twice daily to each test tank. The density of the culture used for feeding was approximately 246 x 10<sup>4</sup> cells/mL.</p>
Test system: _	<p>One test chamber, containing 20 oysters, was exposed to each treatment concentration and control (total of 20 oysters in the dilution water control, solvent control and 20 oysters in each test concentration).</p> <p>The test vessels were 11.3 L glass tanks filled to a depth of 6 cm with 5.4 L of dilution water or test solution.</p> <p>A continuous-flow diluter was used to provide each concentration of the test substance, a negative (dilution water) control and a solvent (DMF) control. A pump was used to deliver the test substance stock solutions and DMF for the solvent control into the mixing chambers assigned to the treatment groups. The stock solutions were mixed with dilution water in the mixing chambers in order to obtain the desired test concentrations.</p> <p>The test system functioned properly during the exposure except for Day 3 due to a problem with the secondary stock delivery pump, the toxicant delivery rate for the previous 24-hour period was lower than usual (75 percent of nominal). This malfunction only resulted in a decrease in the volume of secondary stock delivered to the highest flufenacet test concentration thereby lowering the turnover rate for that one treatment.</p> <p>It is believed that this lower turnover rate did not adversely affect the oysters in this treatment as dissolved oxygen concentrations remained above 50%. The concentration of flufenacet in this treatment was unaffected since the concentration of the secondary stock is the same as the highest concentration and flufenacet has been shown to be stable in seawater. The diluter system operated correctly during this period delivering the proper volumes of toxicant and</p>

dilution water to all other treatments. The problem was corrected and the stock delivery pump functioned properly for the remainder of the exposure period.

Prior to test start, the periphery of the shell margin of each oyster was ground (approximately 2-5 mm) with a fine grit grinder in order to establish a baseline for new shell growth.

Test parameter:

Test organisms were observed daily for survival. An additional effect criterion determined was new shell growth, measured at test termination.

Test water quality was monitored each day during the test. Salinity of the dilution water was measured once daily in the dilution water control. Throughout the test, water temperature was measured and recorded hourly in the dilution water control chamber. Dissolved oxygen concentrations and pH were measured daily in all test treatments.

Analytical measurements:

During the test, water samples were collected from the controls and from each flufenacet test solution on days 0 and 4 to monitor actual exposure concentrations. Samples were analyzed on each collection day using HPLC analysis.

Statistic:

The mean new shell growth for the control and solvent control was compared using Student's T-test. If there was no statistically significant difference in shell growth for the two controls, then they were pooled prior to comparison to the treated oysters. If a significant difference did exist, then only the solvent control was used in subsequent analysis. Possible statistical differences in growth between the control oysters and the six flufenacet exposure concentrations were evaluated by analysis of variance (ANOVA) and Dunnett's procedure for multiple comparisons of means with a control. Based on the results of the test, the 96-hour EC<sub>50</sub> values and their 95 percent confidence limits were calculated. These values were estimated by a computer program using the following statistical methods: moving average angle, probit, logit and non-linear interpolation.

Findings:

Analytical data:

The mean, measured concentration of FOE 5043 during the test period ranged from 91-96 % of the nominal concentration.

Biological effects: No mortalities of eastern oyster exposed to the active substance flufenacet were observed throughout the test duration in the controls and test concentration up to 8.4 mg a.s./L. Only at the highest test concentration 13.9 mg a.s./L caused 5% mortality of *Crassostrea virginica*.

**Table B.9.2.4.2.3-1: New shell growth in eastern oyster *Crassostrea virginica*.**

Mean, measured concentration (mg a.s./L)	Mean, shell growth (mm)	Shell Growth difference vs pooled controls (mm)	% Reduction relative to the pooled control
Water control	2.15± 0.77	-	-
Solvent control	1.93±0.81	-	-
Pooled control	2.04±0.79	-	-
1.2	2.17±0.62	0.13	6.4
1.7	2.15±0.80	0.11	5.4
3.0	2.17±0.74	0.13	6.4
4.9	1.79± 0.42	-0.25	-12.3
8.4	1.96± 0.59	-0.08	-3.9
13.9	0.76± 0.46	-1.28	-62.8*

±SD standard deviation

\*Statistically significant compared to the pooled controls (Anova,  $\alpha=0.05$ )

Conclusion: The 96-hour  $EC_{50}$  for *Crassostrea virginica* based on the mean, measured test concentrations of flufenacet and shell growth was estimated to be 12.6 mg a.s./L (95% CI: 8.37 - 13.9 mg a.s./L). The no-observed-effect concentration was 8.4 mg a.s./L based upon the lack of statistically significant reduction in new shell growth at this concentration. The  $EC_{50}$  based on mortality is estimated by RMS to be >13.9 mg a.s./L.

**Comments RMS:**

The study was conducted according to the US EPA /FIFRA 72-3 guideline. The study protocol is in line with the draft test guideline according US EPA (OPPTS 850102,1996).

The following validity criteria outlined in the test guideline US EPA (1996) were considered to evaluate the validity of the results of the study.

-The mortality in the controls should not exceed 10% at the end of the test.

During the whole study period no mortality in the controls was observed.

-The dissolved oxygen concentration should be at least 60%.

The dissolved oxygen concentration decreased on Day 1 in all test concentrations and controls. The lowest dissolved oxygen concentration of 1.9 mg/L, representing 26% saturation, was then recorded in the study. The dissolved oxygen concentration remained > 26% saturation until test termination and ranged from 36 to 42% saturation in the solutions with the test item and from 51 to 76% saturation in the controls. The low oxygen concentration resulted from a combination of the low volume turnover rate and the presence of solvent. The study director stated that the low oxygen concentration did not influence on shell growth. The reduction in new shell growth for oysters exposed to the highest treatment level was correlated more with the test concentration, than with DO concentration as demonstrated by the lack of significant reduction in new shell growth at lower test concentrations with lower associated DO concentrations. According to study director further evidence that the low dissolved oxygen concentrations did not affect the results of this study is demonstrated by the fact that mean new shell growth in the control treatment which had a mean DO concentration of 5.0 mg/L (for days 1 through 4) was 2.15 mm.

Treatment 3 mg a.s./L exhibited the lowest mean DO concentration of 1.9 mg/L had a mean new shell growth of 2.17 mm. If low dissolved oxygen concentrations had impacted this study then it would have been expressed as reduced shell growth in treatments exhibiting low dissolved oxygen concentrations.

- The temperature should be 20 °C. Temporary fluctuations (less than 8 h) within  $\pm 0.5$  °C are permissible.

The test temperature exceeded the range  $20 \pm 1$  °C on day 3, between 1525 and 1625 hour by 0.05 °C.

However, the mean temperature was  $20.3 \pm 0.4$  °C.

- In the controls a minimum of 2 mm of new shell growth should be observed.

The new shell growth in the controls was between 1.2 and 4.2 mm with mean values of 2.15 mm (control), 1.93 mm (solvent control) and 2.04 mm (pooled control). Even though, the validity criterion consider the % of air saturation was not met, the results of the study are considered acceptable. The mean values of new shell growth in pooled control was 2.04 mm and organism. The RMS is of the opinion that the results of the study should be used in the risk assessment.

**Agreed endpoints:**

96-hour  $EC_{50 \text{ shell growth}} = 12.6$  mg a.s./L (CI: 8.37-13.9 mg a.s./L), based on mean measured concentration

96 h  $NOEC_{\text{shell growth}} = 8.4$  mg a.s./L, based on mean measured concentration

96 h  $EC_{50} > 13.9$  mg a.s./L (mortality), based on mean measured concentration

**Metabolites:****B.9.2.4.2.4. Thiadone metabolite of FOE 5043. A 96 hour flow-through acute toxicity test with the saltwater mysid (*Mysidopsis bahia*).**


---

<b>Reference:</b>	Thiadone metabolite of FOE 5043. A 96 hour flow-through acute toxicity test with the saltwater mysid ( <i>Mysidopsis bahia</i> ).
<b>Author(s), year:</b>	Palmer S.J., Henry O. Krueger, 1998
<b>Report/Doc. number:</b>	Study No: 108488, Reference BCS No: M-005110-01-1
<b>Guideline(s):</b>	US EPA/ FIFRA 72-3 test guideline: Shrip acute toxicity test.
<b>GLP:</b>	Yes

---

**Material and methods:**

Test substance:	Thiadone (metabolite FOE 5043), Batch No: M-90-10-76, Purity: 99.6%
Test species:	<i>Mysidopsis bahia</i> , saltwater mysid
Number of organism:	2 replicates each with 10 individuals, 20 per treatment and control and solvent control
Age:	< 24 hours old
Type of test:	96 hours, flow-through test
<b><u>Applied concentrations:</u></b>	
Nominal:	0 (control and solvent control), 1.94, 3.24, 5.40, 9.0 and 15 mg metabolite /L
Mean Measured:	0 (control and solvent control), 2.01, 3.36, 5.45, 9.09 and 15.1 mg metabolite/L
Solvent:	DMF, Dimethylformamide 0.10 mL/L
<b><u>Test condition:</u></b>	
Water quality:	Dilution water (Natural seawater, salinity 21 ‰)
Temperature:	Test start: 24.9-25.0 °C Test end: 24.8- 25°C
pH:	Test start: 8.2-8.3 Test end: 8.1-8.2
Salinity:	21 ‰
O <sub>2</sub> content	Ranged between 5.9 and 6.4 mg/L during the test corresponding to 80 to 87 % of air saturation
Light regime:	16 hours light /8 hours darkness, 283 Lux

Feeding:	The juvenile mysids were fed live brine shrimp ( <i>Artemia</i> sp.) nauplii <i>ad libitum</i> twice daily during the test.
Test parameters:	Dissolved oxygen and pH measurements were made at approximately 24-hour intervals. Salinity was measured in the dilution water at test initiation and termination. Temperature was measured at the beginning and end of test. Temperature also was monitored continuously in one negative control replicate. All test vessels were monitored for mortality and sub-lethal effects after 3.5, 24, 48, 72 and 96 hours.
Analytical data:	Test solution samples were collected from all test concentrations, including controls, on Day 0 and Day 4 to measure actual exposure concentrations using HPLC analysis.
Statistic:	There were no mortalities in the controls and treatment groups. Therefore, statistical analyses were not necessary, and the LC <sub>50</sub> values at 24, 48, 72 and 96 hours were estimated to be greater than the highest concentration tested. The no mortality concentration and NOEC were determined by visual inspection of the mortality and clinical observation data.
<b>Findings:</b>	
Analytical measurements:	Samples collected at 0 and 96 hours had measured concentrations that ranged from 99 to 104% of nominal. The mean measured concentration of FOE-Thiadone during the test period was 102-104% of the nominal concentration.
Biological effects:	There was no mortality in the controls and all test concentrations and no sublethal effect of <i>Mysidopsis bahia</i> were observed during the test

**Table B.9.2.4.2.4-1: Effects on mysid shrimp *Mysidopsis bahia* exposed to FOE-Thiadone.**

Mean measured concentration (mg p.m./L)	Cumulative mortality [%]				
	3.5 h	24 h	48 h	72 h	96 h
Control	0	0	0	0	0
Solvent control	0	0	0	0	0
2.01	0	0	0	0	0
3.36	0	0	0	0	0
5.45	0	0	0	0	0
9.09	0	0	0	0	0
15.1	0	0	0	0	0

**Conclusion:** Based on mortality the 96 hour LC<sub>50</sub> was >15 mg p.m./L based on mean measured concentration and NOEC based on mortality and sublethal effects was >15.1 mg p.m. L.

**Comments RMS:**

The study was conducted according to US EPA/FIFRA 72-3 guideline.

The validity criteria given in the test guideline US EPA, OPPTS 850.1035 are met.

The mortality in the controls was <10 % (measured: 0%).

The dissolved oxygen throughout the test was > 60% of air saturation (measured: 80 to 87 % of air saturation).

The test conditions parameters such as: temperature (measured: 24.8-25° C) or salinity (measured: 20 ‰) are with line with recommendation given the US EPA test guideline (temperature: 25 ± 2C, salinity: 20 ±3 ‰, respectively).

The following deviation from guideline was noted:

-The photoperiod was 16 h light /8 h darkness, instead of 14 h light/10 h darkness.

Indicated deviations is however, considered as having no impact on the study results, since all validity criteria were met. The study is considered acceptable.

**Agreed endpoints:**

96 h LC<sub>50</sub> > 15.1 mg p.m./L, based on mean measured concentration

96 h NOEC > 15.1 mg p.m./L, based on based on mean measured concentration

**Table B.9.2.4.2.5. Thiadone metabolite of FOE 5043: 96 hour Shell Deposition test with new shell Eastern oyster (*Crassostrea virginica*).**

<b>Reference:</b>	Thiadone metabolite of FOE 5043: 96-Hour Shell Deposition test with new shell Eastern oyster ( <i>Crassostrea virginica</i> ).
<b>Author(s), year:</b>	Palmer, S. J.; Krueger, H. O.; 1998
<b>Report/Doc. number:</b>	Study No: 108489, Reference BCS No: M-005108-01-1
<b>Guideline(s):</b>	US EPA/ FIFRA 72-3 guideline.
<b>GLP:</b>	Yes

**Material and methods:**

Test substance: Thiadone (a metabolite of flufenacet ), Reference No: M-90-10-76,  
Purity: 99.6%

---

Test species:	Crassostrea virginica
Number of organism:	20 organisms per treatment and control groups
Length at initiation:	Range: 25 - 31 mm (mean, 27 mm)
Acclimatization:	10 days
Type of test, duration:	Flow-trough, 96 hours

Applied concentrations:

Nominal:	Control (0), Solvent (control 0), 2.50, 5.00, 10.0, 20.0 and 40.0 mg p.met./L
Mean Measured:	Control (0), Solvent (control 0), 2.71, 5.51, 10.7, 22.1 and 47 mg p.m. met./L
Solvent:	0.50 mL dimethylformamide/L

Test conditions:

Water quality:	Dilution water (natural seawater diluted to 20 ‰)
Temperature:	Test start: 21.6-22.3°C Test end: 21.7-22.2°C
pH:	Test start: 7.9-8.1 Test end: 7.7-8.1
O <sub>2</sub> content:	6.5-7.1 mg a.s./L representing 82-90 % air saturation
Salinity:	20 ‰
Light regime:	16 hours light/8 hours darkness. Light intensity: 327 Lux
Feeding:	To supplement the diet of the oysters and enhance their condition and growth, the oysters were provided with an algal suspension of <i>Thalassiosira</i> sp., <i>Skeletonema</i> sp., <i>Chaetoceros</i> sp., and <i>Isochrysis</i> sp. (Coast Seafoods Company, Quilcene, Washington). This suspension was provided continuously during holding and testing at a nominal rate of $2.9 \times 10^9$ cells/oyster/day.
Test system:	One test chamber, containing 20 oysters, was exposed to each treatment concentration and control (total of 20 oysters in the dilution water control, solvent control and 20 oysters in each test concentration).  Test chamber 52 L stainless steel aquaria filled with approximately 13 L of test solution. The depth of the test water in a representative test chamber was approximately 6.4 cm.  A continuous-flow diluter was used to provide each concentration of the test substance, a negative (dilution water) control and a solvent (DMF) control. A peristaltic pump was used to deliver the test substance stock solutions and DMF

	<p>for the solvent control into the mixing chambers assigned to the treatment groups. The stock solutions were mixed with dilution water in the mixing chambers in order to obtain the desired test concentrations. The flow of dilution water to the mixing chambers was controlled by rotameters.</p> <p>Prior to test start, the periphery of the shell margin of each oyster was ground with a fine grit grinder in order to establish a baseline for new shell growth.</p>
Test parameter:	<p>Temperature was measured in each test chamber at the beginning and end of the test. Temperature also was measured continuously in the negative control chamber. Dissolved oxygen and pH were measured in each test chamber at test initiation, at approximately 48 hours and at test termination. Dilution water salinity was measured at test initiation and at test termination.</p> <p>Oysters were visually observed at approximately 1.75, 24, 48, 72 and 96 hours after test initiation for mortality and clinical signs of toxicity.</p> <p>At the end of the test, the longest finger of new shell growth was measured to the nearest 0.05 mm using calipers.</p>
Analytical measurements:	<p>Water samples were also collected from each chamber at the beginning and end of the test for analysis. Samples were analyzed on each collection day using HPLC analysis.</p>
Statistic:	<p>Mean shell growth was calculated for each treatment and control group using "TOXSTAT Release 3.5". The data was evaluated for normality and homogeneity of variance using the Chi-Square test and Bartlett's test, respectively. Upon meeting these assumptions, the negative control and solvent control data were compared using a t-test to assess statistical differences in shell growth. When no statically significant differences were found at the 95% level of confidence, the control groups were pooled and growth inhibition was evaluated on the basis of the pooled control data using Bonferroni's t-test. The EC<sub>50</sub> value, the concentration of test substance that induced a 50% inhibition in shell deposition, was calculated using linear interpolation (4). The no-observed-effect-concentration was determined based on statistical analysis of the dose-response data. The EC<sub>50</sub> value, the concentration of test substance that induced a 50% inhibition in shell deposition, was calculated using linear interpolation. The no-</p>

observed-effect-concentration was determined based on statistical analysis of the dose-response data.

#### Findings:

**Analytical data:** Samples collected at 0 and 96 hours had measured concentrations that ranged from 10 to 122 % of nominal.  
The mean, measured concentration of thiadone during the test period ranged from 107 to 118 % of the nominal concentration.

**Biological effects:** No mortalities or sublethal effects of eastern oyster exposed to the thiadone were observed throughout the test duration in the controls and any concentration tested.

**Table B.9.2.4.2.5-1: Shell deposition and shell growth inhibition of *Crassostrea virginica* during 96 hour test.**

Mean measured concentration (mg p.m. /L)	Mean, shell deposition <sup>1</sup> (mm)	Shell Growth Inhibition
Water control	2.66 ± 0.405	-
Solvent control	2.63 ± 0.455	-
Pooled control	2.65 ± 0.426	-
2.71	2.34 ± 0.392	11.7
5.51	2.23 ± 0.5622*	15.8*
10.7	2.03 ± 0.6472*	23.4*
22.1	1.32 ± 0.7692*	50.2*
47	0.598 ± 0.4462*	77.4*

<sup>1</sup> Mean and standard deviation for 20 oysters.

\* Statistically significant different compared to control (using Bonferroni's t-test, p < 0.05)

**Conclusion:** The 96-hour EC<sub>50</sub> for *Crassostrea virginica* based on the mean, measured test concentrations of thiadone and shell deposition was estimated to be 22 mg met./L (95% CI: 17.8-29.9 mg p.m./L). The no-observed-effect concentration was estimated to be 2.71 mg p.m./L based upon the lack of statistically significant shell deposition and shell growth inhibition.

**RMS comments:**

The study was conducted according to the US EPA/FIFRA Guideline 72-3 (1985).

The study is in line with the test guideline US EPA, OPPTS 850.1025 (1996).

The following validity criteria outlined in the test guideline US EPA, OPPTS 850.1025 (1996) were considered to evaluate the validity of the results of the study.

- The mortality in the controls should not exceed 10% at the end of the test.

During the whole study period no mortality in the controls was observed.

-The dissolved oxygen concentration should be at least 60%.

The dissolved oxygen during the study test ranged from 82 to 90 % of air saturation.

-The concentration of the test substance was maintained over the test period (being 107-118% of nominal concentration).

-The environmental conditions (temperature, dissolved oxygen, salinity and pH) were measured at the beginning and at the end of the test in test chamber.

-The temperature should be 20 °C. Temporary fluctuations (less than 8 h) within  $\pm 5$  °C are permissible.

The test temperature was at range of  $22 \pm 1$  during the study.

-In the controls a minimum of 2 mm of new shell growth should be observed.

The new shell growth in the controls was between 1.80 and 3.80 mm with mean values of 2.66 mm (control) and 2.63 mm (solvent control) and 2.65 mm (pooled control).

The following deviation was noted from the guideline:

- No information on spawning was given in the study report.

Therefore, it can be assumed that no spawning was observed during the whole study period.

Although, the validity criterium consider new shell growth was not met, the results of the study are considered acceptable.

**Agreed endpoints:**

96 h  $EC_{50 \text{ shell growth}}$  = 22 mg Thiadone/L (95%CI: 17.8-29.9 mg p.m./L), based on mean measured test concentration

96h  $NOEC_{\text{shell growth}}$  = 2.71 mg Thiadone/L, based on mean measured test concentration

**B.9.2.5. Long-term and chronic toxicity to aquatic invertebrates****B. 9.2.5.1. Reproductive and development toxicity to Daphnia**

One prolonged study (exposure over 21 days) has been performed with *Daphnia magna*. This study was already submitted and accepted during the first EU evaluation of flufenacet. The study is still valid and appropriate for the re-evaluation of flufenacet.

**Table B.9.2.5.1-1: Chronic toxicity data of flufenacet to Daphnia magna**

Organism	Test substance	Endpoint (type of the test)	Value (mg a.s./L)	Reference
<i>Daphnia magna</i> Waterflea	Flufenacet	21- day NOEC (static-renewal reproduction)	3.26 mg	Gagliano G.G. & Bowers L.M. (1994) M-003795-01-1

**B.9.2.5.1.1. Chronic toxicity of FOE 5043 technical to the waterflea (*Daphnia magna*) under static renewal condition.**

<b>Reference:</b>	Chronic toxicity of FOE 5043 technical to the Waterflea ( <i>Daphnia magna</i> ) under static renewal condition.
<b>Author(s), year:</b>	Bowers L.M., Gagliano G.G., 1994
<b>Report/Doc. number:</b>	Study No: 106762, Reference BCS No: M-003795-01-1
<b>Guideline(s):</b>	US EPA /FIRA Guideline 72-4: <i>Daphnia magna</i> Life cycle chronic toxicity test.
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet, Batch No: FL-036, Purity 96.8%
Test species:	<i>Daphnia magna</i>
Age:	First instars < 24 hour old
Number of organism:	Thirteen test chambers were used for each of the concentrations and controls.
Loading:	The loading rate for each reproduction test vessel was approximately 1 daphnid per 200 ml of test solution. The loading rate for each survival test vessel was approximately 1 daphnid per 180 ml of test solution.
Type of test:	Static- renewal, 21 day life cycle

Applied concentrations:

Nominal:	Control (0), Solvent Control, (0), 0.9, 1.8, 3.5, 7, 14 mg a.s./L
Mean measured:	Control (0), Solvent Control (0), 0.82, 1.62, 3.26, 6.33, 12.8 mg a.s./L

---

Solvent:	Aceton, 100 µL/L
<u>Test conditions:</u>	
Water quality:	Dilution water (blended spring water)
Temperature:	19.2 to 21.8 °C (mean, 20.3°C)  For 32 hours, during Day 18 and 19 of the study, the test temperature was slightly higher than $20 \pm 1.0^\circ\text{C}$ . The temperature changes during this period were gradual and ranged from 21.1 to 21.8°C.
pH:	7.6-8.3
Alkalinity:	104-133 mg/L as $\text{CaCO}_3$
Hardness:	162-182 mg/L as $\text{CaCO}_3$
Conductivity:	379-424 µmhos/cm
O <sub>2</sub> content:	6.5-9.1 mg/L corresponding to 71 to 100 % of air saturation  The test solution was not aerated during the study.
Light regime:	16 hours light /8 hours darkness, The light intensity: approximately 590 Lux
Feeding:	Daphnids were fed algae ( <i>Selenastrum capricornutum</i> and/or <i>Ankistrodesmus falcatus</i> ) supplemented with a yeast, trout chow, cereal leaf suspension (YTC) during the study.
Test chamber:	Survival: 1-liter borosilicate glass beakers filled to an approximate volume of 900 ml test solution at a depth of approximately 10 cm.  Reproduction and growth: 250-ml borosilicate glass beakers filled to an approximate volume of 200 ml test solution at a depth of approx. 6 cm.
<u>Test parameters:</u>	Temperature, conductivity, total alkalinity, total hardness and pH were measured in alternating replicates of the survival vessels for the control, solvent control, low, middle and high concentrations.  Temperature was measured hourly from a centrally located test chamber. Manual temperatures were also recorded daily.  Dissolved oxygen was measured in one replicate of each test concentration. All of these parameters were measured in freshly made test solutions on Day 0, 4, 11, and 16, and in the old test solutions on Day 7, 14, and 21.  Samples of freshly prepared test solutions, including controls, were taken on Day 0, 4, 11, and 18. Samples of the old test solutions, including controls, were taken on Day 7, 14, and 21.  Test concentrations were renewed approximately every 72 hours.  High-performance liquid chromatography (HPLC) was used as analytical method.

Observation:	<p>Parent daphnids in the reproduction chambers were monitored daily until release of first broods, after which observations for the number of offspring produced were made on a Monday, Wednesday, Friday schedule. In renewal day, the neonates from each reproductive chamber were counted as soon as the parent daphnid was removed from the chamber. The neonates were then counted, the count was recorded, and the neonates were discarded. Observations for sublethal and behavioral effects of parent <i>Daphnia</i> were made on each day of the study. The body length of parent daphnids from all reproduction chambers was measured at test termination. Daphnids were measured from the apex of the helmet to the base of the posterior spine. Following body length determination, parent daphnids were dried at 60°C for at least 24 hours and individually weighed. Daphnids in the survival chambers were monitored on the same schedule as the daphnids in the reproduction chambers.</p>
Statistic:	<p>Data for the replicate chambers for each concentration were grouped together for analysis. For each analyzed parameter the following statistical tests were conducted:</p> <ol style="list-style-type: none"><li>1) t-test or Fisher's Exact test to determine if control and solvent control data could be pooled;</li><li>2) chi-square test to test the normality of the data set;</li><li>3) Bartlett's test for homogeneity of variances.</li></ol> <p>Control data were pooled if the t-test or Fisher's Exact Test criteria was met, otherwise only solvent control data were used for further analyses. Survival data of adult daphnids was analyzed using analysis of variance (ANOVA). The data from each of the three survival chambers per concentration was used for the statistical analysis for survival.</p> <p>Reproduction data was analyzed by a one-way analysis of variance (ANOVA) to determine if there was a significant difference between the treatment groups and control groups. If the results of the ANOVA showed a significant difference (<math>\alpha = 0.05</math>) then a Dunnett's one-tailed multiple means comparison test was conducted to identify which treatment group(s) were significantly different from the control groups.</p> <p>Time to first brood and parent daphnid growth (length and dry weight) data were assessed in the same way as the reproduction data.</p>

Findings:

Analytical data: The mean measured concentrations of flufenacet ranged from 90 to 93 % of the nominal.

A summary of effects following exposure of *Daphnia magna* are presented in the Table B.9.2.5.1.1-1 below.

**Table B.9.2.5.1.1-1: Summary of effects following exposure of *Daphnia magna* to flufenacet for 21 days.**

Mean measured concentration (mg a.s./L)	Mean adult survival <sup>a</sup> %	Time to 1 <sup>st</sup> brood (days, mean)	Mean total live young <sup>b,d</sup>	Mean Young/Reprod. Days <sup>c,d</sup>	Mean adult length (mm) <sup>e</sup>	Mean adult dry weight (mg) <sup>e</sup>
Control	87	8-10 9.7 ± 0.7	178	16.4 ± 1.0	5.39 ± 0.15	1.061 ± 0.292
Solvent control	100	9-10 9.9 ± 0.3	189	15.7 ± 2.2	5.35 ± 0.22	1.355 ± 0.176
Pooled control	93	9.8 ± 0.5	na	16.0 ± 1.7	5.36 ± 0.19	na
0.82	100	9-10 9.9 ± 0.3	196 <sup>d</sup>	16.2 <sup>1d</sup> ± 1.3	5.32 ± 0.12	1.401 ± 0.138
1.62	100	8-10 9.4 ± 0.8	207 <sup>d</sup>	16.5 <sup>2d</sup> ± 2.0	5.41 ± 0.08	1.422 ± 107
3.26	60*	8-10 9.6 ± 0.7	189 <sup>d</sup>	15.1 <sup>3,4d</sup> ± 1.9	5.37 ± 0.15	1.445 ± 0.150
6.33	100	10-13 11.3 ± 1.2**	148**	13.9** ± 3.1	5.32 ± 0.11	1.561 ± 0.184
12.8	100	-**	0	-**	5.28 ± 0.14	1.431 ± 0.194

± SD standard deviation, na not applicable

- No neonates were produced at this test concentration.

a Percent of adult alive at the end of test.

b Mean number of live young produced in 21 days/ adult. Sum of all replicates.

c Mean number of young /adult, divided by number of reproduction days.

d Based on the total number of surviving young present. Daphnids that died prior to the start of the reproduction phase or died prior to test termination and the young that they produced were excluded from the calculation of the mean percent of adults producing young and the mean number of young per reproduction days.

<sup>1</sup> The daphnid in replicate 10, died on Day 12. Since the daphnid was alive for only two reproduction days, the average value for 10.0 young/adult/repro day this replicate was not included in the statistical analysis.

<sup>2</sup> The daphnid in, replicate 12, died on Day 7. Since the daphnid died prior to start of the reproduction phase, it was not included in the reproduction phase statistical analysis.

<sup>3</sup> The daphnid replicate 8 died on Day 2. Since the daphnid died prior to start of the reproduction phase it was not included in the reproduction phase statistical analysis.

<sup>4</sup> The daphnid in, replicate 9, died on Day 13. Since the daphnid was alive for only 3 reproduction days, this average value (4.7 young/adult/repro day) was not included in the statistical analysis.

<sup>e</sup> No statistically significant differences in mean total length or in mean dry weight from the pooled control.

\* Statistically significantly different from pooled control (Fisher's exact test,  $\alpha=0.05$ ) however, it did not follow a dose responsive pattern.

\*\* Statistically significant from pooled control (Fisher's exact test,  $\alpha=0.05$ ).

**Conclusion:**

Statistically significant difference compared to pooled control in survival adults was observed at test concentration of 3.26 mg a.s./L. This effect is, however, considered as non-treatment related, since no statistically significant effects on this parameter were observed at two higher concentrations.

The 21-day NOEC values for *Daphnia magna* based on adult survival and growth (total length and dry weight), and mean measured concentrations of flufenacet were estimated to be 12.8 mg a.s./L and the 21-day NOEC value based on reproduction and mean, measured test concentration was 3.26 mg a.s./L.

The 21-day EC<sub>50</sub> value for adult immobility was >12.8 mg a.s./L, the highest mean, measured test concentration.

**RMS comments :**

The study is in general agreement with the current valid test guidelines 850.1350 OPPTS and OECD 211 (2008).

The validity criteria given in test guidelines US EPA, OPPTS 850.1350 and OECD 211 (2008) are met.

- The controls mortality of parent animals not exceeded 20% (being: 7-13%).
- The mean number of living offspring produced per parent animal surviving at the end of test was >60% (being: 71-100%).

The following deviation from test guideline were noted:

- For 32 hours, during Day 18 and 19 of the study the test temperature was slightly higher than  $20 \pm 1.0^{\circ}\text{C}$ , which is recommended by guidelines US EPA, OPPTS 850.1350 and OECD 211 (2008) are met.

The temperature changes during this period were gradual and ranged from 21.1 to 21.8°C.

- On Day 3, the daphnids were fed only yeast, trout chow, and cereal leaf suspension without algae.
- Light intensity at the test initiation was 590 Lux (range 1000-1500 Lux is recommended by the guideline OECD 211, 2008).
- No identification of sexes of young was performed.

Since all validity criteria were met, these deviations are considered as having no impact on results of the study.

Hence, the study is considered acceptable.

**Agreed endpoint:**

NOEC<sub>survival</sub> = 12.8 mg a.s./L, based on mean measured concentration

NOEC<sub>growth</sub> = 12.8 mg a.s./L, based on mean measured concentration

NOEC<sub>reproduction</sub> = 3.26 mg a.s./L, based on mean measured concentration

**B.9.2.5.2. Reproductive and developmental toxicity to an additional aquatic invertebrates species.**

The new prolonged study (exposure over 28 days) has been performed with *Americamysis bahia* for the process of the renewal of flufenacet.

**B.9.2.5.2-1. Reproductive and development toxicity to an additional aquatic invertebrate species.**

Organism	Test substance	Endpoint (type of the test)	Value (mg a.s./L)	Reference
<i>Americamysis bahia</i> Mysid shrimp	Flufenacet	NOEC 28 d (flow-through, mortality, reproduction)	<b>0.221 mm</b>	Claude, M.B. et al. (2013) M-452207-01-1

**B.9.2.5.2.1. A flow-through life-cycle toxicity test with the saltwater mysid (*Americamysis bahia*).**

<b>Reference:</b>	Flufenacet: A flow-through life-cycle toxicity test with the saltwater mysid ( <i>Americamysis bahia</i> ).
<b>Author(s), year:</b>	Claude M.B., Martin K.H., Gallagher S.P., Krueger H.O., 2013
<b>Report/Doc. number:</b>	Report No: EBFOL243, Reference BCS No: M-452207-01-1
<b>Guideline(s):</b>	US EPA, OPPTS 850.1350, ASTM Standard E1191-033a
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet tech. Batch No: AE F133402-01-17, Purity 97.49%
Test species:	<i>Americamysis bahia</i> , saltwater mysid
Number of organism:	4 replicates each with 15 organism per treatment and control
Age:	< 24 hours old
Type of test:	flow-through test, 31 days

Applied concentrations:

The effects of flufenacet on the survival, growth and reproduction of *Americamysis bahia* were determined in one exploratory non-GLP pilot study in a flow-through, 31-day test (presented together with the main study) Nominal concentrations selected for use were 20, 50 and 500 µg a.s./L. Based on the effects on reproduction observed in the 500 µg a.s./L treatment group, the NOEC was 50 µg a.s./L. Based on this study results the following application doses were estimated for the current study:

Nominal:	0 (control), 30, 60, 120, 240, and 480 µg a.s./L
Mean Measured:	0 (control), 33, 68, 126, 221, and 469 µg a.s./L

---

Solvent:	None
<u>Test condition:</u>	
Water quality:	Dilution water: (Natural sea water, mean salinity 20 ‰)
Temperature:	24.9-26.2°C
pH:	7.9
Salinity:	19-20 ‰
O <sub>2</sub> content:	7.3-7.4 mg/L corresponding to 99-100 % of air saturation.
Light regime:	14 hours light /10 hours darkness. Light intensity: 220 Lux
Feeding:	During the test, the mysids were fed live brine shrimp nauplii ( <i>Artemia</i> sp.) up to four times daily. In addition, the mysids were fed the enriched brine shrimp for one of the daily feedings during the test, when available.
Test parameters:	<p>Measurements of pH were made in one replicate test chamber of each treatment and control group at the beginning and end of the test, and approximately weekly during the test, with measurements typically rotating among the replicates in each group at each measurement interval.</p> <p>Salinity was measured daily in one replicate of the negative control, with measurements typically rotating among the replicates in the group at each measurement interval. When 100% mortality occurred in a test chamber, measurements of temperature, dissolved oxygen and pH were taken in that test chamber and then discontinued.</p> <p>Prior to pairing, dissolved oxygen was measured in one replicate test chamber of each treatment and control group at the beginning of the test and approximately weekly during the test period, with measurements typically rotating among the replicates in each group at each measurement interval. After mysids attained sexual maturity and were paired on Day 14, dissolved oxygen was measured daily until the end of the test in one replicate test chamber of each treatment and control group, with measurements typically rotating among the replicates in each group at each measurement interval.</p>
Test chamber:	<p>Juvenile Phase: 9-L glass aquaria containing approximately 2.5 L of test solution.</p> <p>Juvenile compartments were 2-L glass containers measuring approximately 12 cm in diameter and 19 cm in height, with two nylon mesh-covered holes on opposite sides of the container</p>

---

	<p>Adult Phase: 19-L glass aquaria filled with approximately 14.5 L of test solution, which contained a self-starting siphoning system to exchange test solution.</p> <p>The reproductive compartments were approximately 10-cm diameter glass petri dishes with sides of nylon mesh screen.</p>
Methods:	<p>A total of 4 replicates, each containing fifteen &lt;24-hour-old neonates, were tested per compartment, one compartment per concentration and control group (60 neonates/concentration). On Day 14 of the test, after mysids attained sexual maturity, male and female adults were paired in each treatment and control group, with a maximum of five reproductive pairs per replicate.</p> <p>The test was terminated on Day 31, which was at least seven days past the median time of first brood release for the negative control (Day 22).</p> <p>At test termination, the sex of each surviving first-generation mysid was confirmed and the total length of each mysid was measured using calipers. The mysids then were placed in a drying oven at approximately 60° C for approximately 42 to 46 hours to obtain dry weight data.</p>
Observation:	<p>Observations were made daily of the survival and behaviour of each first-generation mysid. After the mysids were sexually identified and paired, and with the onset of reproduction, young mysids that were produced were counted, recorded and removed daily. Second-generation mysids were also observed for abnormal development and aberrant behaviour. At test termination (on Day 31), the total body lengths and dry weights of all surviving first-generation mysids were measured.</p>
<u>Analytical data:</u>	<p>Water samples also were collected from alternating replicate test chambers in each treatment and control group at the beginning of the test, approximately weekly during the test and at test termination to measure concentrations of the test substance.</p> <p>The analytical method consisted of samples water analyzing by direct injection high performance liquid chromatography HPLC-UV.</p>
Statistic:	<p>Survival and percent of surviving females producing young data was considered to be discrete-variable data, while the number of young produced per reproductive day, the number of young produced per surviving female and growth data were considered continuous-variable data.</p> <p>Discrete-variable data were analyzed using Chi-square and Fisher's Exact tests to identify treatment groups that showed a statistically significant difference from</p>

the negative control ( $p \leq 0.05$ ). All continuous-variable data were evaluated for normality using the Shapiro-Wilk's test and for homogeneity of variance using Levene's test ( $p = 0.01$ ). The data for all parameters passed the assumptions of normality and homogeneity of variance. Those treatment means that were significantly different from the negative control means were identified using Dunnett's test ( $p \leq 0.05$ ).

#### Findings:

Analytical results: The mean measured concentration of flufenacet during the test period ranged from 92 to 113% of nominal.

A summary of percent juvenile survival, adult survival, young per reproductive day, young produced per surviving female, percent of surviving females producing young, and male and female total body length and dry weight of surviving adults is shown in Table B.9.2.5.2.1-1 below.

**Table B.9.2.5.2.1-1: Summary of effects on survival and reproduction following exposure of *Americamysis bahia* to flufenacet for 31 days.**

Mean measured concentration ( $\mu\text{g a.s./L}$ )	Mean % juvenile survival <sup>a</sup>	Mean % adult survival <sup>b</sup>	Mean number of young produced per reproductive day <sup>3</sup>	Mean % of surviving females producing young <sup>4,5</sup>	Mean number of young per surviving female <sup>3, 5</sup>
Control	91.7	91.8	$0.641 \pm 0.189$	95	$9.8 \pm 2.53$
33	75.0* <sup>1</sup>	89.7	$0.832 \pm 0.250$	100	$13.0 \pm 3.97$
68	100	74.5* <sup>1</sup>	$0.708 \pm 0.171$	100	$10.9 \pm 2.39$
126	98.3	85.3	$0.593 \pm 0.174$	100	$8.9 \pm 2.62$
221	95.0	88.9	$0.726 \pm 0.087$	100	$9.8 \pm 2.07$
469	100 <sup>2</sup>	84.6	$0.453 \pm 0.064$ <sup>6</sup>	100	$6.8 \pm 0.96$ <sup>6</sup>

$\pm$  SD standard deviation

\* Statistically significant decrease in survival in comparison the control (Fisher's Exact test ,  $p \leq 0.05$ ).

a Percent of juvenile mysids alive at pairing on Day 14 of the test.

b Percent of adult mysids alive from Day 15 to the end of the test.

1 While the decrease in survival was statistically significant in comparison to the control, it was not considered to be treatment-related since the difference was not dose-responsive. There were no other unusual sublethal observations in the group, which is further evidence that survivorship does not appear to be dose related.

2 There were 16 mysids were observed in Replicate C, and it was inferred that Replicate C was initiated with 16 mysids. Percent survival for Replicate C was calculated based on 16 mysids initially exposed.

- 3 There were no statistically significant decreases in reproduction and mean number of young per surviving female in comparison to the negative control (Dunnett's test,  $p > 0.05$ ).
- 4 There were no statistically significant decreases in percent of females producing young in comparison to the control (Fisher's Exact test,  $p > 0.05$ ).
- 5 Calculated based on the total number of surviving females present at test termination. Females that died prior to test termination and the young that they produced were excluded from the calculation of the mean percent of surviving females producing young and the mean number of young per surviving female.
- 6 While the decreases in the mean number of young produced per reproductive day and mean number of young per surviving female were not statistically significant in comparison to control, there was an apparent decrease evident in the 469 µg a.s./L treatment group and, therefore, a treatment-related effect could not be precluded for this level.

**Table B.9.2.5.2.1-2: Summary of growth of *Americamysis bahia* following exposure to flufenacet on Day 31<sup>1</sup>.**

Mean measured concentration (µg a.s./L)	Mean male total length (mm)	Mean female total length (mm)	Mean male dry weight (mg)	Mean female dry weight (mg)
Water control	7.43 ± 0.120	7.52 ± 0.261	1.10 ± 0.075	1.35 ± 0.167
33	7.32 ± 0.18	7.41 ± 0.23	1.16 ± 0.054	1.33 ± 0.140
68	7.32 ± 0.199	7.39 ± 0.221	1.11 ± 0.085	1.41 ± 0.145
126	7.42 ± 0.197	7.34 ± 0.166	1.15 ± 0.062	1.35 ± 0.187
221	7.33 ± 0.077	7.48 ± 0.120	1.13 ± 0.111	1.47 ± 0.130
469	7.31 ± 0.124	7.47 ± 0.076	1.20 ± 0.106	1.47 ± 0.078

±SD Standard deviation

<sup>1</sup> No statistically significant decreases in comparison to control (Dunnett's test,  $p > 0.05$ )

#### Conclusion:

Reproduction, measured the mean number of young per surviving female and the mean number of young per reproductive day, was the most sensitive biological endpoint measured.

While no statistical difference were noted in the reproduction endpoints, there was a decrease in reproduction in the 469 µg a.s./L treatment group, and hence a treatment-related effect could not be precluded for this level.

Consequently, the NOEC<sub>reproduction</sub>, was determined to be 221 µg a.s./L, NOEC<sub>survival</sub> was 469 µg a.s./L and NOEC<sub>growth</sub> was determined to be 469 µg a.s./L. The LOEC was estimated to be 469 µg a.s./L and the MATC was 322 µg a.s./L.

In accordance with the new data requirement (Commission Regulation EU No 283/2013), the EC<sub>10</sub>, EC<sub>20</sub>, values should be calculated. Where they can not be estimated, an explanation shall be provided.

**RMS comments:**

The study was conducted according to OPPTS 850.1350 test guideline.

The validity criteria given in the test guidelines US EPA, OPPTS 850.1350 are met.

- The dissolved oxygen throughout the test ranged between 99 and 100 % of air saturation (the range between 60-105% is recommended in test guideline).
- The test conditions parameters ( e.i.: temperature, salinity, pH) were in line with recommendations given in the test guideline.
- In the control 95% of first-generation females produce the average  $9.8 \pm 2.53$  of young (at least 75% of the first-generation females in the control(s) must produce young and that the average be at least three young produced per first-generation female is recommended, according to recommendation given in test guideline US EPA, OPPTS 850.1350.

The study is considered acceptable.

**Agreed endpoints:**

NOEC<sub>reproduction</sub> = 221 µg a.s./L, based on mean measured concentration

NOEC<sub>survival</sub> = 469 µg a.s./L, based on mean measured concentration

NOEC<sub>growth</sub> = 469 µg a.s./L, based on mean measured concentration

**B.9.2.5.3. Development and emergence in Chironomus species**

For propose of the renewal toxicity study was performed according to the OECD with Chironomus riparius.

Details of study are provided briefly in the Table B.9.2.5.3-1.

Summaries of the study is provided thereafter.

**Table B.9.2.5.3-1. Effects of flufenacet to *Chironomus riparius*.**

Organism	Test substance	Endpoint (type of the test)	Value (mg a.s./L)	Reference
<b>Sediment dwelling organism</b>				
<i>Chironomus riparius</i>	Flufenacet	28 d NOEC (emergence,development) Static test	5 mg a.s./L	Bruns E. (2010) M-372857-01-1

**B.9.2.5.3.1. *Chironomus riparius* 28-day chronic toxicity test with flufenacet (tech) in water-sediment system using spiked water.**


---

<b>Reference:</b>	<i>Chironomus riparius</i> 28-day chronic toxicity test with flufenacet (tech.) in water-sediment system using spiked water.
<b>Author(s), year:</b>	Bruns E., 2010
<b>Report/Doc. number:</b>	Study No: EBFOL153, Reference BCS No: M-372857-01-1
<b>Guideline(s):</b>	OECD 219 (adopted 13 April 2004).
<b>GLP:</b>	Yes

---

Material and methods:

Test substance:	Flufenacet (tech), Batch No: K664078, Purity 97.5%
Test species:	<i>Chironomus riparius</i>
Age:	The first instars, approx. 2-3 day old
Type of test:	Static water-sediment test, 28 days
Test medium:	M7-medium
Number of organism:	4 replicates each with 20 larvae per treatment and control groups. Additional replicates (with chironomids) were used for chemical analysis of the test item on day 0 and day 7 for all test concentrations and the controls (2 replicates). For the chemical analysis on day 28 one beaker of the four beakers for biological evaluations was used. A further replicate (n=1) of each test concentration was prepared with chironomids to measure the temperature, pH and oxygen content in the test water during the study.

Applied concentrations:

Nominal: 0 (control), 1.25, 2.50, 5.00, 10.0 and 20.0 mg a.s./L  
(spiked water application)

Solvent: None

Test conditions:

Water quality:	M7-medium, based on deionised water: total hardness 271.45 as CaCO <sub>3</sub> mg/L (mean), alkalinity 151.3 as CaCO <sub>3</sub> mg/L (mean), ammonia (1-1-1.2 in the control) and 21.3-31.9 in the highest test concentration
Temperature:	20.4-20.8 °C
pH:	8.3-8.7
O <sub>2</sub> content:	7.2-8.3 mg/L corresponding to 81 to 93 % of air saturation
Light regime:	16 hours light /8 hours darkness. Light intensity: 826 Lux (mean)
Test sediment:	Artificial sediment was used in the test, prepared 8 days before the start of the

exposure period. It consists of 75.8 % quartz, 4% sphagnum moss peat (pH 5.0-6.5, particle size of < 1 mm, air dried), 20 % kaolinite (kaolinite content of 30.2 %, pH value 5.5 - 7.5, "Kaolin W"), 0.1% calcium carbonate to adjust the pH value to  $7 \pm 0.5$  and 45% deionised water.

Sediment characterization:

Experimental Day -7:

pH: 6.9

Water content: 31.4%

Organic carbon: 2.0 %

Feeding:

During the study the larvae were fed at least about three times per week with a commercial ornamental fish food extract (trade name etra Phyll®) as used for the breeding. An appropriate amount of this suspension (about 0.5 - 1 mg Tetraphyll® /Larvae/day) was added to each test container.

Methods:

A layer of ca. 1,5 cm of wet sediment ( according to about 140 g) was added to each test vessel ( 600 mL glass beakers). Thereafter 6 cm of M-7 medium was added to the sediment. Thus the height ratio sediment: overlying water was 1:4. For acclimatization and equilibration the test vessels were prepared 7 days before the study commenced. One day prior to treatment (Day -1) the test organisms (L1-larvae) were transferred with pipette in a randomized procedure into the test vessels. Number of larvae per replicate was 20.

One day after adding the test substance was added to the water column using a pipette (Day 0). Gentle aeration was provided through a glass pipette situated about 2.5 cm above the sediment layer throughout the complete study over 28 days. Test beakers were covered by clear plastic plates, preventing evaporation.

Test parameters:

Because all test vessels are placed under isothermal\_conditions, a continuous temperature measurement in one vessel was done to calculate the mean, min, and max figures for water temperature (based on continuously (hourly means) measured values). Temperature values are measured in one control beaker and recorded hourly. Additionally the temperature is measured once a week in the overlying water of the additional test vessels.

One day prior to the start of the study and later on once a\_week, samples of the water column of the additional test vessels for water parameter measurements of each test level incl. control(s) were taken, and the pH of these samples were measured. At the end of the test (day 28) pH was measured in all test vessels additionally. Dissolved oxygen in the overlying water phase of the additional test

vessels for water parameter measurements of each test level incl. control(s) was measured twice per week. At the end of the test (day 28) dissolved oxygen concentration was measured in all test vessels additionally. Measurements of total hardness of the control and the highest test concentration (water phase) were performed on day 0 and day 28. Measurements of ammonia of the control and the highest test concentration (water phase) were performed on day 0 and day 28.

Observation:

The test vessels were observed at least three times per week to make a visual assessment of any behavioural differences compared to the control. The sex, time point of emergence and number of emerged midges was recorded daily during the period of emergence. As only fully emerged adults are relevant for the endpoints of this study, larvae which did not yet mature were not taken into account for emergence rates and development time. To determine number and sex of emerged adults, the covering plates of each test container were carefully moved and the midges, which mostly stayed at the sides of the vessels, were enumerated; after identification of the sex (male midges have feathered antennae) midges were removed.

Statistic:

ECx values and confidence intervals after 28 days were calculated by probit (or logit, weibit, etc.) analysis or in case of failure by non parametric-methods from the appropriate parameters (endpoints), using a commercial program. The LOEC determinations from the appropriate parameters (endpoints) were done, using the ANOVA procedure ( $\alpha = 0.05$ , one sided) and properly selected multiple t-tests a commercial program.

In case of a limit test (comparison of control and one treatment group only) the STUDENT t-test can be used.

Calculations were carried out using Microsoft Excel® spreadsheets. All further statistical evaluations were done using the commercial program ToxRat Professional.

Analytical data:

The concentration of flufenacet was analysed in the overlying water column and the pore water of the sediment at 1 hour, 7 days and 28 days after application in an additional prepared test vessels of all test concentrations and controls. Additional analyses in the sediment were considered to be not necessary because the partitioning of the active ingredient between water and sediment is known from water/sediment studies done under comparable conditions. The analysed

concentrations over time in the overlying water and the pore water reflect the expected distribution of the active ingredient between the compartments.

High-performance liquid chromatography HPLC-UV and was used as analytical method.

#### Findings:

Analytical measurements: The mean measured concentrations of the active substance in the overlying water were between 83 % (1 hour after spiking) and 14 % (28 days after spiking). The recovered activity in the pore water was between 1.4% (one hour after spiking) and 0.7% (after 28 days).

#### Biological effects:

**Table B.9.2.5.3.1-1: Influence on emergence and development rate after 28 days exposure of flufenacet.**

Initial nominal concentration (mg a.s./L)	Number of introduced midges	Number of emerged midges	Start and end of emergency (days)	Emergence of inserted larvae			Development pooled sex rate (1/d)
				total %	male %	Female %	
Controls (pooled) <sup>1</sup>	160	148	14-27	92.5	50.6	41.9	0.057
1.25	80	70	14-27	87.5	38.8	48.8	0.057
2.5	80	74	14-27	92.5	48.8	43.8	0.059
5	80	69	14-27	86.3	40.0	46.3	0.054
10	80	64*	14-27	80.0*	42.5	37.5	0.053*
20	80	2	18-27	2.5*	2.5	0	0.056

<sup>1</sup> No statistically different distribution between sexes compared to the assumption of 50% females and 50% males. Therefore male and female results were pooled (Ch2 2-2 Test,  $\alpha > 0.05$ , two-side).

\* Statistically significant reductions on number of emerged midges, emergence of inserted larvae (Chi<sup>2</sup>-2 x 2 Test with Bonferroni Correction,  $\alpha = 0.05$ , one-sided) and development rate (Dunnett's multiple t-test,  $\alpha = 0.05$  one-sided smaller).

Conclusion: There were treatment related effects observed for emergence ratios between the pooled control group and the 10 and 20 mg a.s./L treatment groups. There was also a treatment related effect observed for development rate between the pooled control and the 10 mg a.s./L treatment group.

Therefore, the 28-day LOEC was 10 mg a.s./L and the NOEC was 5 mg a.s./L based on nominal test concentration.

The 28-day EC<sub>50</sub> value based on emergence rate (pooled sex) of *Chironomus riparius* exposed to water-sediment system was 12.9 mg a.s./L (95% CI: 9.87 -

17.1 mg a.s./L) and the EC<sub>15</sub> value was estimated to be 10.2 mg a.s./L ( CI: 6.03-12.5 mg a.s./L). Based on development rate (pooled sex) - EC<sub>15</sub> and EC<sub>50</sub> values were determined as >20 mg a.s./L (based on nominal concentration).

**RMS comments:**

The study was conducted in line with test guidelines OECD 219 (2004).

The validity criteria given in test guideline are met.

-The emergence in the controls was at least 70% (being 92.5%, pooled control).

- The emergence to adults from control vessels should occur between 12 and 23 days after their insertion into vessels.

In the present study the emergence to adults from control vessels started on Day 14 and last emergence occurred on Day 27. Taken into consideration that 86.6 % of the controls emerged before day 23, the slightly delayed emergence of 4.7% , in opinion of RMS is having no negative impact on the outcome of the study. In addition, it should be noted that emergence in the control was above 70% (being 92.5% in pooled control).

- The water temperature should not differ by more than  $\pm 1^{\circ}\text{C}$ . The water temperature in the test vessels was in line with the validity criterion (being 20.4-20.8°C).

- At the end of the test, pH and the dissolved oxygen concentration should be measured in each vessel.

The oxygen concentration should be at least 60% of the air saturation at the temperature used, and the pH of overlying water should be in the 6 – 9 range in all test vessels.

In the study the oxygen concentration was >60% (ranged between 81 and 93% of air saturation, and the pH between 8.3 and 8.7 were recorded during the study).

Sediment used in the study fulfilled the guideline OECD 219, 2004 requirements.

The study is considered acceptable.

**Agreed endpoints:**

EC<sub>50</sub>( emergence rate) = 12.9 mg a.s./L (95% CI: 9.87 - 17.1 mg a.s./L), based on nominal concentration

EC<sub>50</sub>( developmental rate) > 20 mg a.s./L

NOEC<sub>(emergence, development)</sub> = 5 mg a.s./L, based on nominal concentration

**B.9.2.6. Effects on algal growth**

Potential effects of flufenacet on algal growth were investigated with different algal species, a green alga, a blue-green alga, marine and estuarine diatom. *Pseudokirchneriella subcapitata* was identified as the most sensitive species. For this species two standard laboratory studies were performed. The older study revealed the lowest endpoint of 96 h  $E_rC_{50}$ =0.00315 mg a.s./L and the new one study with 72 h  $E_rC_{50}$ =0.0212 mg a.s./L. In addition, in one non standard study for this species a relatively low endpoint of 96 h  $E_rC_{50}$  of 0.00645 mg a.s./L was determined. The studies for *Skeletonema costatum* were considered not acceptable.

For tested metabolites such as FOE oxalate, FOE methylsulfide, FOE methylsulfone, TFA and FOE-Trifluoroethanesulfonic acid performed with green alga *Pseudokirchneriella subcapitata* the lower toxicity in comparison to parent compound were recorded.

For metabolites FOE 5043-Thiadone, the studies for *Pseudokirchneriella subcapitata* were considered not fully reliable but an acceptable indication of low concern.

Potential effects of product DFF+FFA SC 600 on algal growth were also investigated with green alga and the study *Pseudokirchneriella subcapitata* resulted in a 72 h  $E_rC_{50}$  of 0.0063 mg a.s./L.

**Table B.9.3-1: Toxicity of Flufenacet to aquatic organism.**

Organism	Test substance	Endpoint (type of the test)	Value (mg a.s./L)	Reference
<b>Algae</b>				
Green algae <i>Pseudokirchneriella subcapitata</i>	Flufenacet	96 h $E_rC_{50}$ NOE <sub>r</sub> C 72 h $E_yC_{50}$ NOE <sub>y</sub> C (static test)	<b>0.00315 im</b> 0.00064 im 0.001783 im 0.00064 im	Bowers L.M,1995 Dorgerloh M M-086475-01-1
Green algae <i>Pseudokirchneriella subcapitata</i>	Flufenacet	72 h $E_rC_{50}$ NOE <sub>r</sub> C 72 h $E_yC_{50}$ NOE <sub>y</sub> C ( static test)	<b>0.0212 geom</b> 0.000138geom 0.00538 geom 0.000138geom	Bruns E (2010) M-363891-03-1
Green algae <i>Pseudokirchneriella subcapitata</i>	Flufenacet	<b>72/96h</b> Geomean*	<b><math>E_rC_{50}</math></b> <b>0.00755*</b>	Bruns E (2010) M-363891-03-1 Dorgerloh M (1998) M-086475-01-1
Green algae <i>Desmodesmus subspicatus</i>	Flufenacet	72 h $E_rC_{50}$ NOE <sub>r</sub> C 72 h $E_yC_{50}$ NOE <sub>y</sub> C (static test)	0.675 geom 0.0084 geom 0.07696 geom 0.0084 geom	Bruns E. (2011) M-415813-01-1
Green algae	Flufenacet	96 h $E_rC_{50}$	<b>0.00645 nom.</b>	Anderson, J. P. E. (1997)

Organism	Test substance	Endpoint (type of the test)	Value (mg a.s./L)	Reference
<i>Pseudokirchneriella subcapitata</i>		(Static)		M-002343-01-1 <sup>2</sup>
Green algae <i>Chlorella vulgaris</i>	Flufenacet	72 h ErC <sub>50</sub> NOE <sub>r</sub> C 72 h E <sub>y</sub> C <sub>50</sub> NOE <sub>y</sub> C (static test)	11.1 nom 0.98 nom 3.71 nom 0.98 nom	Bruns E. (2011) M-416169-01-1
Blue algae <i>Synechococcus leopoliensis</i>	Flufenacet	72 h ErC <sub>50</sub> NOE <sub>r</sub> C 72 h E <sub>y</sub> C <sub>50</sub> NOE <sub>y</sub> C ( static test)	>10 nom 0.307 nom >10 nom 0.096 nom	Bruns E. (2011) M-415814-01-1
Blue-green algae <i>Anabaena flos-aquae</i>	Flufenacet	96h E <sub>r</sub> C <sub>50</sub> 96h E <sub>y</sub> C <sub>50</sub> NOE <sub>r</sub> C NOE <sub>y</sub> C (statitc test)	>53.2 mm 26.65 mm 3.77mm < 1.930 mm	Hugens&Alexander (1993) M-002423-01-1
Freshwater diatom <i>Navicula pelliculosa</i>	Flufenacet	96h E <sub>r</sub> C <sub>50</sub> 96h E <sub>y</sub> C <sub>50</sub> NOE <sub>r</sub> C NOE <sub>y</sub> C (statitc test)	5.044 mm 2.13 mm 1.120 mm 1.120 mm	Bowers, L. M.; Dobbs, M. G. (1995) M-002355-01-1 <sup>1a</sup>
<i>Chlamydomonas terricola</i>	Flufenacet	216 h E <sub>r</sub> C <sub>50</sub> NOE <sub>r</sub> C 216 E <sub>y</sub> C <sub>50</sub> NOE <sub>y</sub> C	0.657 nom 0.096 nom 0.332 nom 0.0960 nom	Sobczyk H (2011) M-418627-01-1
Green algae <i>Pseudokirchneriella subcapitata</i>	FOE oxalate	72 h ErC <sub>50</sub> NOErC 72 h EbC <sub>50</sub> NOEbC ( static test)	<b>&gt;100 nom</b> >100 nom >100 nom >100 nom	Bruns E. (2009) M-358823-011
Green algae <i>Pseudokirchneriella subcapitata</i>	FOE methylsulfide	72 h ErC <sub>50</sub> NOErC 72 h EbC <sub>50</sub> NOEbC	<b>83.8 nom</b> 10 nom 30.5 nom 10 nom	Dogerlorh (1998) M-002341-01-1
Green algae <i>Pseudokirchneriella subcapitata</i>	FOE methylsulfone	72 h ErC <sub>50</sub> NOErC (static test)	<b>&gt;10 nom</b> >10 nom	Bruns (2010) M-364591-01-1
Green algae <i>Desmodesmus subspicatus</i>	FOE sulfonic acid	72 h ErC <sub>50</sub> 72 h EbC <sub>50</sub> NOEbC NOErC (static test)	<b>&gt;86.7 nom</b> >86.7 nom ≥86.7 nom ≥86.7 nom	Anderson (1995) M-004931-01-1
Green algae	TFA	72 h ErC <sub>50</sub>	192.48 nom	Groeneveld et al.

Organism	Test substance	Endpoint (type of the test)	Value (mg a.s./L)	Reference
<i>Pseudokirchneriella subcapitata</i>		72 h E <sub>y</sub> C <sub>50</sub> NOE <sub>r</sub> C NOE <sub>y</sub> C (static test)	4.19 nom 0.36 nom <0.36nom	(1992) M-247820-01-1
Green algae <i>Pseudokirchneriella subcapitata</i>	TFA	72 h E <sub>r</sub> C <sub>50</sub> 72 h E <sub>b</sub> C <sub>50</sub> NOE <sub>b,r</sub> C	> <b>1.2 nom</b> >1.2 nom 0.12 nom	Berends & Molenaar (1993) M-247818-02-1
Green algae <i>Scenedesmus supspicatus</i>	TFA	EC <sub>50</sub>	>120 nom	Berends , Keetelaar-Jansen, van Dijk(1995) M-247825-01-1 <sup>1</sup>
Green algae (various species)	TFA	E <sub>r</sub> C <sub>50</sub>	>112 to >2400	Berends (1996) M-247818-02-1 <sup>2</sup>
Green algae <i>Pseudokirchneriella subcapitata</i>	FOE5043- (Trifluoroethane sulfonic acid)	96 h E <sub>r</sub> C <sub>50</sub> 96 h E <sub>y</sub> C <sub>50</sub> NOE <sub>y</sub> C NOE <sub>r</sub> C	> <b>100 nom</b> >100 nom >100 nom >100 nom	Bruns E. (2012) M-444217-01-1
Green algae <i>Pseudokirchneriella subcapitata</i>	FOE Thiadone	72 h E <sub>r</sub> C <sub>50</sub> 72 h NOE <sub>r</sub> C 72 h E <sub>b</sub> C <sub>50</sub> 72 h NOE <sub>b</sub> C	15.0 nom 2.10 nom 4.10 nom 0.66 nom	Hall, A. T.; Lam, C. V., (1999) M-009214-01-1 <sup>1</sup>

mm ..... mean measured concentration, n... nominal, geom ..... geometric measured concentration,  
im .....initial measured concentration

<sup>1</sup> The study is not fully reliable but can be used as supportive information indicating that metabolite is clearly less toxic than active substance.

<sup>1a</sup> The study is not fully reliable but can be used as supportive information.

<sup>2</sup> The study is considered as supplementary information.

\* geometric mean value E<sub>r</sub>C<sub>50</sub> of three laboratory studies with *Pseudokirchneriella subcapitata* (Dorgerloh M, 1998; M-086475-01-1, Bruns E., 2010; M-363891-03-1 and Anderson, J. P. E. 1997; M-002343-01-1)

**Effects on growth of green algae:****Active substance****B.9.2.6.1. Effects of flufenacet to green alga (*Selenastrum capricornutum* )**

<b>Reference:</b>	Toxicity of <sup>14</sup> C-FOE 5043 to the Green Alga <i>Selenastrum capricornutum</i>
<b>Author(s), year:</b>	Bowers L.M., 1995
<b>Report/Doc. number:</b>	Study no. F3883501, Reference BCS No:M-002348-02-1
<b>Guideline(s): I</b>	FIFRA Guideline 123-2, Growth and Reproduction of Aquatic Plants (Tier 2)
<b>GLP:</b>	Yes

**Material and methods:**

Test substance:	<sup>14</sup> C-FOE 5043; Batch: C-583A; Purity 99.4% a.s.( analysed)
Test species:	Green alga ( <i>Selenastrum capricornutum</i> )
Number of organisms:	3,000 cells/mL; 3 replicates per treatment, control and solvent control group
Type of test, duration:	Static test, 120 hours

**Applied concentrations:**

Nominal (measured) :	0.63 (0.64), 1.25 (1.31), 2.5 (2.52), 5 (5.04) and 10 (9.64) µg a.s./L
Solvent:	Dimethylformamide( DMF), 500 ul dimethylformamide/L

**Test conditions:**

Water quality:	APP nutrient medium according to EPA guideline
Temperature:	23.6-24.4°C
pH:	7.1 – 7.4 (0 h), 7.6 – 9.5 (120 h)
Conductivity	338-370 umhos/cm (0) ,244-378 (120 h)
Incubation:	Continuous lighting, approximately-4600 Lux

Analytical measurements: Each day, density was determined in three replicates at each test concentration using a light microscope and an Improved Neubauer hemocytometer. Each hemocytometer was placed on the viewing stage of the microscope for cell counting.

A calibrated data logger and thermocouple were used to monitor the test system temperature each hour in the environmental chamber. In addition, manual temperature readings were recorded. The thermometer and thermocouple were immersed in a centrally located, 250-ml flask of water in the environmental chamber. The pH and conductivity were measured in the controls and in the low,

middle and high test solutions on Day 0 and Day 5. Samples of  $^{14}\text{C}$ -FOE 5043 test solutions, including controls, were taken on Day 0 and Day 5 to measure actual exposure concentrations.

Findings:

Analytical data:	<p>The Day 0 measured concentrations of <math>^{14}\text{C}</math>-FOE 5043 were 9.64, 5.04, 2.52, 1.31 and 0.64 <math>\mu\text{g a.s./L}</math>, which represents 96 to 105% of the nominal test concentrations. On Day 5, the <math>^{14}\text{C}</math>-FOE 5043 measured concentrations were 9.71, 4.85, 2.54, 1.29 and 0.63 <math>\mu\text{g a.s./L}</math> which represents 97 to 103% of the nominal test concentrations.</p> <p>All results are based on nominal test concentration.</p>
Statistical data evaluation:	<p>Growth data for 120 h was analyzed using the following statistical tests:</p> <ol style="list-style-type: none"><li>1) t-test to determine if control and solvent control data could be pooled;</li><li>2) Chi-square test for normality and Levene's test for homogeneity of variance;</li><li>3) ANOVA followed by the Dunnett's Test;</li><li>4) probit analysis to estimate the slope of the dose-response curve, the <math>\text{EC}_{25}</math>, and the <math>\text{EC}_{50}</math>.</li></ol> <p>Statistical analyses were conducted using PC based computer programs (SAS ver. 6.10, EPA ICp ver. 2.0, and TOXSTAT ver. 3.3).</p>
Morphological effects:	<p>No morphological change in algae was observed in any test concentration.</p>

Below, on the figure B.9.2.6.-1, are presented the raw data obtained in the study – the measured algal cell densities in function of time (the table was copied from the study report).

Figure B.9.2.6.-1: The raw data obtained in the study.

Measured Algae Densities During the <sup>14</sup> C-FOE 5043 <i>Selenastrum capricornutum</i> Growth Test						
Measured Concentration (µg/L)	REP	DENSITY (cells/ml)				
		DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
Control	A	0.75 x 10 <sup>4</sup>	1.42 x 10 <sup>4</sup>	6.44 x 10 <sup>4</sup>	53.25 x 10 <sup>4</sup>	162.75 x 10 <sup>4</sup>
	B	1.00 x 10 <sup>4</sup>	1.53 x 10 <sup>4</sup>	7.47 x 10 <sup>4</sup>	43.63 x 10 <sup>4</sup>	118.00 x 10 <sup>4</sup>
	C	0.70 x 10 <sup>4</sup>	2.06 x 10 <sup>4</sup>	7.50 x 10 <sup>4</sup>	36.63 x 10 <sup>4</sup>	96.25 x 10 <sup>4</sup>
Solvent Control	A	0.53 x 10 <sup>4</sup>	1.64 x 10 <sup>4</sup>	6.97 x 10 <sup>4</sup>	35.63 x 10 <sup>4</sup>	156.50 x 10 <sup>4</sup>
	B	0.70 x 10 <sup>4</sup>	1.25 x 10 <sup>4</sup>	6.57 x 10 <sup>4</sup>	46.88 x 10 <sup>4</sup>	124.00 x 10 <sup>4</sup>
	C	0.78 x 10 <sup>4</sup>	1.97 x 10 <sup>4</sup>	8.22 x 10 <sup>4</sup>	56.50 x 10 <sup>4</sup>	121.25 x 10 <sup>4</sup>
0.64	A	0.75 x 10 <sup>4</sup>	1.42 x 10 <sup>4</sup>	6.42 x 10 <sup>4</sup>	38.63 x 10 <sup>4</sup>	168.50 x 10 <sup>4</sup>
	B	1.06 x 10 <sup>4</sup>	1.44 x 10 <sup>4</sup>	5.92 x 10 <sup>4</sup>	47.00 x 10 <sup>4</sup>	143.50 x 10 <sup>4</sup>
	C	1.00 x 10 <sup>4</sup>	1.86 x 10 <sup>4</sup>	7.20 x 10 <sup>4</sup>	51.50 x 10 <sup>4</sup>	147.75 x 10 <sup>4</sup>
1.31	A	0.75 x 10 <sup>4</sup>	1.97 x 10 <sup>4</sup>	6.86 x 10 <sup>4</sup>	33.50 x 10 <sup>4</sup>	146.00 x 10 <sup>4</sup>
	B	0.89 x 10 <sup>4</sup>	1.83 x 10 <sup>4</sup>	5.17 x 10 <sup>4</sup>	28.00 x 10 <sup>4</sup>	146.50 x 10 <sup>4</sup>
	C	0.78 x 10 <sup>4</sup>	1.53 x 10 <sup>4</sup>	5.86 x 10 <sup>4</sup>	35.25 x 10 <sup>4</sup>	117.50 x 10 <sup>4</sup>
2.52	A	0.39 x 10 <sup>4</sup>	1.33 x 10 <sup>4</sup>	0.47 x 10 <sup>4</sup>	15.69 x 10 <sup>4</sup>	104.75 x 10 <sup>4</sup>
	B	0.45 x 10 <sup>4</sup>	0.72 x 10 <sup>4</sup>	0.39 x 10 <sup>4</sup>	2.58 x 10 <sup>4</sup>	26.25 x 10 <sup>4</sup>
	C	0.58 x 10 <sup>4</sup>	1.14 x 10 <sup>4</sup>	0.97 x 10 <sup>4</sup>	20.34 x 10 <sup>4</sup>	152.75 x 10 <sup>4</sup>
5.04	A	0.36 x 10 <sup>4</sup>	0.61 x 10 <sup>4</sup>	<0.19 x 10 <sup>4</sup>	0.72 x 10 <sup>4</sup>	6.43 x 10 <sup>4</sup>
	B	0.47 x 10 <sup>4</sup>	0.61 x 10 <sup>4</sup>	<0.25 x 10 <sup>4</sup>	0.56 x 10 <sup>4</sup>	5.06 x 10 <sup>4</sup>
	C	0.53 x 10 <sup>4</sup>	0.56 x 10 <sup>4</sup>	0.25 x 10 <sup>4</sup>	0.78 x 10 <sup>4</sup>	10.08 x 10 <sup>4</sup>
9.64	A	0.19 x 10 <sup>4</sup>	<0.11 x 10 <sup>4</sup>	<0.11 x 10 <sup>4</sup>	<0.11 x 10 <sup>4</sup>	<0.11 x 10 <sup>4</sup>
	B	0.30 x 10 <sup>4</sup>	<0.14 x 10 <sup>4</sup>	<0.14 x 10 <sup>4</sup>	<0.11 x 10 <sup>4</sup>	<0.17 x 10 <sup>4</sup>
	C	0.28 x 10 <sup>4</sup>	<0.17 x 10 <sup>4</sup>	<0.19 x 10 <sup>4</sup>	<0.14 x 10 <sup>4</sup>	<0.14 x 10 <sup>4</sup>

These values were used to calculate the toxicity endpoint being the final outcome of the study. RMS verified these calculations and stated that they were not in line with the recommendations of the relevant OECD Guidelines but with those given by US EPA ( $E_rC_{50}$ ,  $E_bC_{50}$  and NOEC were calculated for 120 hours).

It shall be indicated that that set of data was subjected to the repeated mathematical analysis performed by the, Dorgerloh, aimed on the derival of the toxicity endpoints in line with the recommendations provided by OECD 201. These calculations were presented in a separated report and are summarised below, in a separate summary.

**B.9.2.6.2. Toxicity of  $^{14}\text{C}$ -FOE 5043 – to Green Alga *Selenastrum capricornutum*.**

<b>Reference:</b>	Toxicity of $^{14}\text{C}$ -FOE 5043 – to Green Alga <i>Selenastrum capricornutum</i> .
<b>Author(s), year:</b>	Dorgerloh, M. 1998,
<b>Report/Doc. number:</b>	Study no. F3883501, Reference BCS No:M-002348-02-1, Report ID: DOM 98092, M-086475
<b>Guideline(s): I</b>	OECD 201 (June 1984)

Aims and methods:

**Aim of the study:** To calculate the toxicity of  $^{14}\text{C}$ -FOE 5043 for the green alga *Selenastrum capricornutum* using the data obtained in the study by [Bowers L.M., 1995], presented on Figure B.9.2.6 -1.

**Methods:**

Growth data was analyzed using the following statistical tests:

- 1) t-test to determine if control and solvent control data could be pooled;
- 2) Chi-square test for normality and Levene's test for homogeneity of variance;
- 3) ANOVA followed by the Dunnett's Test;
- 4) probit analysis to estimate the slope of the dose-response curve, the  $\text{EC}_{50}$  and the  $\text{EC}_{10}$ .

Result:

The results of the calculations were verified by the RMS. It was stated that 96-hours NOEC for biomass was not realistic, being higher than the determined  $\text{E}_b\text{C}_{50}$ . For that reason RMS repeated calculations. All results – those obtained by Dorgerloh and by the RMS, are presented below.

The results obtained by Dorgerloh (1998) :

96 h  $\text{E}_r\text{C}_{50} = 3.10 \mu\text{g/L}$  (95% CI: n.d. )

96 h  $\text{NOE}_r\text{C} = 1.31 \mu\text{g/L}$

96 h  $\text{E}_b\text{C}_{50} = 1.82 \mu\text{g/L}$  (95% CI: 1.77-1.87  $\mu\text{g s.a./L}$ ),

$\text{NOE}_b\text{C} = 5.04 \mu\text{g/L}$

Dorgerloh (1998) as the reliable toxicity endpoint obtained from this study proposed the value obtained for the time point of 96 hours instead of that for 72 hours. No explanation for that fact was provided in the study, only a statement was made that the data for 72-hours time-point were considered an outlier.

The Applicant on RMS's request carried out an additional analysis of the raw data used in calculations. It was noticed that although the concentrations of the test item were considered to be stable with time, no explanation could have been given for the effects observed at the concentrations of 2.52 and 5.04 µg flufenacet/L. For these two concentrations a decrease in cell numbers on day 3 was observed, followed by a subsequent increase of that parameter. That concerned in particular the replicate B for the test concentration of 2.52 µg/L, in comparison to the results obtained for replicates A and C, where very low number of the cells was recorded. Due to that fact it was very difficult to interpret the results for 72-hours time point. Hence the results of the growth rate for 96 hours were taken into account.

Due to the fact that the 96-hours NOEC for biomass calculated by Dorgerloh (1998) was above  $E_bC_{50}$  value (one-side, Dunnet test,  $p \leq 0.05$ ). RMS verified the statistical calculations of toxicity endpoints for 96 hours period, using TOXRAT program. The recalculation was provided by a probit analysis effect concentrations ( $EC_{10}$ ,  $EC_{20}$  and  $EC_{50}$ ) for yield and growth rate were derived, including confidence limits (95% and 99%). NOECs and LOECs were recalculated using Williams' Multiple Sequential t-test with significance level  $\alpha = 0.05$ , one-sided smaller.

The results are presented below:

Results of effects of flufenacet on the *Selenastrum carpiconatum* was performed in the Table B. 9.2.6.2-1.

**Table B. 9.2.6.2-1: Effects of technical flufenacet on the *Selenastrum capricornutum*.**

Flufenacet (initial measured [µg/L])	Yield		Growth rates $\mu$ [1/day] and % inhibition of $\mu$	
	0-96 hours		0-96 hours	
	Y	%	$\mu$	%
Control	45.120	-	1.25	-
0.64	45.410	-0.6	1.25	-0.3
1.31	31.950	29.2*	1.17	6.6
2.52	12.570	72.1*	0.86	31.2*
5.04	0.387	99.1	0.20	83.6*
9.64	-0.180	100.4*	-0.23	118.4*

\* Statistically significant compared to control (Wilimas Multiple Sequestial procedure test procedure)

New calculation provided by RMS :

96 h  $E_y C_{10} = 0.862$  µg a.s./L ( 95% CI: 0.56-1.077 µg a.s./L)

96 h  $E_y C_{20} = 1.107$  µg a.s./L (95% CI: 0.816-1.316 µg a.s./L)

96 h  $E_y C_{50} = 1.783$  µg a.s./L (95%CI: 1.542-2.060 µg a.s./L)

96 h NOE<sub>y</sub>C = 0.64 µg a.s./L

96 h E<sub>r</sub>C<sub>10</sub> = 1.679 µg a.s./L (95% CI: 1.177-2.033 µg a.s./L)

96 h E<sub>r</sub>C<sub>20</sub> = 2.084 µg a.s./ L (95% CI: 1.612-2.423 µg a.s./L)

96 h E<sub>r</sub>C<sub>50</sub> = 3.15 µg a.s./L ( 95% CI: 2.765-3.612 µg a.s./L)

96 h NOE<sub>t</sub>C < 0.64 µg a.s./L

The 96 h E<sub>r</sub>C<sub>50</sub> of 3.15 µg a.s./L, was included in the geometric mean value for *P. subcapitata*.

**Overall comments RMS to the study:**

The study (Bowers L.M., 1995) was conducted according to the FIFRA Guideline 123-2. To fulfill the requirements of the OECD 201 test guideline, the new calculations of  $E_rC_{50}$ ,  $E_bC_{50}$  and NOEC values based on the original raw data, together with their statistical evaluation, were provided by Dorgerloh, (1998). RMS verified these calculations and decided to repeat them because of the low reliability of NOEC for biomass and lack of confidential intervals.

Also RMS verified the acceptability of the study using the following validity criteria set by OECD 201 test guideline, with the relevant results presented in:

- The mean cell density in the control should increase by a factor  $\geq 16$  (measured cell density increased by the factor of 24 for 0-72 hours and by the factor of 148 for 0-96 hours).
- The mean coefficient of variation for section-by-section specific growth rates in the control cultures shall not exceed 35% (measured values: 39.6 % for 72 hours and 41.5 % for 0-96 hours).
- The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7% (measured values: 2.74 % for 0-72 h and 3.75% for 0-96 h).
- pH in the control should not increase by more than 1.5 units during the study (measured: for the study period 0-120 h by 2.3 units).
- Initial cell concentration, recommended by the guideline, shall be between  $5 \times 10^3$  and  $5 \times 10^4$  (measured: initial cell concentration in the control was only  $3 \times 10^3$  cell/mL).

One of these criteria – the CV criterion, was not met.

Despite the fact the CV criterium was not met, RMS decided to consider study results in the risk assessment.

The the reason of that was that in the peculiar study M-002343-01-1 in the third test, a 96 h  $E_rC_{50}$  of 6.45  $\mu\text{g a.s./L}$  was calculated for algae non-previously exposed to the active substance through a design which seems to be similar to OECD 201.

This value suggests that the 96 hour  $E_rC_{50}$  of 3.15  $\mu\text{g a.s./L}$  obtained in the study is not inconsistent even if the study appears to be not fully reliable. Additionally, in the first study of the peculiar study M-002343-01-1, ~60% effects on algae growth rate are observed after 72h and 96 h, suggesting that the  $E_rC_{50}$  at 72h and at 96 h would be relatively close.

**Agreed endpoints:**

96 h  $E_y C_{10}$  = 0.862  $\mu\text{g a.s./L}$  ( 95% CI: 0.56-1.077  $\mu\text{g a.s./L}$ )

96 h  $E_y C_{20}$  = 1.107  $\mu\text{g a.s./L}$  (95% CI: 0.816-1.316  $\mu\text{g a.s./L}$ )

96 h  $E_y C_{50}$  = 1.783  $\mu\text{g a.s./L}$  (95%CI: 1.542-2.060  $\mu\text{g a.s./L}$ )

96 h  $NOE_y C$  = 0.64  $\mu\text{g a.s./L}$

96 h  $E_r C_{10}$  = 1.679  $\mu\text{g a.s./L}$  (95% CI: 1.177-2.033  $\mu\text{g a.s./L}$ )

96 h  $E_r C_{20}$  = 2.084  $\mu\text{g a.s./L}$  (95% CI: 1.612-2.423  $\mu\text{g a.s./L}$ )

96 h  $E_r C_{50}$  = 3.15  $\mu\text{g a.s./L}$  (95% CI: 2.765-3.612  $\mu\text{g a.s./L}$ )

96 h  $NOE_r C$  < 0.64  $\mu\text{g a.s./L}$

All results based on the initial measured concentration.

**B.9.2.6.3. Effects of flufenacet to green alga (*P. subcapitata* / *Selenastrum carpiconantum*)**


---

<b>Reference:</b>	<i>Pseudokirchneriella subcapitata</i> growth inhibition test with flufenacet.
<b>Author(s), year:</b>	Bruns, E. 2010
<b>Report/Doc. number:</b>	Study No.: E 323 3797-4, Report No: EBFOL 150, Reference BCS No: M-363891-03-01 with Amendment No 2 and No1 Amendment No 2 (updated on the new statistical analysis provided by Applicant)
<b>Guideline(s):</b>	OECD 201 (March 2006)
<b>GLP:</b>	Yes

---

Material and methods:

Test substance:	Flufenacet (tech.), Batch No: K664078, Purity: 97.5 % (analysed)
Test species:	<i>Pseudokirchneriella subcapitata</i>
Number of organisms:	10,000 cells/mL
<u>Applied concentration:</u>	
Nominal (geomean measured):	Control (0), solvent control (0), 0.0905 (0.138), 0.289 (0.416), 0.923 (1.25), 2.95(3.71), 9.42 (11.1), 30.1 (34.4), 96.1 (102), 307 (322), 980 (983), 3130 (3127) and 10,000 (8605) µg a.s./L 3 replicate vessels per test level and 6 replicate vessels per control
Solvent:	DMF (dimethyloforamid)
Type of test, duration:	Static test, 3 days
<u>Test conditions:</u>	
Water quality:	Nutrient medium according to OECD guideline 201
Temperature:	21.2 to 22.5°C
pH:	7.9-8.2 (0 h), 7.9- 8.5 (72 h) in the control
Incubation:	Continuous lighting, 7930-8840 Lux, ( mean , 8313 Lux)

## Analytical measurements:

Test parameters:	The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature data was recorded by a data logger that calculated the mean, min and max temperatures (based on continuously (hourly means)
------------------	---

measured values. The pH was measured at each observation time in all test levels and the control.

Cell numbers per volume (as a surrogate for biomass per volume) were estimated photometrically. For this purpose, small samples of treated, inoculated test medium were placed in 5 cm cuvettes on day 1, day 2, and day 3 of the exposure period (without replacing after measurement). The extinctions were determined at a wave length of 578 nm using a single-beam-photometer. The photometer was calibrated using culture medium without algae.

HPLC-UV analyses; in the test medium of all treatment levels and the control on day 0 and 3.

Statistic:

The following statistical analyses were conducted:

Pair-wise comparison between Control and solvent control for Cell Number at 72 h:

- Kolmogorov-Smirnov-test on Normal Distribution

- STUDENT-t test for Homogeneous Variances

Effective Concentrations (ECx) with Growth Rate at 72 h

- Probit analysis using linear max. likelihood regression

Threshold Concentrations (NOEC) with Growth Rate at 72 h

- Kolmogorov-Smirnov-test on Normal Distribution

- Cochran's Test Procedure on Variance Homogeneity

- STUDENT-t test for Homogeneous Variances

Software:

- Microsoft Excel spreadsheets

- Statistical Software ToxRat Professional version 2.09

- ToxRat Validation Report, valid for ToxRat Version 2.09

Findings:

Analytical data:

The concentrations of flufenacet in the treated samples after 0 hours were in range 88 % to 158 % of nominal concentrations (average 121 %) and after 3 days in range 84 - 147 % of nominal concentration (average 113 %). Therefore all results are in relation to the geometric mean measured test concentrations.

Morphological effects: No morphological change in algae was observed in any test concentration.

The numerical results of the experiment are presented below in tables B.9.2.6.3-1 for growth and 9.2.6.3-2 for yield.

**Table B.9.2.6.3-1: Effects of flufenacet on the green algae *Pseudokirchneriella subcapitata*. Growth.**

Geometric mean measured concentration [µg a.s./L]	Cell Number (means) per mL after 72h	Average Specific Growth Rates [days <sup>-1</sup> ] (0-72 h)-	Inhibition of Average Specific Growth Rate [%]	Doubling time of algae cells [days]
Control	801000	1.461	-	0.474
Solvent control	837000	1.475	-	0.470
Pooled control	819000	1.468	-	0.472
0.138	791000	1.457	0.8	0.476
0.416	751000	1.440	1.9	0.481
1.25	712000	1.421	3.2*	0.488
3.71	601000	1.364	7.1*	0.508
11.1	117000	0.819	44.2*	0.846
34.4	67000	0.632	57.0*	1.100

\*Statistically different to pooled control (Williams t-test,  $\alpha < 0.05$ )

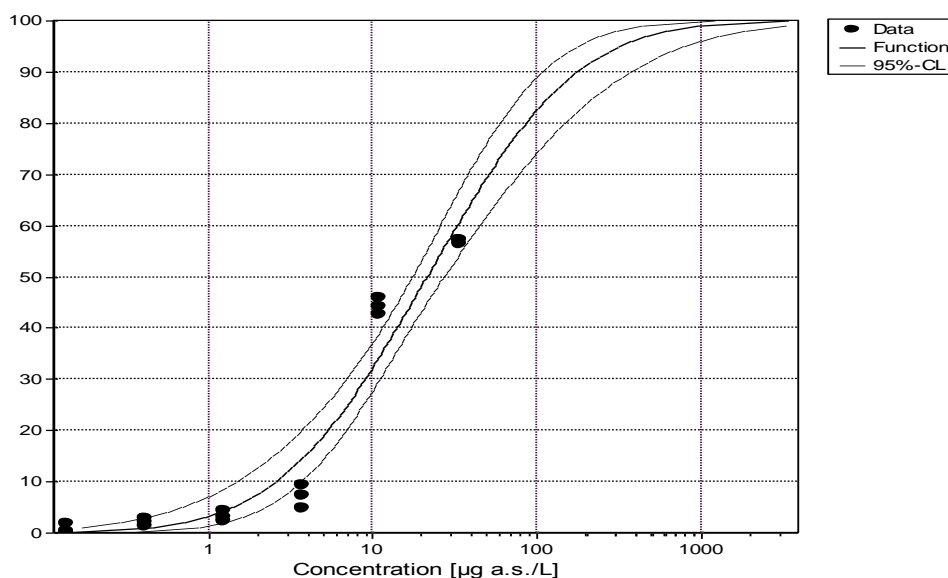
**Table B.9.2.6.3-2: Effects of flufenacet on the green algae *Pseudokirchneriella subcapitata*. Yield.**

Geometric mean measured concentration [µg a.s./L]	Cell Number (means) per mL after 72 h	Average Specific Growth Rates [days <sup>-1</sup> ] (0-72 h)	Inhibition of Average Specific Growth Rate [%]
Pooled control	837000	1.475	-
0.138	791000	1.457	3.4
0.416	751000	1.440	8.3*
1.25	712000	1.421	13.2*
3.71	601000	1.364	26.9*
11.1	117000	0.819	86.8*
34.4	67000	0.632	93.0*

\* Statistically significant in comparison to pooled control (Dunnett's multiple t-test,  $\alpha = 0.05$ , one side)

These data were then subjected to the mathematical analysis aimed on the deriving of the following endpoints:  $E_rC_{50}$ ,  $E_yC_{50}$  for for all concentrations tested during the study. The determined values underwent the statistical analysis. RMS evaluating the calculations stated that the value 72 h  $E_rC_{50}=138 \mu\text{g a.s./L}$  had very wide Confidence Interval (95% CI: 37.14-640.58), what made it highly uncertain. For that reason the value was not accepted and the Applicant was asked to recalculate it. That was done using narrower data set – the initial data set was limited by excluding five highest test concentrations, those above  $34.4 \mu\text{g a.s./L}$ . Therefore the repeated calculations were carried out using following six test concentrations: 0 (control), solvent control (0) 0.905 (0.138), 0.289 (0.416), 0.923 (1.25), 2.95 (3.71), 9.42 (11.1), 30.1 (34.4)  $\mu\text{g a.s./L}$ . That was due to the fact that the dose response around the  $EC_{50}$  was very flat and inhibition at high concentrations did not reach 100%. On the other hand these high concentrations had a high weight in the probit analysis.

The resulting Concentration-effect curve based on concentrations tested (from 0 to 34.4.  $\mu\text{g a.s./L}$ ) shows the influence of the test item on growth rate of the introduced *Pseudokirchneriella subcapitata* as observed after 72 h is presented below on figure B.9.2.6.3-1:



**Fig. B.9.2.6.3-1: Concentration-effect curve showing the influence of the test item on growth rate of the introduced *Pseudokirchneriella subcapitata* as observed after 72 h.**

Conclusion:

Growth inhibition values for *Pseudokirchneriella subcapitata* exposed to flufenacet, based on geometric mean measured concentration of the test item, were following:

Tested parameter	Results
72 h ErC <sub>50</sub>	21.20 µg a.s./L (95% CI: 17.336-27.030)
72 h ErC <sub>20</sub>	5.306 µg a.s./L (95%CI: 3.702-6.859)
72 h ErC <sub>10</sub>	2.571 µg a.s./L (95%CI, 1.500-3.688)
72 h NOErC	0.138 µg a.s./L
72 h EyC <sub>50</sub>	5.385 µg a.s./L (95% CI: 4.547-6.412)
72 h EyC <sub>20</sub>	2.859 µg a.s./L (95% CI: 2.081-3.500)
72 h EyC <sub>10</sub>	2.053 µg a.s./L (95%CI:1.327-2.658)
72 h NOEyC	0.138 µg a.s./L

**RMS comments:**

The study was conducted according to the OECD 201 test guideline (2006).

In general the study is in line with the evoked test guideline and all validity criteria were met. The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period. In the study the cell density increased by a factor of 81.9 (pooled control) which is in line with the evoked OECD test guideline. The mean CV for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control must not exceed 35%. The mean CV was determined to be 10.2 %. The CV of the average specific growth rates during the whole test period in replicate control cultures must not exceed 7%.

The mean CV for the whole test period was 1.1 %.

The RMS is of the opinion that the reliability of the results is therefore proven.

Therefore, the study is considered acceptable.

**Agreed endpoints:**

72 h ErC<sub>50</sub> = 21.20 µg a.s./L (95% CI: 17.336-27.030)

72 h ErC<sub>20</sub> = 5.306 µg/L (95% CI: 3.702-6.859)

72 ERC<sub>10</sub> = 2.571 µg/L (95% CI: 95%, 1.500-3.688)

72 h NOErC = 0.138 µg a.s./L

72 h EyC<sub>50</sub> = 5.385 µg a.s./L (95%CI: 4.547-6.412)

72 h EyC<sub>20</sub> = 2.859 µg a.s./L (95% CI: 2.081-3.50)

72 h EyC<sub>10</sub> = 2.053 µg/L ( 95% CI: 1.327-2.658)

72 NOE<sub>yC</sub> = 0.138 µg a.s./L

All results based on geomean measured concentration.

#### B.9.2.6.4. Effects on *Pseudokirchineriella subcapitata* (formerly *Selenastrum carpiconatum*).

<b>Reference:</b>	Growth of the Green Alga, <i>Pseudokirchineriella subcapitata</i> (formerly <i>Selenastrum capricornutum</i> ), During and After Exposure to High Concentrations of FOE 5043.
<b>Author(s), year:</b>	Anderson, J.P.E. 1997
<b>Report/Doc. number:</b>	Study No.: E 323 1194-3, Reference BCS No.: AJD/157097, M-002343-01-1
<b>Guideline(s): I</b>	OECD 201 (June 1984).
<b>GLP:</b>	Yes

#### Material and methods:

Test substance:	FOE 5043: Flufenacet (tech.); Batch No.: 00157875; Purity: 99.5%
Test species:	<i>Pseudokirchineriella subcapitata</i> (formerly <i>Selenastrum capricornutum</i> )
Number of organisms:	10,000 cells/mL; 4 replicate per control and each test concentrations
Type of test, duration:	Semi-static test, 20 days.

#### Applied concentrations:

Nominal ( measured ) :	Control (0), 2.25, 4.5, 9.0 and 18 µg a.s./L
Solvent:	Acetonitrile, amount added unknown
Test conditions:	
<u>Water quality:</u>	Nutrient medium according to the OECD (1984) guideline
Temperature:	23 ± 2°C, Light intensity: 8000 Lux
pH:	7.95-7.99 (Day 0), 8.10-8.39 (Day 20)
Incubation:	In this study were conducted three types of laboratory tests to study the influence of flufenacet , on growth-inhibition and „recovery“ of the single-celled green alga, <i>P. subcapitata</i> .  The first test was designed to study the growth and reproduction of <i>P. subcapitata</i> during a 20-days exposure to high but decreasing concentrations of

flufenacet. In this test, nutrient medium was inoculated with *P. subcapitata* and treated with 0, 4.50 and 9.00 µg a.s./L. Every 4 days, 16.7 % of the medium (plus cells) was removed and replaced with fresh, flufenacet-free medium. Cell growth in the cultures was measured throughout the experiment.

The second test was designed to compare the rates of growth of *P. subcapitata* cells after 4, 8, 12 and 16 day exposures to flufenacet. For this test, cells removed in 4 day intervals from the cultures of the first test were washed and transferred to fresh, flufenacet-free nutrient medium. Growth in these 4 sets of cultures was followed by determining cell numbers 0, 3 and 4 days after inoculation.

The third test was made to determine if growing *P. subcapitata* cells in culture medium treated with flufenacet produced cells that were resistant to the herbicide. To conduct this test, cells grown for 20 days in cultures of the first test were collected, washed and used to inoculate nutrient medium treated with 0, 2.25, 4.5, 9.0 and 18.0 µg a.s./L after 24, 48, 72 und 96 h and data were used to calculate  $E_rC_{50}$ , NOEC and LOEC values.

Analytical measurements:

HPLC-UV analysis was provided in the test medium of all treatment levels and the control.

Test parameters:

The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature data was recorded by a data logger that calculated the mean, min and max temperatures (based on continuously (hourly means) measured values. The pH was measured at each observation time in all test levels and the control. Cell numbers per volume (as a surrogate for biomass per volume) and possible alterations in algae cells such as unusual cell size were estimated by direct algae cell counting under a microscope at a magnification of 400 times. Recorded light intensity values from the used incubator, measured at nine different places.

#### Findings:

Analytical data:

Measured concentrations of flufenacet in nutrient media during 20 days (test 1) on day 0, before solution on days 4, 8 and 12 and before and after dilution on day 16 was in range between 78 to 98% of nominal (average 91%).

Measured concentrations of flufenacet in nutrient media in the third test ranged between 109 and 112 % of nominal (average 111%).

Due to analytical findings all results are based on nominal test concentrations.

---

Morphological effects:	No morphological change in algae was observed in any test concentration.
Statistic:	Evaluation was made using an analysis of variance ("Dunnett's-Test") at a significance level of 5 % (one-sided).
Findings:	<p>The results of the first test, showed that the cell numbers increased during the first 3 days after each dilution with one exception (day 4-7 at test concentration of 9 µg a.s. /L). In cultures treated with 4.5 µg a.s. /L the increase in cell number per days between days 4 and 7 was ca. 33% less than in the solvent controls. After a second dilution on day 8, the growth rate in these cultures was higher than in the controls. Similar results were obtained with the solvent controls and the 9 µg a.s./L cultures, however, growth rates in the treated cultures were negative between days 4-7, and then positive between days 7and 8. Between 8-11, growth rates were higher than in the controls. At the end of experiment the total number of cell in the 4.5 and 9.0 µg a.s /L cultures were only 14-18 % less than in the solvent control, respectively.</p> <p>The data indicated that extended exposure of <i>P. subcapitata</i> cells to high but decreasing concentrations of flufenacet did not permanently impair their potential to grow and reproduce.</p> <p>The results obtained from the first test are presented in the Table B.9.2.6.4-1 and in Figure 9.2.6.4-1.</p> <p>Figure 1 summarizes the biological part of data presented in the Table B.9.2.6.4-1 and shows growth curves and average number in cultures before and after each dilution with flufenacet-free treatment nutrient. The average growth rates in the cultures (e.i., average increase in cell number per day) between days 4 to7, 8 to 11, 12 to 15 and 16-20 also given.</p>

**Table B.9.2.6.4-1: Growth of *P. subcapitata* cells in the control and flufenacet –treated cultures that were diluted every 4 days with fresh, flufenacet -free nutrient solution (a). (Test 1).**

Days exposed to flufenacet	Days after Transfer to flufenacet -Free Nutrient Medium	Cell Numbers (x10 <sup>4</sup> )/ml (a)		
		Concentration (Nominal) of flufenacet (µg a.s. /L)		
		Days 0 to 4=0	Days 0 to 4=4.5 [µg a.s. /L]	Days 0 to 4= 9.00 [µg a.s. /L]
0		1	1	1
(b)		±0	±0	±0
3		130	46	6.25
(b)		±8.10	±1.96	±3.93
4		315	90.85	9.60
(c)		±11.09	±2.92	±1.12
<b>Dilution, Day 4</b>		<b>Days 4 to 8=0</b>	<b>Days 4 to 8= 3.75 [µg a.s./L]</b>	<b>Days 4 to 8= 7.5 [µg a.s. /L]</b>
4		263±9.7	75.7±2.4	8.0±1.0
(b)				
7		649±46.646	334.00±7.66	6.12±1.89
(d)				
8		670.66±20.72	352.66±27.28	18.12±4.54
(e)				
<b>Dilution, Day 8</b>		<b>Days 8 to 12=0</b>	<b>Days 8 to 12= 3.13 [µg a.s./L]</b>	<b>Days 8 to 12= 9.00 [µg a.s./L]</b>
8		559.5 ±18.5	294.3±23.6	15.1±3.8
(d)				
11		540±109.01	403.00±59.72	121.75±15.35
(b)				
12		523.68±22.28	377.88±35.36	206.60±19.88
(e)				
<b>Dilution, Day 12</b>		<b>Days 12 to 16=0</b>	<b>Days 12 to 16= 2.60 [µg a.s./L]</b>	<b>Days 12 to 16= 5.20 [µg a.s./L]</b>
12		436.4	315.1±29.8	172.2±16.4
(d)		±18.4		
15		536.00	463.00±68.61	506.00±23.21
(b)		±5.66		
16		605.40	497.08±7.52	472.84±8.08
(e)		±16.72		
<b>Dilution, Day 16</b>		<b>Days 16 to 20=0</b>	<b>Days 16 to 20=2.17 [µg a.s./L]</b>	<b>Days 16 to 20=4.33 [µg a.s./L]</b>
16		483.6±34.5	414.1±	393.8±6.8
19		562.00±57.92	470±44.36	515.00±40.71
20		610.04±16.68	524±76	500.24±11.24

- a Dilution were made after 4, 8, 12 and 16 days by removing 25 ml ( plus cells) of 150 ml culture medium (=16.7%) and replacing this 25 ml of fresh , flufenacet -free nutrient medium.
- b Numbers determined by microscopic counting
- c Number determined by photometric analysis
- d Numbers determined by calculation, i.e., numbers for day 4 undiluted controls- (numbers in day 4 undiluted controls x 0.167)=numbers for day 4 controls after dilution.
- e Number determined by photometric analyses after dilution samples (1/1 or ¼) with cell-free medium: values were multiplied by 3 or 4 to calculate cell numbers

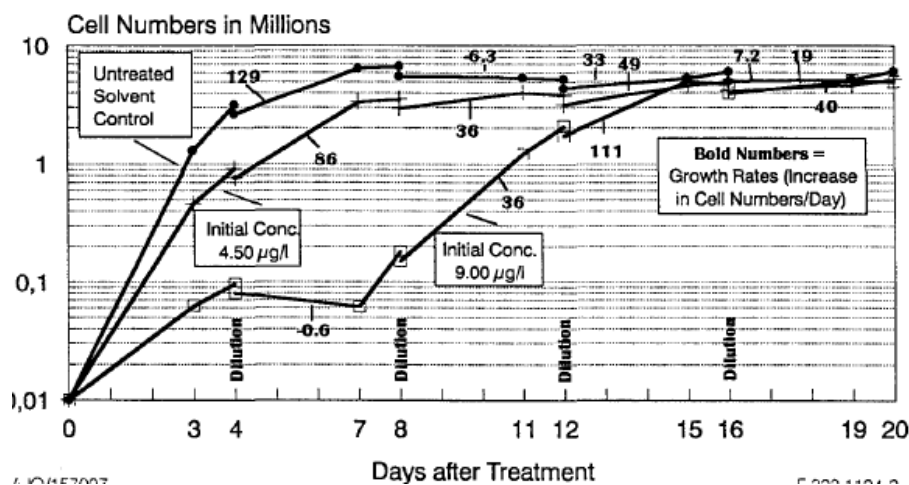


Figure 9.2.6.4-1. Growth of *P. subcapitata* cells during exposure to the high but decreasing concentration of flufenacet (based on the results presented in the Table B.9.2.6.4-1).

In the second test was to run determine if growth rates of *P. subcapitata* cells changed after exposure to high but decreasing concentrations of flufenacet. To run this test, cells removed on days 4, 8, 12 and 16 from cultures of the first test were washed and used to inoculate fresh, flufenacet-free nutrient medium. Growth in these 4 sets of cultures was followed by determining cell numbers 0, 3 and 4 days after inoculation.

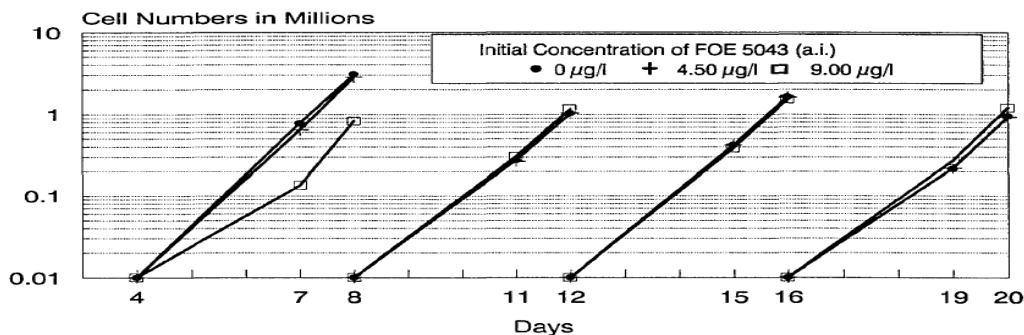
The results are presented in the Table B.9.2.6.4-2. and in Figure 9.2.6.4-2 below: The results indicated that the cells collected on 4, 8, 12 and 16 days after treatment grew at rates were more comparable to those in the control (Fig 2). It can be conducted that exposure of *P. subcapitata* to relatively high concentration of flufenacet did not negatively effect the ability of the cells to grow and reproduce.

**Table B.9.2.6.4-2: Growth of washed *P. subcapitata* cells in flufenacet -free nutrient medium after this exposure to various concentrations of flufenacet for 4, 8, 12, and 16 days. ( Test 2).**

Days exposed to flufenacet	Days after Transfer to flufenacet Free-nutrient medium	Cell Numbers (x10 <sup>4</sup> )/ml (a)		
		Concentration [Nominal] of flufenacet [µg a.s. / l] <sup>(b)</sup>		
		Days 0 to 4=0	Days 0 to 4=4.5 [µg a.s./L]	Days 0 to 4= 9.00 [µg a.s./L]
4	0	1±0	1±0	1±0
	3	77.30±8.03	64.02±7.44	13.42±1.62
	4	306.36±23.40	281.88 ±10.48	82.71±8.45
Dilution, Day 4		Days 4 to 8=0	Days 4 to 8=3.75 [µg a.s. /L]	Days 4 to 8= 7.5 [µg a.s. /L]
8	0			
	3	27.44±2.88	26.58±0.71	30.66±1.55
	4	102.43±16.17	102.76±13.89	116.16±6.89
Dilution , Day 8		Days 8 to 12=0	Days 8 to 12=3.13 [µg a.s./L]	Days 8 to 12= 6.26 [µg a.s. /L]
12	0	1±0	1±0	1±0
	3	42.18±3.62	41.91±0.62	38.76±14.45
	4	161.68±20.42	160.08±19.16	151.16±42.22
Dilution, Day 12		Days 12 to 16=0	Days 12 to 16 =2.60 [µg a.s./L]	Days 12 to 16= 5.20 [µg a.s. /L]
16	0	1±0	1±0	1±0
	3	21.48±0.20	21.87±0.55	26.91±9.04
	4	95.01±3.74	92.62±2.68	120.07±26.47

(a) Dilution were made after 4,8,12 and 16 days by removing 25 ml (plus cell) from the cultures and replacing this with 25 ml of untreated nutrient medium: cell removed during dilution were washed and used as inoculums

(b) Average ±standard deviation from 3 replicates culture : numbers determined by photometric analysis  
At start initiation 10 000 cell/mL.



**Figure 9.2.6.4-2 . Growth of washed *P.subcapitata* cells in Flufenacet -free nutrient medium after their exposure for 4, 8, 12 or 16 days to high but decreasing concentration of herbicide.**

The third test was run to determine if growth of *P. subcapitata* cells in FOE 5043-treated nutrient medium created cells that were resistant to FOE 5043.

To run this test, cells collected on day 20 from untreated and treated cultures of the first test, were washed and used to inoculate nutrient medium treated with 0, 2.25, 4.5, 9.0 and 18.0 µg flufenacet/L.

The influence of 0, 2.25, 4.5, 9.0 and 18.0 ug flufenacet/L on growth in cultures inoculated with cells collected from 0, 4.5 and 9.0 ug a.s. /L, treatments, was presented in the Table B.9.2.6.4-3 and Table B.9.2.6.4-5 below:

**Table B.9.2.6.4-3. The influence of Flufenacet on growth rates and the % inhibition of *P.subcapitata* in cultures inoculated with washed cells from 20 day-old solvent control cultures ( 0 ug flufenacet /L)**

Nominal concentration [µg a.s./L]	Mean cell <sup>a</sup> number per mL [ 0-96 h] x10 <sup>4</sup>	Average specific growth rate [0- 96h]	% inhibition of growth rate compared to control
		µ	%
(0) Solvent control	81.72	1.12	0
2.25	38.97	0.93	17.1*
4.5	12.91	0.66	41.2*
9.0	3.97	0.36	67.7*
18	3.07	0.30	73.3*

\* Statistically significant compared to control (Dunnet's test , one side)

At start 1 x 10<sup>4</sup> cell/ml

<sup>a</sup> Cells used inoculums in this growth inhibition test were collected from 20 day-old solvent control cultures taht had been diluted after 4,8,12, a and 16 days by removing 21 mL ( plus cell) of the 150 mL culture replaing this with 25 mL of fresh , flufenacet free nutrient medium

**Table B.9.2.6.4-4. The influence of Flufenacet on growth rates and the % inhibition of *P.subcapitata* in cultures inoculated with washed cells from 20 day-old culture initially treated with 4.5 ug flufenacet/L(a)**

Nominal concentration [µg a.s./L]	Mean cell <sup>a</sup> number per mL [ 0-96 h] x10 <sup>4</sup>	Average specific growth rate [0- 96 h]	% inhibition of growth rate compared to control
		µ	%
0 (Solvent control)	121.97	1.22	-
2.25	77	1.10	9.6*
4.5	18.36	0.74	39.0*
9.0	4.16	0.38	69.3*

Nominal concentration [µg a.s./L]	Mean cell <sup>a</sup> number per mL [ 0-96 h] x10 <sup>4</sup>	Average specific growth rate [0- 96 h]	% inhibition of growth rate compared to control
		µ	%
18	3.66	0.34	71.9*

\* Statistically significant compared to control (Dunnet's test , one side)

At start 1 x 10<sup>4</sup> cell/ml

<sup>a</sup> Cells used inoculums in this growth inhibition test were collected from 20 day-old solvent control cultures taht had been diluted after 4,8,12, a and 16 days by removing 21 mL ( plus cell) of the 150 mL culture replaing this with 25 mL of fresh, flufenacet free nutrient medium.

**B.9.2.6.4-5: The influence of Flufenacet on growth rates and the % inhibition of *P.subcapitata* in cultures inoculated with washed cells from 20 day-old culture initially treated with 9.0 ug flufenacet/L (a)**

Nominal concentration [µg a.s./L]	Mean cell number per ml [ 0-96h] x10 <sup>4</sup>	Average specific growth rate [0- 96h]	% inhibition of growth rate compared to control
		µ	%
Solvent control	68.04	1.06	-
2.25	37.68	0.92	13.1*
4.5	13.55	0.87	37.4*
9.0	4.22	0.38	64.5*
18	2.6	0.26	75.3*

\* Statistically significant compared to control (Dunnet's test , one side)

<sup>a</sup> Cells used inoculums in this growth inhibition test were collected from 20 day-old solvent control cultures taht had been diluted after 4,8,12, a and 16 days by removing 21 mL ( plus cell) of the 150 mL culture replaing this with 25 mL of fresh , flufenacet free nutrient medium

The growth rate for 0-96 hours for cell collected from 20-days-old cultures initially treated flufenacet with 4.5 µg a.s./L and 9 µg a.s./L is presented below:

**B.9.2.6.4-6: The 96 h E<sub>r</sub>C<sub>50</sub> for cells collected from 20-day-old cultures initially treated with flufenacet.**

		Cultures initially treated with flufenacet at		
		0 µg a.s./L	4.5 µg a.s./L	9.0 µg a.s./L
Endpoints				
Growth rate (0-96 h)	E <sub>r</sub> C <sub>50</sub>	6.45	7.05	6.99
	LOE <sub>r</sub> C	2.25	2.25	2.25
	NOE <sub>r</sub> C	<2.25	<2.25	<2.25

The 96 h  $ErC_{50}$  (growth rate) values of cultures inoculated with cells from the controls or Flufenacet -treated cultures are similar 6.45 in the control, 6.99 and 7.05 for 4.5  $\mu\text{g a.s./L}$ .

The LOEC and NOEC values, which should not be over-evaluated in this experiment because of the lack of very low concentrations of flufenacet, were 2.25 and  $< 2.25$  for all cultures.

### **Conclusion:**

Based on the  $ErC_{50}$  it can be concluded that extended exposure of *P. subcapitata* cells to flufenacet in treated nutrient solution did not change the sensitivity of the cell populations to the herbicide, and did not select for cells that are resistant to flufenacet.

### **Comments RMS:**

The study is a non-standard study. However, it seems that the condition *not previously exposed algae to the active substance* of the third run with 96 h  $ErC_{50} = 6.45 \mu\text{g a.s./L}$  value can be regarded as a standard assay by itself (similar to OECD 201).

Taking into account the validity criteria of the OECD 201 (1984) for the third test (for algae not previously exposed to the active substance) the biomass in the control cultures increased exponentially by a factor of at least 24 within the 72 hour test period, which is in accordance with the validity criteria ( $>16$ ) relevant at the time the study was conducted.

According to OECD 201(2006) test guideline additional validity criteria should be met in the study:

-The mean CV for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control for 72<sup>nd</sup> hour must not exceed 35%. The %CV was determined to be 52.81%. The %CV of the average specific growth rates during the whole test period must not exceed 7%.

The mean CV for the whole test period was 9.6 %. Therefore, two of three validity criteria for 72 hours are not met.

Since the  $ErC_{50}$  is estimated at 96 hours, RMS took into account also the validity criteria for 96 hours.

Due to that fact that increase in cell number at 96<sup>th</sup> hours was 81 ( should be  $>16$ ), the mean %CV for section-by-section specific growth rates (days 0-1, 1-2, 2-3) was above but relatively close to 35% at 96<sup>th</sup> hour (being 43.38%) and %CV of the average specific growth rates during the whole test period was 2.97% (should be  $<7\%$ ) in RMS's opinion the acceptability of the study was demonstrated and despite of identified deficiencies the 96 h  $ErC_{50}$  value of 6.45  $\mu\text{g a.s./L}$  can be used in calculation of geometric mean  $ErC_{50}$  for *P. subcapitata*.

Therefore, RMS would propose to use in the risk assessment a geometric mean  $E_rC_{50}$  value derived from the studies M-002348-02-1 (96 h  $E_rC_{50}$  = 3.1 µg a.s./L) and M-002343-01-1 (96 h  $E_rC_{50}$  = 6.45 µg a.s./L) and from the more recent study M-363891-03-01 (72h  $E_rC_{50}$  = 21.2 µg a.s./L). The resulting geomean  $E_rC_{50}$  would therefore be 7.55 µg/L.

**Agreed endpoints:**

96 h of  $E_rC_{50}$  = 6.45 µg a.s./L

NOER < 2.25 µg a.s./L

**B.9.2.6.5. *Pseudokirchneriella subcapitata* flow-through growth inhibition and recovery test with flufenacet/AE F13340.**

<b>Reference:</b>	<i>Pseudokirchneriella subcapitata</i> flow-through growth inhibition and recovery test with flufenacet/AE F133402.
<b>Author(s), year:</b>	Bruns, E. 2013
<b>Report/Doc. number:</b>	Study no. E 418 4253-7, Report No: EBFOL150, Reference BCS No:M-451657-01-1
<b>Guideline(s): I</b>	None (no standardised test guideline available for this study) based on OECD 201
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet /AE F133402, Batch No: NK61BX0367, Purity: 97.5 %.
Test species:	<i>Pseudokirchneriella subcapitata</i> ( formerly known <i>Selenastrum capricornutum</i> )
Type of the test:	Flow through test, 35 days
<u>Applied concentrations:</u>	3 peaks of flufenacet applied using a flow through test system
Nominal ( measured)	The first peak 4 (3.56) µg a.s./L The second peak: 23 (21.6) µg a.s./L The third peak: 12 (7.98) µg a.s./L.
Solvent:	DMF
Test vessel	Two chemostac reactors of 1500 mL (Replicate A and Replicate B)
Test volume	The value of 1500 mL nutrient medium per replicate (reactor) under continues flow through condition (complete medium exchange of 1500 ml within 48 hours) was used.

---

	<p>The reactors were illuminated with 13 LED panels each placed directly at the reactor wall resulting in a light intensity of ca. 15500 Lux .</p>
Water quality:	Nutrient medium according to OECD 201(1984, 2011) guideline
Temperature:	24 ± 1°C
pH:	7 - 8.2
Incubation:	<p>The test started with a cell density of 40 000 cells/mL.</p> <p>After 5 days a steady state of about 400 000 cells/mL was reached.</p> <p>The first peak was applied after the cell density reached steady state on day 6.03 2012 (Day 0).</p> <p>The second peak was applied to the reactors seven days later ( Day 7). The third peak was applied additional 10 day later ( Day 17).</p> <p>The nutrient media in the chemostat-flasks was stirred by magnetic stirrers and percolated using sterile air and a ring made of Accurel PP V8/2 HF tube membrane. Constant aeration of 1 L/min ± 0.1 L compressed air was reached by a flow adjuster. The aeration plus stirring prevented the sedimentation of algae cells. All operations were conducted under sterile conditions to handle an axenic1 algae culture.</p>
<u>Test parameters:</u>	<p>Morphological examinations of cells using a microscope were performed daily during the entire test period.</p> <p>Temperature both reactors were connected with a closed water circuit thermostat that allows control of the water temperature over the whole testing time.</p> <p>The pH was measured daily in the outflow samples using an electronic pH meter. Orthophosphate und total phosphate were measured daily. The measurement was carried out using a LANGE test kit. The measurement was performed using a photometer.</p>
<u>Findings:</u>	
Analytical data:	<p>Samples were analyzed for the actual concentration of flufenacet present before the first peak and on the days of application as well as on the succeeding days following the three applications. Based on the chemical analysis all results are given as measured concentrations of the test item in the test medium.</p> <p>The measured concentrations for the three peak exposure events ranged between 53.0-(66) % to 94% of nominal values of flufenacet.</p>
Morphological effects:	No morphological change in algae was observed in any test concentration.
Statistic:	All calculations were carried out using Microsoft Excel spreadsheets.

Findings:

The experiment started on the Day 24.02.2102 ( the first day of the study) and ended on the day 30.03.2012 ( 35 day of the study, 21 days after Day 0).

The cell density (number of counted cell \*10<sup>4</sup>/mL) in two reactors (A and B) used in the study was presented below:

		cell density (number of counted cells) [*10 E4 / ml]				Mean cell density x 10 <sup>4</sup> cells/ ml		Mean cell density x 10 <sup>4</sup> cells/ ml  A + B
		replicate A		replicate B				
2012	day	1	2	1	2	replicate A	replicate B	
24.2	-11	-	-	-	-	4.00	4.00	4.00
25.2	-10	42.0	48.0	61.0	58.0	45.0	60.0	52.5
26.2	-9	109	98.0	129	107	103	118	111
27.2	-8	210	206	214	247	208	231	220
28.2	-7	306	319	305	327	313	316	315
29.2	-6	439	423	413	444	431	429	430
1.03	-5	469	489	464	464	479	464	472
2.03	-4	423	437	424	433	430	429	430
3.03	-3	426	439	444	443	433	444	439
4.03	-2	434	425	403	440	430	422	426
5.03	-1	401	409	399	431	402	415	409
<b>6.03</b>	<b>0</b>	<b>440</b>	<b>392</b>	<b>410</b>	<b>405</b>	<b>416</b>	<b>408</b>	<b>412</b>
7.03	1	470	408	408	442	439	425	432
8.03	2	400	411	391	401	406	396	401
9.03	3	372	378	374	367	375	371	373
10.03	4	391	380	399	405	386	402	394
11.03	5	404	462	418	385	433	402	418
12.03	6	404	432	400	398	418	399	409
<b>13.03</b>	<b>7</b>	<b>424</b>	<b>408</b>	<b>454</b>	<b>412</b>	<b>416</b>	<b>433</b>	<b>425</b>
14.03	8	244	268	297	282	256	290	273
15.03	9	214	241	274	236	228	255	242
16.03	10	181	204	226	213	193	220	207
17.03	11	170	166	185	179	168	182	175
18.03	12	130	159	126	164	145	145	145
19.03	13	231	213	233	231	222	232	227
20.03	14	279	281	251	260	280	256	268
21.03	15	362	336	354	332	349	343	346
22.03	16	364	411	371	399	388	385	387
<b>23.03</b>	<b>17</b>	<b>392</b>	<b>402</b>	<b>381</b>	<b>412</b>	<b>397</b>	<b>397</b>	<b>397</b>
24.03	18	284	304	275	234	294	255	275
25.03	19	359	408	301	349	384	325	355
26.03	20	468	383	406	387	426	397	412
27.03	21	401	398	411	402	400	407	404
28.03	22	444	458	371	377	451	374	413
29.03	23	412	454	373	345	433	359	396
30.03	24	405	395	318	364	400	341	371

The results of the experiment are presented in the Table B 9.2.6.5-1 and Figure 9.2.6.5-1.

The chemical analysis of the first peak (4.00 µg a.s./L ) resulted in a measured concentration of 3.91 µg a.s./L for reactor A and 3.21 µg a.s./L for reactor B. The mean measured concentration of 3.56 µg/L for both reactors was estimated on

the day one.

The analysis of the second peak (nominal 23.0 µg a.s. /L) revealed a measured concentration of 21.8 for reactor A and 21.4 µg a.s. /L for reactor and B. The mean measured concentration of two reactors was 21.6 µg a.s. /L.

The chemical analysis of the third peak (nominal 12.0 µg a.s. /L) resulted in 8.11. µg a.s./L on reactor A and 7.84 mg /L for reactor B. The mean measured concentration for two reactors was calculated to be 7.98 µg a.s. /L

The first peak ( 3.56 µg a.s. /L) was applied after the cell density reached steady state on day 0. After exposure the cell density increased slightly on the next day, followed by a slight decline within the previously observed variability for two days (97.3 % on day 1 and 90.5 % on day two\*). On day 7 the second peak (21.6 µg a.s./L) was applied, resulting in a decrease of cell density for five days (maximum growth inhibition 34.3 %\*). After five days the population recovered and reached the steady state cell density within five days. The last peak (7.98 µg a.s./L) was applied on day 17, resulting in a cell density reduction of about 30 % (69.2 %\*) for one day, followed by a fast recovery of cell density one day later.

**Table B 9.2.6.5-1: Cell density in % compared to cell density of the related steady state after exposure of 3 peaks of flufenacet .**

Date	Days	Peak concentration of flufenacet (nominal)	Concentration Flufenacet1 [mg/L] [measure] <sup>1</sup>	% of nominal	Mean cell density x 10 <sup>4</sup> cells/ ml A + B	[%]*
6.03	0	4.00	<b>3.56</b>	<b>89</b>	<b>412</b>	<b>100</b>
7.03	1		0.111	3	432	105
8.03	2		<LOQ	0	401	92.8
9. 03	3		<LOQ	0	373	86.3
10. 03	4		-	0	394	91.2
11. 03	5		-	0	418	101
13. 03	7	<b>23.0</b>	<b>21.6</b>	<b>94.0</b>	<b>425</b>	<b>100</b>
14. 03	8		1.03	4.5	273	64.2
15. 03	9		0.701	3.00	242	56.9
16. 03	10		0.311	1.00	207	48.7
17.03	11		-	1.00	175	41.2
18. 03	12		<LOQ	0	145	34.1
19. 03	13		-	-	227	53.4

Date	Days	Peak concentration of flufenacet (nominal)	Concentration Flufenacet1 [mg/L] [measure] <sup>1</sup>	% of nominal	Mean cell density x 10 <sup>4</sup> cells/ ml A + B	[%]*
20. 03	14		-	-	268	63.1
21. 03	15		-	-	346	81.4
22. 03	16		-	-	387	93.4
23.03	17	12.0	<b>7.98</b>	<b>66.0</b>	<b>397</b>	<b>100</b>
24. 03	18		0.107	1.00	275	69.2
25. 03	19		0.106	1.00	355	84.4
26. 03	20		<LoQ	0	412	104

\* cell density in % compared to cell density of the related steady state

1 mean value of replicate A and replicate B

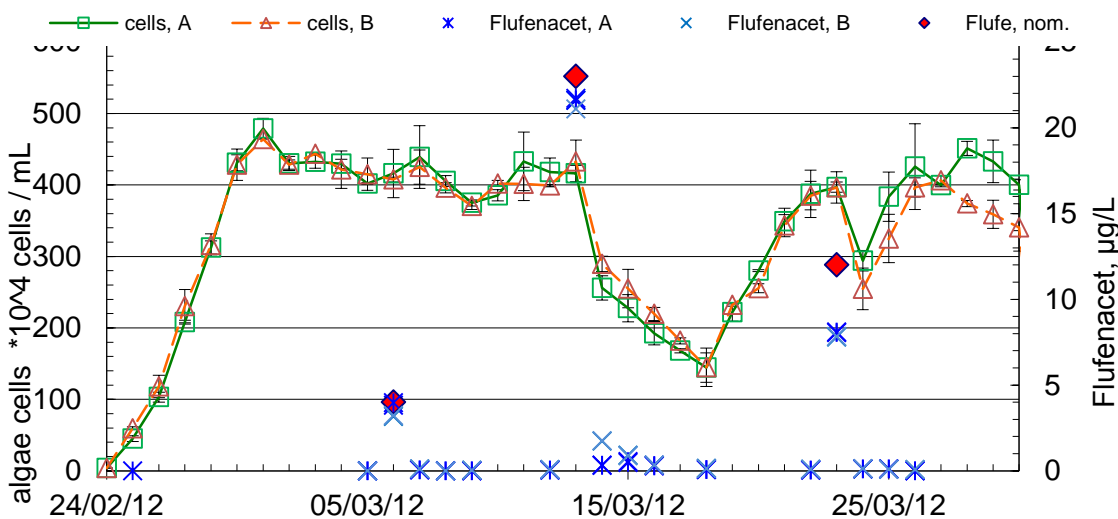


Figure 9.2.6.5-1. The algae cells/ml after exposure of 3 peaks ( 3.56, 21.6 and 7.98) of flufenacet.

#### Conclusion of RMS:

The experiment shows that after short all peaks term the recovery of *Pseudokirchinella sp.* were of up to 21.6 µg/L. However, on the day 27.03.2012 ( 21 days after Day 0, 5 days after application of the third peak application) the cell density started to differ and cell density declined in two reactors.

**Comment RMS:**

Since this is not a standard study no test guideline is available. The endpoint is based on recovery of the algae after three short term peaks exposure concentration up 21.6 µg a.s./L.

In opinion of RMS the study is not considered acceptable.

After the third peak of 7.98 microgram/L the steady state was not achieved to the level comparable to steady state before the first peak.

The lack of the control caused that it was not possible to interpret the study results after the third peak.

According to the EFSA AGD (2013), such study should be designed for the estimation of an  $E_rC_{50}$  under peak exposure condition which is to be compared to the  $E_rC_{50}$  under constant exposure. Given the design of that study and the lack of control condition, the reliability of the study is limited.

Therefore, the study can not be used in the risk assessment.

**The study is not acceptable.**

**B.9.2.6.6. *Desmodesmus subcapitatus* growth inhibition test with flufenacet (tech).**

<b>Reference:</b>	<i>Desmodesmus subspicatus</i> growth inhibition test with flufenacet (tech).
<b>Author(s), year:</b>	Bruns, E. 2011
<b>Report/Doc. number:</b>	Study No. E 323 4056-3, Report No: EBFOL114/3, Reference BCS No: M-415813-01-1
<b>Guideline(s):</b>	OECD 201 (March, 2006)
<b>GLP</b>	Yes

Material and methods:

Test substance:	Flufenacet (tech.), Batch No: K664078, Purity: 97.5 %
Test species:	<i>Desmodesmus subspicatus</i>
<u>Number of organisms:</u>	10,000 cells/mL; 3 replicate vessels per test level and 6 replicate vessels per control
Type of test, duration:	Static test, 3 days

Applied concentrations:

Nominal:	Control (0), Solvent control (0), 0.00288, 0.00921, 0.00294, 0.00940, 0.030, 0.096, 0.307, 0.980, 3.13, 10 mg a.s./L
----------	--

Geometric mean measured : Control (0) Solvent control (0), 0.00246, 0.00845, 0.00256, 0.00840, 0.0255, 0.263, 0.809, 2.525, 7.068 mg a.s./L

Solvent: DMF, dimethylformamid

Test conditions:

Water quality: Nutrient medium according to OECD guideline

Temperature: 21.8-21.9 °C ( mean, 21.8°C)

pH: 7.3-8.0 (0 h), 7.7-7.8 (72 h)

Incubation: Continuous lighting, 7040-7470 Lux, ( mean, 7258 Lux)

Test parameters:

The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature data was recorded by a data logger that calculated the mean, min and max temperatures (based on continuously (hourly means) measured values). The pH was measured at each observation time in all test levels and the control.

Cell numbers per volume (as a surrogate for biomass per volume) were estimated photometrically. For this purpose, small samples of treated, inoculated test medium were placed in 5 cm cuvettes on day 1, day 2, and day 3 of the exposure period (without replacing after measurement).

The extinctions were determined at a wave length of 578 nm using a single-beam-photometer.

To detect possible alterations in algae cells such as unusual cell size were estimated by direct algae cell counting under a microscope at a magnification of 400 times.

Samples were analysed (HPLC-MS/MS) for the actual concentration of flufenacet present in the test medium of all treatment levels and controls on Day 0 and 3.

Findings:

Analytical data: The analytical findings of flufenacet in the treatment levels found after 0 hours were 76 % to 90 % of nominal (average 85 %), after 3 day 66 % to 94 % of nominal (average 84 %) were found. Due to analytical findings all results are based on geometric mean measured test concentrations.

Statistic: The following statistical analysis are performed:  
Comparison between Control and solvent control

for Yield and for Growth Rate at 72h.

- STUDENT-t test for Homogeneous Variances

(ECx) for Growth Rate at 72 h and Yield

-Probit analysis using linear max. likelihood regression

-(NOEC) with Growth Rate at 72 h and Yield

- Shapiro-Wilk's Test on Normal Distribution

- Bartlett's Test Procedure on Variance Homogeneity

- Williams Multiple Sequential t-test Procedure

Software:

-Microsoft Exel spreadsheets

-Statistical Software ToxRat Professional version 2.10.05

#### Findings:

Morphological effects: Small clods of cells after (after 48 and 72 hours) observed at tested concentrations from 25.5 mg a.s./L.

**Table B.9.2.6.6-1: Effects of the flufenacet on *Desmodemus subspicatus*. Growth**

Geometric mean measured [mg a.s. /L]	Mean cell number per ml [ 0-72h]	Average specific growth rate [0- 72h]	% inhibition of growth rate compared to control
		μ	%
Pooled control	237000	1.041	-
0.00246	254000	1.068	-2.6
0.00845	289000	1.109	-6.5
0.0256	289000	1.104	-6.0
0.0840	245000	1.054	-1.2
0.0255	172000	0.932*	10.5
0.0857	93000	0.731*	29.8
0.263	79000	0.676*	35.1
0.809	49000	0.522*	49.8
2.525	24000	0.289*	72.3
7.068	21000	0.241*	76.9

-%

Inhibition: increase in growth relative to the control

\*

Statistically significant compared to control ( $\alpha=0.05$ , one-sided smaller, Williams multiple sequential t-test)

**Table B. B.9.2.6.6-2: Effects of the flufenacet on *Desmodium subspicatus*. Yield**

Geometric mean measured [mg a.s. /L]	Yield [0-72h]	% inhibition of growth rate compared to control
	y	%
Pooled control	22.6	-
0.00246	24.4	-7.9
0.00845	27.8	-22.9
0.0256	27.8	-23.0
0.0840	23.5	-3.8
0.0255	16.1	28.7
0.0857	8.2*	63.6
0.263	6.8*	69.7
0.809	3.9*	82.7
2.525	1.4*	93.7
7.068	1.1*	95.1

\* Statistically significant compared to the pooled control (Welch t-Test,  $\alpha \leq 0.05$ , one-sided)

#### Conclusion:

Growth inhibition values based on geometric mean measured concentration obtained with flufenacet on *Desmodium subspicatus* were as follow:

Tested parameter	Results
72 h E <sub>r</sub> C <sub>50</sub>	675 µg a.s. /L (95% CI: 559.82-818.70 µg a.s./L)
72 h E <sub>r</sub> C <sub>10</sub>	18.71 µg a.s./L (95% CI: 11.90-27.16 µg a.s./L)
72 h E <sub>r</sub> C <sub>20</sub>	64.08 µg a.s./L (95% CI: 46.34-84.25 µg a.s. L )
72 h NOE <sub>r</sub> C	8.40 µg a.s./L (95% CI: 58.55-101.04 µg a.s. /L)
72 h E <sub>y</sub> C <sub>50</sub>	76.96 µg a.s./L
72 h E <sub>y</sub> C <sub>10</sub>	5.96 µg a.s./L (95% CI: 3.06-9.62 µg a.s./L)
72 h E <sub>y</sub> C <sub>20</sub>	14.35 µg a.s./L (95% CI: 8.78-20.72 µg a.s./L)
72 h NOE <sub>y</sub> C	8.4 µg a.s./L

#### **RMS comments:**

The study was conducted according to the OECD 201 test guideline (2006).

In general the study is in line with the stated test OECD 201 test guideline and all validity criteria are met.

The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period. In the study the cell density increased by a factor of 23.7 (pooled control) which is in line with the OECD 201 test guideline. The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control must not exceed 35%. The mean coefficient of variation was determined to be 33.6 %. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%. The mean coefficient of variation for the whole test period was 5.1 %.

The RMS is of the opinion that the reliability of the results is given.

Therefore, the study is considered acceptable.

**Agreed endpoints:**

72 h  $E_rC_{50}$  = 675 µg a.s./L (95% CI: 559.82 - 818.70 µg a.s. /L)

72 h  $E_rC_{10}$  = 18.714 µg a.s./L (95% CI: 11.90-27.16 µg a.s. /L)

72 h  $E_rC_{20}$  = 64.08 µg a.s./L (95% CI: 46.34-84.25 µg a.s. /L)

$NOE_rC$  = 8.40 µg a. s./L

72 h  $E_yC_{50}$  = 76.96 µg a.s./L

72 h  $E_yC_{10}$  = 5.969 µg a.s./L (95% CI: 3.06-9.62 µg a.s./L)

72 h  $E_yC_{20}$  = 14.358 µg a.s./L (95% CI: 8.78-20.72 µg a.s./L)

$NOE_yC$  = 8.4 µg s.a./L (based on biological significant effects (28.7%) at 25.5 µg s.a./L)

All results based on geometric mean measured concentration.

**B.9.2.6.7. *Chlorella vulgaris* growth inhibition test with flufenacet ( tech.)**

<b>Reference:</b>	<i>Chlorella vulgaris</i> growth inhibition test with flufenacet (tech)
<b>Author(s), year:</b>	Bruns, E., 2011
<b>Report/Doc. number:</b>	Study No. E 323 4099-0, Report No: EBFOL114/4 Reference BCS No: M-416169-01-1
<b>Guideline(s):</b>	OECD 201 (March 2006)
<b>GLP</b>	Yes

Material and methods:

Test substance:	Flufenacet (tech.), Batch No: K664078, Purity: 97.5 %
Test species:	<i>Chlorella vulgaris</i> , Strain SAG 211-11b
Number of organisms:	10,000 cells/mL; 3 replicate vessels per test level and 6 replicate vessels per controls
Type of test, duration:	Static test, 3 days

Applied concentrations:

Nominal: Control (0), Solvent control (0), 0.00294, 0.00940, 0.0300, 0.0960, 0.307, 0.980, 3.13, 10.0 mg a.s./L

Solvent: DMF, dimethylformamid

Test conditions:

Water quality: Nutrient medium according to OECD guideline

Temperature: 21.3-22.2°C (mean, 21.5°C)

pH: 7.9 (0 h), 7.8-7.9 (72 h)

Incubation: Continuous lighting, 7800-8550 Lux ( mean, 8141 Lux)

Test parameters: The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature data was recorded by a data logger that calculated the mean, min and max temperatures (based on continuously (hourly means) measured values). The pH was measured at each observation time in all test levels and the control.

Cell numbers per volume (as a surrogate for biomass per volume) were estimated photometrically. For this purpose, small samples of treated, inoculated test medium were placed in 5 cm cuvettes on day 1, day 2, and day 3 of the exposure period (without replacing after measurement).

The extinctions were determined at a wave length of 578 nm using a single-beam-photometer.

To detect possible alterations in algae cells such as unusual cell size were estimated by direct algae cell counting under a microscope at a magnification of 400 times.

Samples were analysed (HPLC-MS/MS) for the actual concentration of flufenacet present in the test medium of all treatment levels and controls on

Day 0 and 3.

Statistic: Comparison between Control and solvent control for Yield and for Growth Rate at 72h :

STUDENT-t test for Homogeneous Variances

ECx for Growth Rate at 72 h

-Probit analysis using linear max. likelihood regression

NOEC with Growth Rate at 72 h

-Shapiro-Wilk's Test on Normal Distribution

-Levene's Test on Variance Homogeneity (with Residuals)

-Williams Multiple Sequential t-test Procedure

Software:

Microsoft Excel spreadsheets

Statistical Software ToxRat Professional version 2.10.05

ToxRat Validation Report, valid for ToxRat Version 2.10

Findings:

Analytical data:

The analytical findings of flufenacet in the treatment levels found after 0 hours were 81 % to 96 % of nominal (average 86 %), after 3 day 80 % to 101 % of nominal (average 87 %) were found.

Due to analytical findings all results are based on nominal test concentrations.

Morphological effects:

No morphological change in algae was observed in any test concentration.

**Table B.9.2.6.7-1: Effects of the flufenacet on *Chlorella vulgaris*. Growth.**

Nominal concentration [mg a.s. /L]	Mean cell number per mL [ 0-72h]	Average specific growth rate [0- 72h]	% inhibition of growth rate compared to control
		μ	%
Pooled control	1143000	1.565	-
0.00294	1375000	1.636	-4.5
0.00940	1442000	1.651	-5.5
0.0300	1495000	1.662	-6.2
0.096	1126000	1.563	0.1
0.307	175000	1.716	-9.7
0.980	1181000	1.584	-1.2
3.13	67000	1.396*	10.8
10.0	13000	0.845*	46

-%

inhibition: increase in growth relative to the control

\*

Statistically significant compared to control ( $\alpha=0.05$ , one-sided smaller, Williams multiple sequential t-test)

**Table B.9.2.6.7-2: Effects of the flufenacet on *Chlorella vulgaris*. Yield.**

Nominal concentration [mg a.s. /L]	Yield [0-72h]	% inhibition of growth rate compared to control
	y	%
Pooled control	113.2	-
0.00294	136.5	-20.5
0.00940	143.2	-26.4
0.0300	148.4	-31.1
0.096	111.6	1.5
0.307	174.0	-53.6
0.980	117.1	-3.4
3.13	66.0*	41.7
10.0	12.0*	89.4

\* Statistically significant compared to control ( $\alpha=0.05$ , one-sided smaller, Williams multiple sequential t-test).

Growth inhibition values based on nominal concentration obtained with flufenacet on *Chlorella vulgaris*, were as follow:

Tested parameter	Results
72 h E <sub>r</sub> C <sub>50</sub>	11.1 mg s.a./L (95%CI:10.33-11.96 mg s.a./L)
72 h E <sub>r</sub> C <sub>20</sub>	4.70 mg s.a./L (95%CI: 4.23-5.11 mg s.a./L)
72 h E <sub>r</sub> C <sub>10</sub>	3.00 mg s.a./L (95%CI:2.55-3.41 mg s.a./L)
72 NOE <sub>r</sub> C	0.98 mg s.a./L
72 h E <sub>y</sub> C <sub>50</sub>	3.71 mg s.a./L (95%CI:3.21-4.31 mg s.a./L )
72 h E <sub>y</sub> C <sub>20</sub>	1.932 mg s.a./L (95%CI:1.44-2.33 mg s.a./L )
72 h E <sub>y</sub> C <sub>10</sub>	1.373 mg s.a./L (95%CI:0.91-1.75 mg s.a./L)
72 h NOE <sub>y</sub> C	0.980 mg s.a./L

**RMS comments:**

The study was conducted according to the OECD 201 test guideline (2006). In general the study is in line with the stated test guideline and all validity criteria are met.

The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period. In the study the cell density increased by a factor of 114.3 (pooled control) which is in line with the OECD 201 test guideline. The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control must not exceed 35%. The mean coefficient of variation was determined to be 32.9 %.

The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%. The mean coefficient of variation for the whole test period was 5.4 %.

The RMS is of the opinion that the reliability of the results is given.

Therefore, the study is considered acceptable.

**Agreed endpoints:**

72 h  $E_rC_{50}$  = 11.1 mg a.s./L (95% CI: 10.33-11.96 mg a.s./L)

72 h  $E_rC_{20}$  = 4.70 mg a.s./L (95% CI: 4.23-5.11 mg a.s./L)

72 h  $E_rC_{10}$  = 3.00 mg a.s./L (95% CI: 2.55-3.41 mg a.s./L)

72 h  $NOE_rC$  = 0.980 mg a.s./L

72h  $E_yC_{50}$  = 3.71 mg a.s./L (95% CI: 3.21-4.31 mg a.s./L)

72 h  $E_yC_{20}$  = 1.93 mg a.s./L (95% CI: 1.44-2.33 mg a.s./L)

72 h  $E_yC_{10}$  = 1.373 mg a.s./L (95% CI: 0.91-1.75 mg a.s./L)

72 h  $NOE_yC$  = 0.980 mg a.s./L

All results based on nominal concentration.

**B.9.2.6.8. *Chlamydomonas terricola* growth inhibition test with flufenacet (tech.).**

<b>Reference:</b>	<i>Chlamydomonas terricola</i> growth inhibition test with flufenacet (tech.).
<b>Author(s), year:</b>	Sobczyk, H. ,2011.
<b>Report/Doc. number:</b>	Study No. E 323 3853-7, Report No: EBFOL114/1, Reference BCS No: M-418627-01-1
<b>Guideline(s):</b>	OECD 201, (March 2006)
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet (tech.), Batch No: K664078, Purity: 97.5 %
Test species:	<i>Chlamydomonas terricola</i>
Number of organisms:	10,000 cells/mL, 3 replicate vessels per test level and 6 replicate vessels per control
Type of test, duration:	Static test, 9 days
<u>Applied concentrations:</u>	
Nominal:	Control (0), Solvent control (0), 0.00940, 0.0300, 0.0960, 0.307, 0.980, 3.13, 10.0 mg a.s./L
Solvent:	DMF, Dimethyloformamid

Test conditions:

Water quality:	Nutrient medium according to OECD 201 guideline
Temperature:	22.5± 22.8°C (mean: 22.5 °C)
pH:	6.0 (0 h), 5.9-6.7 (216 h)
Incubation:	Continuous lighting, 5070-5470 Lux, (mean 5233 Lux)
Test parameters:	<p>The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature data was recorded by a data logger that calculated the mean, min and max temperatures (based on continuously (hourly means) measured values)). The pH was measured at each observation time in all test levels and the control. Cell numbers per volume (as a surrogate for biomass per volume) were estimated photometrically.</p> <p>The extinctions were determined at a wave length of 578 nm using a single-beam-photometer.</p> <p>Samples were analysed (HPLC-MS/MS) for the actual concentration of flufenacet present in the test medium of all treatment levels and controls on Day 0, 4 and 9.</p>

Statistic:	<p>The following statistical test were performed:</p> <p>Comparison between Control and solvent control</p> <p>STUDENT-t test for Homogeneous Variances (ECx) for Growth Rate at 216 h</p> <p>Probit analysis using linear max. likelihood regression</p> <ul style="list-style-type: none"><li>- (NOEC) with Growth Rate</li><li>- Shapiro-Wilk's Test on Normal Distribution</li><li>- Levene's Test on Variance Homogeneity (with Residuals)</li><li>- Williams Multiple Sequential t-test</li></ul> <p>Software:</p> <ul style="list-style-type: none"><li>- Microsoft Excel spreadsheets</li><li>- Statistical Software ToxRat Professional version 2.10.05</li><li>- ToxRat Validation Report, valid for ToxRat Version 2.1</li></ul>
------------	---

Findings:

Analytical data:	<p>The analytical findings of flufenacet in the treatment levels found after 0 hours were 94 % to 107 % of nominal (average 102 %), after 96 h 87 % to 107 % of nominal (average 96 %) and after 216 hours 92 % to 105 % of nominal (average 98 %).</p>
------------------	---

Due to analytical findings all results are based on mean measured concentration test concentrations.

Morphological effects: No morphological change in algae was observed in any test concentration.

**Table B. B.9.2.6.8-1: Effects of the flufenacet on *Chlamydomonas terricola*. Mean cell number after 72 h, 96 h and 216 hours.**

Nominal concentration [mg a.s./L]	Mean cell number per mL [72 h]	Mean cell number per mL [96 h]	Mean cell number per mL [120 h]	Mean cell number per mL [168 h]	Mean cell number per mL [192 h]	Mean cell number per mL [216 h]
Pooled control	30000	43000	66000	161000	234000	304000
0.00940	30000	44000	67000	173000	253000	330000
0.0300	32000	47000	72000	174000	253000	327000
0.0960	30000	42000	62000	136000	203000	263000
0.307	28000	36000	50000	100000	145000	184000
0.980	22000	22000	23000	25000	25000	26000
3.13	19000	17000	16000	15000	13000	15000
10.0	19000	17000	16000	15000	15000	15000

**Table B.9.2.6.8-2: Effects of the flufenacet on *Chlamydomonas terricola*. Growth.**

Nominal concentration [mg a.s. /L]	Average specific growth rate [0-168 h]	% inhibition of growth rate compared to control	Average specific growth rate [0-192 h]	% inhibition of growth rate compared to control <sup>1</sup>	Average specific growth rate [0-216 h]	% inhibition of growth rate compared to control <sup>1</sup>
	μ	%	μ	%	μ	%
Pooled control	0.396	-	0.393	-	0.378	-
0.00940	0.406	-2.7	0.403	-2.4	0.387	-2.4
0.0300	0.407	-2.8	0.403	-2.6	0.387	-2.3
0.0960	0.372*	6.1	0.375	4.5	0.362	4.3
0.307	0.372*	17.4	0.333*	15.2	0.322*	14.8
0.980	0.130*	67.2	0.116*	70.5	0.105*	72.2
3.13	0.055*	85.2	0.034*	91.4	0.042*	89.0
10.0	0.059*		0.048*	87.9	0.043*	88.6

-% Inhibition: increase in growth relative to the control

\* Statistical significantly compared to control (p=0.05, one-sided smaller, Williams multiple sequential t-test)

Table B.9.2.6.8-3: Effects of the flufenacet on *Chlamydomonas terricola*. Yield.

Nominal concentration [mg a.s./L]	Yield [0-168 h]	% inhibition of growth rate compared to control	Yield [0-192h]	% inhibition of growth rate compared to control	Yield [0-216 ])	% inhibition of growth rate compared to control
	y	%	y	%	y	%
Pooled control	15.102	-	22.402	-	29.366	-
0.00940	16.326	-8.1	24.304	-8.5	31.979	-8.9
0.0300	16.353	-8.3	24.279	-8.4	31.697	-7.9
0.0960	12.567	16.8*	19.269	14.0	25.275	13.9
0.307	8.955	40.7*	13.544*	39.5	17.412*	40.7
0.980	1.485	90.2*	1.529*	93.2	1.581*	94.6
3.13	0.745	96.9*	0.309*	98.6	0.456*	98.4
10.0	0.511	96.6*	0.465*	97.9	0.474*	98.4

-% Inhibition: increase in growth relative to the control

\* Statistically significantly compared to control ( $\alpha=0.05$ , one-sided smaller, Williams multiple sequential t-test,  $p \leq 0.05$ )

#### Conclusion:

Growth inhibition values based on nominal concentration obtained with flufenacet on *Chlamydomonas terricola* were as follow:

Tested parameter	Results
216 h $E_rC_{50}$	0.657 mg s.a./L (95% CI:0.561-0.762)
216 h $E_rC_{20}$	0.328 mg s.a./L (95% CI:0.248-0.398)
216 h $E_rC_{10}$	0.229 mg s.a./L (95% CI:0.156-0.293)
216 h $NOE_rC$	0.096 mg s.a./L
216 h $E_yC_{50}$	0.332 mg s.a./L (95% CI:0.272-0.404)
216 h $E_yC_{20}$	0.155 mg s.a./L (95% CI:0.104-0.198)
216 h $E_yC_{10}$	0.1042 mg a.s./L (95% CI: 0.060-0.142)
216 h $NOE_yC$	0.096 mg s.a./L

**RMS comments:**

The study was conducted according to the OECD 201 test guideline (2006).

According to the validity criteria given in the test guideline OECD 201(2006) the biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period.

However, this the criterium may be not met when species that grow slower then mentioned in the test guideline are used. In the study with *Chlamydomonas terricola* in the control cultures tested the increase of the cell density was determined to be only 3.4 (after 72 h) and 4.3 (after 96 h). The 16 fold increase of cell density in the control was observed after 168 hours and was 16.1.

Taken into consideration the validity criteria given in the test guideline OECD 201 (2006) for less frequent species tested, the coefficient of variation of average specific growth rates during the whole test period in replicate control cultures was 4.4%, which is in line with the guideline OECD 201 ( should be <10%).

In the RMS opinion the study is considered valid.

**Agreed endpoints:**

216 h  $E_rC_{50}$  = 0.657 mg s.a./L (95% CI:0.561-0.762)

216 h  $E_rC_{20}$  = 0.328 mg s.a./L (95% CI:0.248-0.398)

216 h  $E_rC_{10}$  = 0.229 mg s.a./L (95% CI:0.156-0.293)

216 h  $NOE_rC$  = 0.0960 mg s.a./L

216 h  $E_yC_{50}$  = 0.332 mg s.a./L (95% CI:0.272-0.404)

216 h  $E_yC_{20}$  = 0.155 mg s.a./L (95% CI:0.104-0.1980)

216 h  $E_yC_{10}$  = 0.104 mg a.s./L (95% CI: 0.060-0.142)

216 h  $NOE_yC$  = 0.096 mg s.a./L

All results are based on nominal test concentration.

**B.9.2.6.9. *Synechococcus leopoliensis* growth inhibition test with flufenacet (tech.)**


---

<b>Reference:</b>	<i>Synechococcus leopoliensis</i> growth inhibition test with flufenacet (tech.)
<b>Author(s), year:</b>	Bruns, E. 2011
<b>Report/Doc. number:</b>	Study No. E 323 3796-3, Report No: EBFOL114EBFOL 114 , Reference BCS No: M-415814-01-1
<b>Guideline(s):</b>	OECD 201 (March 2006)
<b>GLP:</b>	Yes

---

Material and methods:

Test substance:	Flufenacet (tech.), Batch No: K664078, Purity: 97.5 %
Test species:	<i>Synechococcus leopoliensis</i>
Number of organisms:	60,000 cells/mL; 3 replicate vessels per test level and 6 replicate vessels per control
Type of test, duration:	Static test, 3 days

Applied concentrations:

Nominal: Control (0), solvent control (0), 0.00940, 0.0300, 0.0960, 0.307, 0.980, 3.13 and 10.0 mg a.s./L

Solvent: DMF, Dimethylformamid

Test conditions:

Water quality: Nutrient medium according to OECD guideline

Temperature: 21.4 to 22.9°C (mean: 21.5°C)

pH: 7.9-8.1 (0 h), 8.0-8.1 (72 h)

Incubation: Continuous lighting, 7930-8670 Lux, (mean: 8219 Lux)

Test parameters: The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature data was recorded by a data logger that calculated the mean, min and max temperatures (based on continuously (hourly means) measured values). The pH was measured at each observation time in all test levels and the control. Cell numbers per volume (as a surrogate for biomass per volume) cell size were estimated by direct algae cell and possible alterations in

algae cells such as unusual counting under a microscope at a magnification of 400 times.

Samples were analysed (HPLC-UV) for the actual concentration of flufenacet present in the test medium of all treatment levels and controls on Day 0 and Day 3.

Statistic:

Comparison between control and solvent control for Yield at 72h, Comparison between control and solvent control for Growth Rate at 72h:

- STUDENT-t test for Homogeneous Variances

ECx for Growth Rate and Yield at 72h

- Probit analysis using linear max. likelihood regression

Effects on Growth Rate and Yield

- Shapiro -Wilk's Test on Normal Distribution

- Levene's Test on Variance Homogeneity (with Residuals)

- Welch-t test for in homogeneous Variances with Bonferroni-Holm Adjustment

- Williams Multiple Sequential t-test Procedure

Software:

- Microsoft Exel spreadsheets

- Statistical Software ToxRat Professional version 2.10.05

- ToxRat Validation Report, valid for ToxRat Version 2.10

Findings:

Analytical data:

The analytical findings of flufenacet in the treatment levels found after 0 hours were 95 % to 115 % of nominal (average 104 %), after 3 day 91 % to 113 % of nominal (average 102 %) were found.

Due to analytical findings all results are based on nominal test concentrations.

Morphological effects: No morphological change in algae was observed in any test concentration

**Table B.9.2.6.9-1: Effects of the flufenacet on *Synechococcus leopoliensis*. Growth.**

Nominal concentration [mg a.s./L]	Mean cell number per mL [72 h]	Average specific growth rate [0-72h]	% inhibition of growth rate compared to control
		$\mu$	%
Pooled control	1371 000	1.037	-
0.00940	1342000	1.035	0.2
0.0300	1345000	1.037	0.1
0.0960	1305000	1.026	1.1
0.307	1277000	1.019	1.8
0.980	1123000	0.976*	5.9
3.13	87000	0.891*	14.1
10.0	94300	0.91*	11.5

\* Statistically significant compared to control (Williams multiple sequential t-test,  $\alpha=0.05$ , one-sided smaller,  $p \leq 0.05$ ).

**Table B.9.2.6.9-2: Effects of the flufenacet on *Synechococcus leopoliensis*. Yield.**

Nominal concentration [mg a.s. /L]	Yield [0-72 h]	% inhibition of growth rate compared to control
		%
Pooled control	128.958	-
0.00940	128.167	0.6
0.0300	128.500	0.4
0.0960	124.500	3.5
0.307	121.667*	5.7
0.980	106.333*	17.5
3.13	81.000*	37.2
10.0	88.333*	31.5

\* Statistically significant difference compared to control to control (Williams multiple sequential t-test,  $\alpha=0.05$ , one-sided smaller,  $p \leq 0.05$ ).

## Conclusion:

Growth inhibition values based on nominal concentration obtained with flufenacet on *Synechococcus leopoliensis* were as follows:

Tested parameter	Results
72 h E <sub>r</sub> C <sub>50</sub>	>10 mg s.a./L
72 h E <sub>r</sub> C <sub>10</sub>	3.75 mg s.a./L (95%CI: 2.27-6.32 mg s.a./L )
NOE <sub>r</sub> C	0.307 mg s.a./L
72 h E <sub>y</sub> C <sub>50</sub>	>10 mg s.a./L
72 h E <sub>y</sub> C <sub>20</sub>	1.44 mg s.a./L (95%: CI: 0.788-2.27 mg s.a./L )
72 h E <sub>y</sub> C <sub>10</sub>	0.293 mg s.a./L (95% CI: 0.08-0.57 mg s.a./L )
72 h NOE <sub>y</sub> C	0.096 mg s.a./L

**RMS comments:**

The study was conducted according to the OECD test guideline (OECD 201, 2006). In general the study is in line with the stated test guideline and all validity criteria are met.

The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period. In the study the cell density increased by a factor of 22.8 (pooled control) which is in line with the OECD 201 test guideline. The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control must not exceed 35%. The mean coefficient of variation was determined to be 25.6%. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%. The mean coefficient of variation for the whole test period was 1.7%.

The RMS is of the opinion that the reliability of the results is given.

Therefore, the study is considered acceptable.

**Agreed endpoints:**

72 h E<sub>r</sub>C<sub>50</sub> > 10 mg a.s./L

72 h E<sub>r</sub>C<sub>10</sub> = 3.75 mg s.a./L (95% CI: 2.27-6.32 mg s.a./L)

NOE<sub>r</sub>C = 0.307 mg a.s./L

72 h E<sub>y</sub>C<sub>50</sub> > 10 mg a.s./L

72 h E<sub>y</sub>C<sub>20</sub> = 1.44 mg s.a./L (95%: CI: 0.788-2.27 mg s.a./L)

72 h E<sub>y</sub>C<sub>10</sub> = 0.293 mg s.a./L (95% CI: 0.08-0.57 mg s.a./L)

NOE<sub>y</sub>C = 0.096 mg a.s./L

All results based on nominal test concentration.

**Blue green alga****B.9.2.6.10. Acute Toxicity of FOE 5043 (technical) to *Anabaena flos-aqua*.**

<b>Reference:</b>	Acute Toxicity of FOE 5043 (technical) to <i>Anabaena flos-aquae</i> .
<b>Author(s), year:</b>	Hughes, J.S., Alexander, M.M., 1993
<b>Report/Doc. number:</b>	Study no. B059-022-1, Reference BCS No: M-002423-01-1
<b>Guideline(s):</b>	FIFRA Guideline 123-2; Tier 2 Non-target Aquatic Plant Toxicity
<b>GLP</b>	Yes

It should be noted that the new statistiactal analysis of the raw data was performed by the Applicant specially for the pupurpose of the current evaluation assessment and on request by the RMS. That was done in order to get endpoints compliant with the current requirement (EC<sub>10</sub>, EC<sub>20</sub>, EC<sub>50</sub> NOEC for growth rate and yield).

**Material and methods:**

Test substance:	FOE 5043 (tech.), Batch No.: 2030032, Purity: 97.5 %
Test species:	<i>Anabaena flos-aquae</i>
Number of organisms:	3,000 cells/mL; 3 replicate vessels per test level and 3 replicate per control
Type of test, duration:	Static test, 5 days
<b><u>Applied concentrations:</u></b>	
Nominal:	Control (0), solvent control (0), 1.90, 3.80, 7.60, 15.2, 30.3, 60.5 mg a.s./L
Solvent:	DMF
<b><u>Test conditions:</u></b>	
Water quality:	AAP nutrient medium
Temperature:	23.59-24.26°C
pH:	pH:7.42-7.59 (0 h), 7.01-7.45 (120 h)
<b><u>Incubation:</u></b>	Continuous illumination, 2153±215 Lux
Test parameters:	Cell counts were made using the Coulter Counter (Model ZBI with C-1000 Channelyzer and MCV Computer, Model MHR) on Day 3, 4 and 5. Cell counts were not performed on days 1 and 2 due to the difficulty in obtaining accurate data during the lag phase of growth where the initial inoculum is 3,000cells/mL. Counts performed on days 3, 4 and 5 are sufficient to define the shape of the growth curve.

Samples were analysed (HPLC-UV) for the actual concentration of flufenacet present in the test medium of all treatment levels and controls on Day 0 and 5.

Findings:

Analytical data:

The analytical findings of flufenacet in the treatment levels found after 0 hours were 91.4 % to 107 % of nominal and after 5 day 84.5 % to 103 % of nominal were found. All results are based on mean measured test concentrations.

Statistic:

The repeated calculations was provided by Applicant by a probit analysis effect concentrations (EC<sub>10</sub>, EC<sub>20</sub> and EC<sub>50</sub>) for yield and growth rate were derived, including confidence limits (95% and 99%). To visualize the concentration-response, growth rates and yields were plotted against the log concentrations.

NOECs and LOECs were recalculated using Williams' Multiple Sequential t-test (provided the precondition of homogeneity of variance and normal distribution were met) with significance level  $\alpha = 0.05$ , one-sided smaller.

Findings:

Morphological effects:

No morphological change in algae was observed in any test concentration.

Growth inhibition values based on mean measured concentration obtained with flufenacet on *Anabaena flos aquae* were as follow:

**Table B.9.2.6.10-1: Effects of the flufenacet on *Anabena flos-aquae*. Growth.**

Mean measured concentration (mg a.s./L)	Average specific growth rate (0-72h)	% inhibition of growth rate compared to control	Average specific growth rate (0-96h)	% inhibition of growth rate compared to control
	$\mu$	%		%
Pooled control	0.031	-	0.042	-
1.930	0.027*	12.4	0.041	3.6
3.770	0.027*	8.2	0.042	1.4
7.360	0.027*	12.9	0.038*	11.1
15.300	0.025*	20.0	0.040	6.6
31.200	0.018*	42.6	0.036*	14.0
53.200	0.018*	46.8	0.028*	34.7

\* Statistically significant compared to control (Williams multiple sequential t-test,  $\alpha=0.05$ , one-sided smaller,  $\alpha=0.05$  ).

**Table B.9.2.6.10-2: Effects of the flufenacet on *Anabena flos-aquae*. Yield.**

Mean measured concentration (mg a.s. /L)	Yield (0-72 h)	% inhibition of growth rate compared to control	Yield (0-96h)	% inhibition of growth rate compared to control
		%		%
Pooled control	25.833	-	172.167	-
1.930	19.00*	26.5	148.333*	13.8
3.770	21.33*	17.4	176.333*	-2.4
7.360	18.667*	27.4	108.33*	37.1
15.300	15.333*	40.6	134.00*	22.2
31.200	8.00*	69.0	96.00*	44.2
53.200	7.00*	72.9	40.00*	76.8

\* Statistically significant difference compared to control (Williams multiple sequential t-test,  $\alpha=0.05$ , one-sided smaller,  $\alpha=0.05$ ).

Tested parameter	Results
72 h E <sub>r</sub> C <sub>10</sub>	4.32 mg a.s./L (95% CI: 1.76-7.02 mg a.s./L)
72 h E <sub>r</sub> C <sub>20</sub>	10.67 mg a.s./L (95% CI: 6.42-14.67 mg a.s./L)
72 h E <sub>r</sub> C <sub>50</sub>	60.04 mg a.s./L (95% CI 42.47-107.44 mg a.s./L)
NOE <sub>r</sub> C	< 1.93 mg a.s./L
72 h E <sub>y</sub> C <sub>10</sub>	1.38 mg a.s./L (95% CI: 0.26-2.92 mg a.s./L)
72h E <sub>y</sub> C <sub>20</sub>	3.30 mg a.s./L (95% CI:1.09-5.67 mg a.s./L)
72 h E <sub>y</sub> C <sub>50</sub>	17.30 mg a.s./L (95% CI:11.47 -28.46 mg a.s./L)
NOEC <sub>y</sub>	<1.93 mg a.s./L
96 h E <sub>r</sub> C <sub>10</sub>	13.70 mg a.s./L (95% CI: 6.39-19.48 mg a.s./L)
96 h E <sub>r</sub> C <sub>20</sub>	30.38 mg a.s./L (95% CI: 22.09-40.18 mg a.s./L)
96 h E <sub>r</sub> C <sub>50</sub>	>53.20 mg a.s./L
NOE <sub>r</sub> C	3.77 mg a.s./L
96 h E <sub>y</sub> C <sub>10</sub>	1.74 mg a.s./L (95% CI: 0.18-4.01 mg a.s./L)
96 h E <sub>y</sub> C <sub>20</sub>	4.44 mg a.s./L (95% CI: 1.09-8.02 mg a.s./L)
96 h E <sub>y</sub> C <sub>50</sub>	26.65 mg a.s./L (95% CI: 16.50-58.64 mg a.s./L)
NOE <sub>y</sub> C	<1.93 mg a.s./L
120h E <sub>r</sub> C <sub>10</sub>	24.48 mg a.s./L (95%:17.06-29.54)
120 h E <sub>r</sub> C <sub>20</sub>	40.79 mg a.s./L (95%:35.06-46.98)
120 h E <sub>r</sub> C <sub>50</sub>	>53.20 mg a.s./L
NOE <sub>r</sub> C	15.30 mg a.s./L
120 E <sub>y</sub> C <sub>10</sub>	4.46 mg a.s./L (95%:4.91.- 4.69)
120 E <sub>y</sub> C <sub>20</sub>	8.45 mg a.s./l (95%:4.91-11.68)
120 h E <sub>y</sub> C <sub>50</sub>	28.69 mg a.s./L (95%:22.29-39.24)
NOE <sub>y</sub> C	3.70 mg a.s./L

**Comment RMS:**

The study was conducted according to the FIFRA Guideline 123-2.

According to validity criteria given in test guideline OECD 201 (2006, 2011) the biomass in the control cultures should increase exponentially by a factor of at least 16 within the 72 hour test period. In the study the cell density increased by a factor of 9.6 within 72 hours, but by factor 58 within the 96 hour test period.

According to validity criteria set in the OECD 201 (2006) guideline the mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control must not exceed 35%.

In opinion of RMS this criterium should be not applied to *Anabena flos-aquae* due to that the criterium was derived using data from studies done with other green algae species, such as *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*.

According to OECD 201 (2006) test guideline, for other, less frequently tested species such as *Anabena flos-aquae*., the coefficient of variation of average specific growth rates during the whole test period in replicate control cultures should not exceed 10%. In this study the coefficient of variation of average specific growth rates during the whole test was 3.46 %. (96h).

The following deviations from the OECD 201 (2006) test guideline were noticed:

- The test design should include preferably three replicates at each test concentration and ideally twice number of controls. In this study the three replicates for control and three replicates for solvent control were used.
- Initial cell concentration range between  $10^4$  is recommended by the guideline. In this study measured the initial cell concentrations in the control was  $3 \times 10^3$  cell/mL.

In opinion of RMS the study is considered valid.

Due to the fact that cell density was not achieved the factor >16 fold, the results obtained for 96 hour was considered acceptable.

**Agreed endpoints:**

96 h  $E_rC_{10}$  = 13.70 mg a.s./L (95% CI: 6.39-19.48 mg a.s./L)

96 h  $E_rC_{20}$  = 30.38 mg a.s./L (95% CI: 22.09-40.18 mg a.s./L)

96 h  $E_rC_{50}$  > 53.20 mg a.s./L

$NOE_rC$  = 3.77 mg a.s./L

96  $E_yC_{10}$  = 1.74 mg a.s./L (95% CI: 0.18-4.01 mg a.s./L)

96  $E_yC_{20}$  = 4.44 mg a.s./L (95% CI: 1.09-8.02 mg a.s./L)

96 h  $E_yC_{50}$  = 26.65 mg a.s./L (95% CI: 16.50-58.64 mg a.s./L)

$NOE_yC$  < 1 93 mg a.s./L

All results based on mean measured concentration.

**Freshwater diatom****B.9.2.6.11. Acute toxicity of <sup>14</sup>FOE 5043 to the Freshwater Diatom (*Navicula pelliculosa*)**

<b>Reference:</b>	Acute toxicity of <sup>14</sup> FOE 5043 to the Freshwater Diatom ( <i>Navicula pelliculosa</i> )
<b>Author(s), year:</b>	Bowers, L.M., Dobbs, M.G. 1995
<b>Report/Doc. number:</b>	Study No. F3883401, Report ID: 107113, M-002355-01-1
<b>Guideline(s):</b>	ASTM 1990, US EPA 123-1; 1982, 1985, 1986, 1989, 1994
<b>GLP</b>	Yes

It should be noted that the new statistical analysis of the raw data was performed by the Applicant specially for the purpose of the current evaluation assessment and on request by the RMS. That was done in order to get endpoints compliant with the current requirement (EC<sub>10</sub>, EC<sub>20</sub>, EC<sub>50</sub> NOEC for growth rate and yield).

**Material and methods:**

Test substance:	<sup>14</sup> C-FOE 5043; Batch No: C-583A, Purity: 99.4% FOE 5043 Technical, Batch 303-0057, Purity: 98.8%
Test species:	<i>Navicula pelliculosa</i>
Number of organisms:	3,000 cells/mL; 3 replicate vessels per test level and control and 3 replicate vessels per high level
Type of test, duration:	Static test, 120 hours

**Applied concentrations:**

Nominal:	Control (0), solvent control (0) 1.25, 2.5, 5, 10 and 20 mg a.s./L
Measured:	Control (0), solvent control (0), 1, 1.2, 2.85, 4.69, 8.57, 19.2 and 20 mg a.s./L
Solvent:	DMF, dimethylformamide

**Test conditions:**

Water quality	APP nutrient medium according to (EPA)
Temperature:	23.8-24.6°C
pH:	7.0-7.3 (0 h), 7.3-8.2 (120 h)
Incubation:	Continuous lighting, approximately 4500 Lux
Test parameters:	Each day, density was determined in three replicates at each test concentration using a light microscope and an Improved Neubauer hemocytometer. The temperature was measured hourly. The pH and conductivity were measured on Day 0 and Day 5.

Samples were analysed (HPLC-UV) for the actual concentration of flufenacet present in the test medium of all treatment levels and controls on Day 0 and Day 5.

#### Findings:

Analytical data:

The analytical findings of  $^{14}\text{C}$ -flufenacet in the treatment levels found were 86 % to 114 % of nominal (Day 0) and 90% to 97% (Day 5).

Due to analytical findings all results are based on nominal test concentrations.

Statistic:

The recalculation performed by Applicant was provided by a probit analysis effect concentrations ( $\text{EC}_{10}$ ,  $\text{EC}_{20}$  and  $\text{EC}_{50}$ ) for yield and growth rate were derived, including confidence limits (95% and 99%). To visualize the concentration-response, growth rates and yields were plotted against the log concentrations.

NOECs and LOECs were recalculated using Williams' Multiple Sequential t-test (provided the precondition of homogeneity of variance and normal distribution were met) with significance level  $\alpha = 0.05$ , one-sided smaller.

#### Findings:

Morphological effects:

No morphological change in algae was observed in any test concentration.

#### B.9.2.6.11-1: Effects of technical flufenacet on the *Navicula pelliculosa*. Growth.

Mean measured concentration [mg a.s. /L]	Average specific growth rate [0- 72h]	% inhibition relative to the controls	Average specific growth rate [0- 96 h]	% inhibition relative to the controls	Average specific growth rate [0- 120 h]	% inhibition relative to the controls [120 h]
	$\mu$	%	$\mu$	%	$\mu$	%
Pooled control	0.636	-	0.763	-	0.994	
1.12 <sup>1</sup>	0.427	32.86	0.692	9.28	0.898	9.60
2.85	0.500	21.34	0.553*	27.57	0.848*	14.63
4.69	0.307*	51.68	0.527	30.94	0.740*	25.54
8.57	-0.033*	105.3	-0.056	107.300	0.521*	47.600
19.2	-0.122*	119.1	-0.211	127.600	-0.100*	110.00

\* Statistically significant compared to pooled control (Williams Multiple t –test,  $\alpha=0.05$ )

<sup>1</sup> Replicate A at the 1.12 mg/L test level was not included in the statistical analysis of the data

**B.9.2.6.11-2: Effects of technical flufenacet on the *Navicula pelliculosa*. Yield.**

Nominal concentration [mg a.s./L]	Yield 72 h x 10 <sup>4</sup>	% inhibition relative to the controls	Yield 96 h x 10 <sup>4</sup>	% inhibition relative to the controls	Yield 120 h x 10 <sup>4</sup>	% inhibition relative to the controls [ 120 h]
Pooled control	1.887	-	6.582	-	44.958	
1.12 <sup>1</sup>	0.800	57.60	4.56	30.72*	27.035	39.87*
2.85	1.037	43.11	2.467	62.52*	22.680	49.5*
4.69	0.517	72.61**	2.467	62.52*	13.187	70.67*
8.57	-0.0030	100.2**	-0.003	100.10*	3.77	91.61*
19.2	-0.087	104.60**	-0.170	102.60*	-0.107	100.2*

\* Statistically significant compared to pooled control (Williams Multiple t –test,  $\alpha=0.05$ )

\*\* Statistically significant compared to pooled control (Welch-t-test, Bonferroni-Holm Adjustment,  $\alpha=0.05$ , one side-smaller)

<sup>1</sup> Replicate A at the 1.12 mg/L test level was not included in the statistical analysis of the data

Growth inhibition values based on nominal concentration obtained with flufenacet on *Navicula pelliculosa* were as follows:

Tested parameter	Results
120 E <sub>r</sub> C <sub>10</sub>	3.09 mg a.s./L (95% CI: 1.868-4.025 mg a.s./L)
120 E <sub>r</sub> C <sub>20</sub>	4.232 mg a.s./L (95% CI: 2.947-5.192 mg a.s./L)
120 E <sub>r</sub> C <sub>50</sub>	7.722 mg a.s./L (95% CI: 6.48-9.435 mg a.s./L)
120 h NOE <sub>r</sub> C	1.120 mg a.s./L
120 E <sub>y</sub> C <sub>50</sub>	2.172 mg a.s./L
120 h E <sub>y</sub> C <sub>10</sub>	n.d.
120 E <sub>y</sub> C <sub>20</sub>	0.723 mg a.s./L (95% CI: 0.09-1.366 mg a.s./L)
NOE <sub>y</sub> C	<1.12 mg a.s./L
96 h E <sub>r</sub> C <sub>10</sub>	2.470 mg a.s./L (95%CI:1.044-3.308 mg a.s./L)
96 h E <sub>r</sub> C <sub>20</sub>	3.156 mg a.s./L
96 E <sub>r</sub> C <sub>50</sub>	5.044 mg a.s./L (95%CI: 4.020-6.493 mg a.s./L)
96 h NOE <sub>r</sub> C	1.120 mg a.s./L
96 h E <sub>y</sub> C <sub>10</sub>	n.d
96 h E <sub>y</sub> C <sub>20</sub>	0.830 mg a.s./L (95%:CI:0.185-1.404 mg a.s./L)
96 E <sub>y</sub> C <sub>50</sub>	2.173 mg a.s./L (95%CI:1.007-3.194 mg a.s./L)
96 h NOE <sub>y</sub> C	1.120 mg a.s./L

**RMS comments:**

The study was conducted according to US EPA/FIFRA 123-1 test guideline.

The following validity criteria given in the test guideline OECD 201 (2006) should be met in the study:

- The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period. In the study the cell density increased by a factor of 8.1 for 72 hours and by a factor about 23 after 96 hours. According to validity criteria stated in the OECD 201 (2006) test guideline the mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control must not exceed 35%.

In opinion of RMS this criterium should be not applied to *Navicula* sp. due to that fact that the criteria were derived using data from studies done with green algae species such as *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*.

However, according to OECD 201 (2006) test guideline for less frequently tested species, like *Navicula* sp. the coefficient of variation of average specific growth rates during the whole test period in replicate control cultures should not exceed 10% In this study the coefficient of variation of average specific growth rates during the whole test was 16.85 %. This criterium was not met.

**Therefore, the study is considered as supportive information.**

**The agreed endpoints:**

96 h  $E_rC_{50}$  = 5.044 mg a.s./L (95%CI: 4.020-6.493 mg a.s./L), based on nominal concentration

96 h  $NOE_rC$  = 1.120 mg a.s./L, based on nominal concentration

96 h  $E_yC_{50}$  = 2.173 mg a.s./L (95%CI: 1.007-3.194 mg a.s./L), based on nominal concentration

$NOE_yC$  = 1.120 mg a.s./L, based on nominal concentration

**Marine diatom****B.9.2.6.12. Toxicity of  $^{14}C$ -FOE 5043 to the Marine Diatom *Skeletonema costatum*.**


---

Reference:	Toxicity of $^{14}C$ -FOE 5043 to the Marine Diatom <i>Skeletonema costatum</i> .
Originally Reported:	
Authors:	Bowers, L. M. (1995): Acute Toxicity of $^{14}C$ -FOE 5043 to the Marine Diatom <i>Skeletonema costatum</i> .
Guideline(s):	FIFRA § 123-2 Tier 2, OECD 201 (June 1984)
Report No:	Study No 107115: BCS Report No:M-002353-02-1
<u>Amendment:</u>	Recalculation to the original report
Author:	Dorgerloh, M. (1998)

---

Report/Doc. number:	Report Nr: DOM 98097, Reference BCS No: M-086470-01-1
Guideline(s):	OECD-Guideline No. 201: "Alga, Growth Inhibition Test" (June 7, 1984)
GLP	Yes

---

The original study presented above ( Bowers L.M., 1995) was performed according to US EPA guideline 123-2. To fulfill the European reporting requirements an additional statistical calculation of data was done by Dorgerlorh (1998) based on raw data of this study. The calculations were included in the separate report (Dorgerloh D M. 1998) The summary of the the original study with the results obtained for Dorgerlorh ( 1998) is presented below:

#### Material and methods:

Test substance:	<sup>14</sup> FOE 5043; Batch No: C-583A, Purity: 99.4%
Test species:	<i>Skeletonema costatum</i> .
<u>Number of organisms:</u>	10,000 cells/mL; 3 replicate vessels per test level and 6 replicate vessels per control
Type of test, duration:	Static test, 120 hours
<u>Applied concentrations:</u>	
Nominal ( measured)	Control (<0.1), Solvent Control (<0.1), 3.75 (3.57), 7.5 (7.47), 15, (14.3), 30 (29.1) and 60 (55.5) µg a.s./L
Solvent:	Dimethylformamide, DMF
<u>Test conditions:</u>	
Water quality:	Enriched salt (ES) media
Temperature:	19.8-21.1°C
Conductivity:	31.4-36 mhos/cm (0 h), 36.1-36.8 mhos/cm (120 h)
pH:	7.0-7.9 ( 0 h ) , 7.8-9.0 (120 h)
Incubation:	16 hour light: 8 hour dark, approximately 4400 Lux
Test parameters:	Each day, density was determined in three replicates at each test concentration using a light microscope and an Improved Neubauer hemocytometer. Temperature was hourly. The pH and conductivity was measured on Day 0, and Day 5.

#### Findings:

Analytical data: The percent of parent <sup>14</sup>C-FOE 5043 in the test solution was 100% on Day 0 and Day 5. Due to analytical findings all results are based on nominal test concentrations.

Statistic: To fulfill new data requirement the original data were recalculated using the OECD data requirement.

Growth data was analyzed using the following statistical tests:

-Probit analysis by Finney (1971)

- Dunnett's Test

#### Findings:

Morphological effects: No morphological change in algae was observed in any test concentration.

The measured algae densities during the 14 C-FOE 5043 *Skeletonema costatum* Growth test is presented below:

Measured Algae Densities During the <sup>14</sup> C-FOE 5043 <i>Skeletonema costatum</i> Growth Test						
Measured Concentration (µg/L)	REP	DENSITY (cells/ml)				
		DAY 1	DAY 2	DAY 3	DAY 4	DAY 5
Control	A	5.11 x 10 <sup>4</sup>	31.38 x 10 <sup>4</sup>	87.00 x 10 <sup>4</sup>	155.75 x 10 <sup>4</sup>	189.75 x 10 <sup>4</sup>
	B	4.33 x 10 <sup>4</sup>	29.17 x 10 <sup>4</sup>	97.00 x 10 <sup>4</sup>	130.00 x 10 <sup>4</sup>	213.00 x 10 <sup>4</sup>
	C	4.94 x 10 <sup>4</sup>	26.25 x 10 <sup>4</sup>	92.75 x 10 <sup>4</sup>	138.50 x 10 <sup>4</sup>	201.75 x 10 <sup>4</sup>
Solvent Control	A	4.22 x 10 <sup>4</sup>	21.50 x 10 <sup>4</sup>	98.75 x 10 <sup>4</sup>	146.25 x 10 <sup>4</sup>	210.00 x 10 <sup>4</sup>
	B	4.42 x 10 <sup>4</sup>	30.50 x 10 <sup>4</sup>	94.00 x 10 <sup>4</sup>	147.50 x 10 <sup>4</sup>	213.00 x 10 <sup>4</sup>
	C	5.00 x 10 <sup>4</sup>	23.63 x 10 <sup>4</sup>	93.50 x 10 <sup>4</sup>	141.25 x 10 <sup>4</sup>	222.00 x 10 <sup>4</sup>
3.57	A	3.42 x 10 <sup>4</sup>	21.25 x 10 <sup>4</sup>	99.50 x 10 <sup>4</sup>	156.00 x 10 <sup>4</sup>	222.75 x 10 <sup>4</sup>
	B	3.44 x 10 <sup>4</sup>	19.75 x 10 <sup>4</sup>	79.75 x 10 <sup>4</sup>	126.25 x 10 <sup>4</sup>	219.75 x 10 <sup>4</sup>
	C	5.06 x 10 <sup>4</sup>	28.42 x 10 <sup>4</sup>	82.50 x 10 <sup>4</sup>	116.50 x 10 <sup>4</sup>	222.00 x 10 <sup>4</sup>
7.47	A	0.94 x 10 <sup>4</sup>	0.44 x 10 <sup>4</sup>	0.53 x 10 <sup>4</sup>	1.06 x 10 <sup>4</sup>	<0.14 x 10 <sup>4</sup>
	B	0.44 x 10 <sup>4</sup>	0.19 x 10 <sup>4</sup>	0.53 x 10 <sup>4</sup>	<0.14 x 10 <sup>4</sup>	0.14 x 10 <sup>4</sup>
	C	0.67 x 10 <sup>4</sup>	<0.11 x 10 <sup>4</sup>	<0.33 x 10 <sup>4</sup>	<0.17 x 10 <sup>4</sup>	<0.14 x 10 <sup>4</sup>
14.3	A	0.92 x 10 <sup>4</sup>	0.50 x 10 <sup>4</sup>	<0.14 x 10 <sup>4</sup>	<0.17 x 10 <sup>4</sup>	<0.11 x 10 <sup>4</sup>
	B	2.06 x 10 <sup>4</sup>	0.92 x 10 <sup>4</sup>	1.19 x 10 <sup>4</sup>	3.06 x 10 <sup>4</sup>	3.42 x 10 <sup>4</sup>
	C	1.75 x 10 <sup>4</sup>	1.67 x 10 <sup>4</sup>	3.08 x 10 <sup>4</sup>	15.50 x 10 <sup>4</sup>	32.63 x 10 <sup>4</sup>
29.1	A	0.64 x 10 <sup>4</sup>	<0.19 x 10 <sup>4</sup>	<0.14 x 10 <sup>4</sup>	<0.39 x 10 <sup>4</sup>	<0.11 x 10 <sup>4</sup>
	B	1.44 x 10 <sup>4</sup>	0.19 x 10 <sup>4</sup>	<0.22 x 10 <sup>4</sup>	<0.44 x 10 <sup>4</sup>	<0.11 x 10 <sup>4</sup>
	C	2.31 x 10 <sup>4</sup>	1.08 x 10 <sup>4</sup>	4.39 x 10 <sup>4</sup>	13.44 x 10 <sup>4</sup>	32.25 x 10 <sup>4</sup>
55.5	A	0.78 x 10 <sup>4</sup>	0.50 x 10 <sup>4</sup>	<0.33 x 10 <sup>4</sup>	<0.19 x 10 <sup>4</sup>	<0.11 x 10 <sup>4</sup>
	B	3.19 x 10 <sup>4</sup>	2.97 x 10 <sup>4</sup>	9.19 x 10 <sup>4</sup>	14.88 x 10 <sup>4</sup>	14.21 x 10 <sup>4</sup>
	C	1.47 x 10 <sup>4</sup>	<0.19 x 10 <sup>4</sup>	<0.42 x 10 <sup>4</sup>	<0.14 x 10 <sup>4</sup>	<0.11 x 10 <sup>4</sup>

Growth inhibition values based on mean measured concentration obtained with flufenacet on *Skeletonema costatum*, were as follow:

**Table B.9.2.6.12-1: Effects of technical flufenacet on the *Skeletonema costatum*.**

Flufenacet [µg s.a./L] nominal	Biomass		Growth Rate per day	
	Biomass integral (0-96 h)	% inhibition relative to the controls	Average specific growth rate (0- 96 h)	% inhibition of growth rate compared to control
control	4636	0.0	1.24	0.0
3.57	4256	8.2	1.22	1.3
7.47	-45	101.0*	-0.31	124.9*

Flufenacet [µg s.a./L] nominal	Biomass		Growth Rate per day	
	Biomass integral (0-96 h)	% inhibition relative to the controls	Average specific growth rate (0- 96 h)	% inhibition of growth rate compared to control
14.30	89	98.1*	0.17	85.9*
29.10	58	98.8*	0.07	94.4*
55.50	129	97.2*	-0.08	106.2*

\* Statistically significant compared to control (Dunnet's one-tailed test,  $p \leq 0.05$ )

Growth inhibition values based mean measured concentration obtained with flufenacet on *Skeletonema costatum* were as follow:

Tested parameter	Results
E <sub>r</sub> C <sub>50</sub> (0 - 96 h) CI 95% -n.d.	9.49
(NOE <sub>r</sub> C, 0-96 h)	3.57
E <sub>b</sub> C <sub>50</sub> (0 - 96 h) CI 95% -n.d.	7.05
NOE <sub>b</sub> C (0-96 h)	3.57

nd- not determined

#### RMS comments:

The original study (Bowers L.M., 1995) was conducted according to the US EPA 123-2 test guideline.

According to the validity criteria given in the OECD 201 (2006) test guideline the mean cell density in the control should increased by a factor  $\geq 16$  (cell density increased by a factor 92 for 0-72 hours and by factor 141 for 0-96 hours).

In addition, according to OECD 201 (2006) test guideline the mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control must not exceed 35%.

In opinion of RMS this criterium should be not applied to *Skeletonema costatum* due to that the criterium were derived using data from studies done with green algae species such as *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*.

However, according to OECD 201 (2006) the coefficient of variation of average specific growth rates during

the whole test period in replicate control cultures should not exceed 10% for less frequently tested species, like *Skeletonema costatum*.

In this study the coefficient of variation of average specific growth rates during the whole test was 1.21% for 0-72 h and 1.85 % for 0-96 h.

To fulfill the requirements stated in OECD 201 test guideline the new statistical re-calculation of growth rate on the original raw data done by Dorgerloh (1998) was performed in the additional report.

In opinion of RMS the recalculation is not considered reliable.

In the source study report by [Huges; 1995] the number of cells in the individual replicates is varying and in some cases not precise (it is reported as a value below a certain level, which also is a variable). That problem was observed for the samples treated with test compound at the levels between 7.74 µg/L and 55.5 µg/L. That variability did not follow any reasonable pattern.

The Applicant calculated the toxicity endpoint for growth rate 96 hours, although that value cannot be considered fully reliable. That is due to the fact that for the indicated above range of concentrations in some replicates the number of cells reported as being below the certain value was brought to that limit in order to calculate the average, what impacted the results (the number of cells was overestimated).

The dose response isn't clear, with one of the lowest concentrations - 7.74 µg/L, having the largest response.

In the same time the test concentrations (7.47 and 55.0 µg/L) with the largest inhibition were excluded from the statistical analysis (which was poor reported in the study protocol). There was no explanation of the obtained results.

**All that taken into account RMS is of the opinion that the endpoints determined in the study cannot be considered reliable. The study is not accepted.**

**Metabolites:****B.9.2.6.13. *Pseudokirchneriella subcapitata* growth inhibition test with flufenacet –oxalate.**

---

<b>Reference:</b>	<i>Pseudokirchneriella subcapitata</i> growth inhibition test with flufenacet –oxalate.
<b>Author(s), year:</b>	Bruns, E., 2009
<b>Report/Doc. number:</b>	Study No.: E 323 3715, Report ID: EBFOL137, Reference BCS Report: M-358823-01-1
<b>Guideline(s):</b>	OECD 201 (March 2006)
<b>GLP:</b>	Yes

---

**Material and methods:**

Test substance:	Flufenacet –oxalate, Batch No: SES10564-3-1, Purity: 95.3 %
Test species:	<i>Pseudokirchneriella subcapitata</i>
Number of organisms:	10,000 cells/ml 3 replicate per treatment group and 6 replicates per control group
Type of test, duration:	Static test, 72 hours

**Applied concentrations:**

Nominal:	Control (0), solvent control (0) 6.25, 12.5, 25.0, 50.0 and 100 mg pure metabolite/L
Solvent:	None

**Test conditions:**

Water quality:	Nutrient medium according to OECD 201 guideline
Temperature:	21.6 to 21.9 °C (mean: 21.7°C)
pH:	6.8-8.1 (0 h), 7.6-8.9 (72 h)

Incubation:	Continuous illumination, 7710-8120 Lux (mean, 7941 Lux)
Test parameters:	Cell numbers per volume (as a surrogate for biomass per volume) were estimated photometrically. For this purpose, small samples of treated, inoculated test medium were placed in 5 cm cuvettes on day 1, day 2, and day 3 of the exposure period (without replacing after measurement).

Possible alterations in algae cells such as unusual cell size were estimated by direct algae cell counting under a microscope at a magnification of 400 times.

The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature data was recorded by a data logger that calculated the mean, min and max temperatures (based on continuously (hourly means) measured values. The pH was measured at each observation time in all test levels and the control.

Samples were analysed HPLC-MS/MS for the actual concentration of flufenacet present in the test medium of all treatment levels and controls on Day 0 and Day 3.

#### Findings:

##### Analytical data:

The analytical findings of flufenacet-oxalate (calculated from flufenacet-oxalate hydrate) in the treatment levels found after 0 hours were 104 % to 107 % of nominal (average 105 %), after 3 day 102 % to 117 % of nominal (average 107 %) were found.

Due to analytical findings all results are based on nominal test concentrations.

##### Statistic:

The statistical analysis were performed to determine:

ECx for Growth Rate at 72 h

Probit analysis using linear max. likelihood regression

NOEC with Growth Rate at 72

Cochran's Test Procedure on Variance Homogeneity

Williams Multiple Sequential t-test Procedure

#### Software:

Statistical Software ToxRat Professional version 2.09

ToxRat Validation Report, valid for ToxRat Version 2.09

#### Findings:

##### Morphological effects:

No morphological change in algae was observed in any test concentration.

**Table B.9.2.6.13-1: Effects of technical Flufenacet –oxalate on the *Pseudokirchneriella subcapitata*.**

Nominal concentration [mg a.s./L]	Mean cell number per ml [ 0-72h]	Average specific growth rate [0- 72h]	% inhibition of growth rate compared to control	Doubling time of algae cell [days]
		μ	%	days
Control	920 000	1.507	-	0.460
6.25	994 000	1.533	-1.7	0.452
12.5	962 000	1.522	-1.0	0.455
25.0	983 000	1.529	-1.5	0.453
50.0	1 003 000	1.536	-1.9	0.451
100	985 000	1.530	-1.5	0.453

-% inhibition: increase in growth relative to the control

Growth inhibition values based on nominal concentration obtained with flufenacet-oxalate, on *Pseudokirchneriella subcapitata* were as follow:

Test substance	Flufenacet-oxalate
E <sub>r</sub> C <sub>50</sub> /E <sub>b</sub> C <sub>50</sub>	>100 mg met./L
NOE <sub>r</sub> C /NO <sub>b</sub> C <sub>50</sub>	≥100 mg met./L

**RMS comments:**

The study was conducted according to the OECD 201 test guideline (2006). In general the study is in line with the stated test guidelines and all validity criteria are met. The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period. In the study the cell density increased by a factor of 92 which is in line with the OECD 201 test guideline. The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control must not exceed 35%. The mean coefficient of variation was determined to be 20.3 %. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%. The mean coefficient of variation for the whole test period was 1.2 %.

The RMS is of the opinion that the reliability of the results is given. Therefore, the study is considered acceptable.

**Agreed endpoints:**

E<sub>r</sub>C<sub>50</sub> > 100 mg Flufenacet-oxalate/L

NOE<sub>r</sub>C ≥ 100 mg Flufenacet-oxalate /L

$E_bC_{50} > 100$  mg Flufenacet-oxalate /L  
 $NOE_bC \geq 100$  mg Flufenacet-oxalate /L  
 All results based on nominal test concentration.

#### B.9.2.6.14. Influence of FOE 5043-sulfonic acid on the growth of the green alga, *Scenedesmus subspicatus*.

<b>Reference:</b>	Influence of FOE 5043-sulfonic acid on the growth of the green alga, <i>Scenedesmus subspicatus</i> .
<b>Author(s), year:</b>	Anderson, J. P. E., 1995
<b>Report/Doc. number:</b>	Study No AJO/132495, Reference BCS No: M-004931-01-1
<b>Guideline(s):</b>	OECD 201 (March 2006)
<b>GLP:</b>	Yes

#### Material and methods:

Test substance: Sodium salt of the FOE 5043-sulfonic acid, Batch No: WAK 6222-3, Purity: 93.60 %

Test species: *Desmodesmus subspicatus* formerly know as *Scenedesmus subspicatus*

Number of organisms: 10,000 cells/mL; 6 replicate vessels per test level and 3 replicate vessels per control

Type of test, duration: Static test, 3 days

#### Applied concentrations:

Nominal: Control (0), solvent control (0), 8.67, 15.6, 27.7, 48.6, 86.7 mg p.m/L

Solvent: No

#### Test conditions:

Water quality:

Temperature: 23±2 °C

pH: 7.89-7.96 (0 h), 9.63-10.07 (72 h)

Incubation: Continuous illumination, 8000 Lux (±20 %)

Test parameters: The pH was measured in all test levels and the control with 24 hour interval. Cell numbers were estimated photometrically. For this purpose, samples of treated, inoculated culture medium were placed in 5 cm cuvettes and the

extinctions were determined at a wave length of 578 nm using a single-beam-photometer.

Possible alterations in algae cells such as unusual cell size were estimated by direct algae cell counting under a microscope at a magnification of 400 times.

Quantitative analyses of FOE 5043-sulfonic acid Na-salt in cell-free samples of the nutrient medium were made at the beginning of the experiment.

Because growing algal cells can adsorb, incorporate and/or metabolize the active ingredient under study, concentrations were not determined at the end of the experiment.

#### Findings:

Analytical data:

The quantities found at the beginning of the test, in reference to the nominal concentrations, were 87 % to 89 %. The average for the measured concentrations was 88 %.

Statistic:

The EC<sub>50</sub> for growth of biomass (E<sub>b</sub>C<sub>50</sub>) and for algal growth rate (E<sub>r</sub>C<sub>50</sub>) were calculated using probit analyses after Finney (1952), and the slopes of the regression lines were calculated following Litchfield and Wilcoxon. The NOEC and LOEC were calculated by an analysis of variance (Dunnett's-Test).

#### Findings:

Morphological effects:

No morphological change in algae was observed in any test concentration.

**Table B.9.2.6.14-1: Effects of flufenacet -sulfonic acid on the *Desmodesmus subspicatus*.**

Nominal concentration (mg p.m. met./L)	Mean cell number per mL [ 0-72h] x 10 <sup>4</sup>	Biomass		Average specific growth rate [0- 72h] μ	% inhibition of growth rate compared to control*
		Area under the curve [0-72]	% inhibition relative to the control		
Control	116.61	2272	0.0	1.58	-
8.67	121.53	2340	-3.0	1.60	-0.9
15.6	118.86	2299	-1.2	1.59	-0.4
27.7	127.63	2437	-7.3	1.61	-1.9
48.6	121.75	2434	-7.1	1.60	-0.0
86.7	112.14	2294	-1.0	1.57	-0.8

\*test initiation with 10,000 cells/mL

Growth inhibition values based on nominal concentration obtained with Flufenacet sulfonic acid on *Desmodesmus subspicatus*.

Test parameter	Flufenacet–sulfonic acid
72 h E <sub>r</sub> C <sub>50</sub>	>86.7 mg pure met./L
NOE <sub>r</sub> C	≥86.7 mg pure met./L
72 h E <sub>b</sub> C <sub>50</sub>	>86.7 mg pure met./L
NOE <sub>b</sub> C	≥86.7 mg pure met./L

**RMS comments:**

The study was conducted according to the OECD 201 test guideline (2006). In general the study is in line with the stated test guideline.

The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period. In the study the cell density increased by a factor of 116.61 which is in line with the OECD 201(2006) test guideline. The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control must not exceed 35%.

The mean coefficient of variation was determined to be 18.44%.

The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%. The mean coefficient of variation for the whole test period was 0.68 %.

The following deviations from the OECD 201(2006) test guideline were noted:

- The pH in the control increased more than 1.5 units during the study (measured: 2.1 units (pH: 7.95-10.01).
- The measurement of test concentration was not done at the end of test.

All biological criteria were met in the study. The concentration of the test item were determined only at the beginning of the study and were not monitored afterwards however, from comfortable short term aquatic studies (e.g. the fish study Dorgerloh (1995) M-004932-01-1) stability of the test item can be deduced and is sufficient to conclude on very low concern of FOE sulfonic acid to algae.

Therefore, in RMS opinion the study is considered acceptable.

**Agreed endpoint:**

72 h E<sub>r</sub>C<sub>50</sub> > 86.7 mg pure metabolite/L

NOE<sub>r</sub>C > 86.7 mg pure metabolite/L

72 h E<sub>b</sub>C<sub>50</sub> > 86.7 mg pure metabolite/L

NOE<sub>b</sub>C > 86.7 mg pure metabolite/L

**B.9.2.6.15. *Pseudokirchneriella subcapitata* growth inhibition test with flufenacet –methylsulfone.**


---

<b>Reference:</b>	<i>Pseudokirchneriella subcapitata</i> growth inhibition test with flufenacet – methylsulfone.
<b>Author(s), year:</b>	Bruns, E. 2010
<b>Report/Doc. number:</b>	Study no. E 323 3711-0, Report No;EBFOL146, Reference BCS No: M-364591-01-1
<b>Guideline(s):</b>	OECD 201 (March 2006)
<b>GLP:</b>	Yes

---

Material and methods:

Test substance:	Flufenacet –methylsulfone, Batch No: SES 10623-5-1,Purity: 97.6 %
Test species:	<i>Pseudokirchneriella subcapitata</i>
Number of organisms:	10,000 cells/mL; 6 replicate vessels per test level and 6 replicate vessels per control
Type of test, duration:	Static test, 3 days
<u>Applied concentrations:</u>	
Nominal:	Control (0), solvent control (0) 10.0 mg met./L
Solvent:	DMF, Dimethyloformamid
<u>Test conditions:</u>	
Water quality:	Nutrient medium according to OECD guideline
Temperature:	22.0-22.7 °C (mean: 21.7°C)
pH:	8.2 (0 h), 8.6-8.7 (72 h)
Incubation:	Continuous illuminati on, 7910-8320 Lux (mean, 8072 Lux)
Test parameters:	The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature data was recorded by a data logger that calculated the mean, min and max temperatures (based on continuously (hourly means) measured values). The pH was measured at each observation time in all test levels and the control.  Cell numbers per volume (as a surrogate for biomass pervolume) were estimated photometrically. For this purpose, small samples of treated, inoculated test

medium were placed in 5 cm cuvettes on day 1, day 2, and day 3 of the exposure period (without replacing after measurement).

The extinctions were determined at a wave length of 578 nm using a single-beam-photometer. Possible alterations in algae cells such as unusual cell size were estimated by direct algae cell counting under a microscope at a magnification of 400 times.

Samples were analysed (HPLC-UV) for the actual concentration of flufenacet – methylsulfone present in the test medium of all treatment levels and controls on Day 0 and Day 3.

#### Findings:

##### Analytical data:

The analytical findings of flufenacet-methylsulfone in the treatment found after 0 hours were 91% of nominal, after 3 day 100 % were found. Due to analytical findings all results are based on nominal test concentrations.

##### Statistic:

The following statistical analysis were conducted:

Effective Concentrations (ECx) with Growth Rate at 72 h

- Probit analysis using linear max. likelihood regression

Threshold Concentrations (NOEC) with Growth Rate at 72 h

- Kolmogorov-Smirnov-test on Normal Distribution

- Cochran's Test Procedure on Variance Homogeneity

- STUDENT-t test for Homogeneous Variances

Software:

- Microsoft Excel spreadsheets

- Statistical Software ToxRat Professional version 2.09

- ToxRat Validation Report, valid for ToxRat Version 2.09

#### Findings:

##### Morphological effects:

No morphological change in algae was observed in any test concentration.

**Table B.9.2.6.15-1: Effects of technical flufenacet –methylosulfone on the *Pseudokirchneriella subcapitata*.**

Nominal concentration [mg p.m./L]	Mean cell number per ml [ 0-72h]	Average specific growth rate [0- 72]	% inhibition of growth rate compared to control*	Doubling time of algae cell [days]
		μ	%	days
Control	844 000	1.478	-	0.469
Solvent control	835 000	1.475	-	0.470
Pooled control	839 000	1.477	-	0.469
10.00	818 000	1.468	0.6	0.472

\*relative to pooled controls

Growth inhibition values based on nominal concentration obtained with flufenacet-methylosulfone on *Pseudokirchneriella subcapitata* were as follows:

Test substance	Flufenacet-methylosulfone
E <sub>r</sub> C <sub>50</sub>	>10 mg metabolite /L
NOE <sub>r</sub> C	≥10.0 mg metabolite /L

**RMS comments:**

The study was conducted according to the OECD 201 test guideline (2006). In general the study is in line with the stated test guidelines and all validity criteria are met. The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period. This corresponds to a specific growth rate of 0.92 per day. In the study the cell density increased by a factor of 83.9 ( pooled control) which is in line with the OECD 201 test guideline. The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) was determined to be 30.9 % (in the control must not exceed 35%).

The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%. The mean coefficient of variation for the whole test period was 0.8 %.

The RMS is of the opinion that the reliability of the results is given.

Therefore, the study is considered acceptable.

**Agreed endpoints:**

E<sub>r</sub>C<sub>50</sub> > 10 mg metabolite /L, based on nominal concentration

NOE<sub>r</sub>C ≥ 10 mg metabolite /L, based on nominal concentration

---

**B.9.2.6.16. FOE 5043-Methylsulfide – Influence on the growth of the Green Alga, *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*).**


---

**Reference:** FOE 5043-Methylsulfide – Influence on the growth of the Green Alga, *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*).

**Author(s), year:** Dorgerloh M., 1998

**Report/Doc. number:** Study no. E 323 1346-2, Report ID: DOM 98011, M-002341-01-1

**Guideline(s):** ASTM E 1218, US-EPA FIFRA§ 123-2 Tier 2, OECD 201 (June 1984)

**GLP:** Yes

---

Material and methods:

**Test substance:** FOE 5043-Methylsulfide; Batch No. WAK 7825-2-1, Purity: 97.7 %

**Test species:** *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*).

**Number of organisms:** 10,000 cells/mL; 3 replicate vessels per test level and 6 replicate vessels per control

**Type of test, duration:** Static test, 4 days

Applied concentrations:

**Nominal:** Control (0), 1.0, 1.8, 3.2, 5.6, 10.0, 18.0, 32.0, 56.0 and 100 mg pure metabolite/L

**Solvent:** None

Test conditions:

**Water quality:** Nutrient medium according to OECD 201 guideline

**Temperature:** 23±2°C

**pH:** 8.04-8.14 (0 h), 8.31-10.16 (72h)

**Incubation:** Continuous illumination, 8000 Lux

**Test parameters:** Cell numbers were estimated photometrically. For this purpose, samples of treated, inoculated culture medium were placed in 5 cm cuvettes and the extinctions were determined at a wave length of 578 nm using a single-beam-photometer. To detect possible alterations in algal cells that might influence extinction measurements, such as unusual cell size, samples from each flask were examined under a microscope at a magnification of 400 times.

Samples were analysed (HPLC-UV) for the actual concentration of flufenacet-methylsulfide present in the test medium of all treatment levels and controls on Day 0 and Day 4. Temperatures and pH values of the cultures were determined using electronic equipment.

Findings:

Analytical data: The analytical findings of FOE 5043-Methylsulfide in the treatment levels found were 87 % to 105 % of nominal. Due to analytical findings all results are based on nominal test concentration.

Statistic: The 72 h EC<sub>50</sub> for biomass (E<sub>b</sub>C<sub>50</sub>) and for algal growth rate (E<sub>r</sub>C<sub>50</sub>) were calculated using probit analyses after Finney and the slopes of the regression lines were calculated following Litchfield and Wilcoxon (1949). Calculations were carried out using commercial software The NOEC's and LOEC's were calculated by an analysis of variance Dunnett's-Test.

Findings:

Morphological effects: Cell swollen after 48, 72 and 96 hours at tested concentrations- 56 mg metabolite/L and 100 mg metabolite/L.

**Table B.9.2.6.16-1: Effects of FOE 5043-Methylsulfide on the *Pseudokirchneriella subcapitata*.**

Nominal concentration [mg p.m. /L]	Mean cell number per ml [0-72h]	Biomass		Growth rate	
		Area under the curve [0-72h]	% inhibition relative to the control	Average specific growth rate [0- 72h]	% inhibition of growth rate compared to control
				μ	%
Control	111 6000	2060	-		
1	112 4700	2044	0.8	1.60	-0.1
1.8	117 7400	2137	-3.7	1.60	-1.1
3.2	97 5000	1803*	12.5	1.61	2.9
5.6	100 48000	1846	10.4	1.55	2.2
10	100 1500	1849	10.3	1.56	2.3
18	848 000	1646*	20.1	1.50*	5.8
32	739100	1260*	38.9	1.46*	8.7
56	254000	500*	75.7	1.10*	30.9
100	391000	122*	94.1	0.47*	70.5

\* Statistically significant compared to control ( Dunnet test, one side smaller, α=0.05)

Growth inhibition values based on nominal concentration obtained with FOE 5043 Methylsulfide on

*Pseudokirchneriella subcapitata* were as follow:

Test parameter	Results
72 h E <sub>r</sub> C <sub>50</sub>	83.8 mg p.m/L
72 h NOE <sub>r</sub> C	10 mg p.m./L
72 h E <sub>b</sub> C <sub>50</sub>	30.5 mg p.m./L
72 h NOE <sub>b</sub> C	10.0 mg p.m /L

**RMS comments:**

The study was conducted according to the US-EPA-FIFRA under § 123-2 and OECD 201 (1984) test guidelines. In general the study is in line with the stated test guidelines and all validity criteria are met.

The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period. In the study the cell density increased by a factor of 111.6 for 72 h which is in line with the OECD 201 test guideline. The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control must not exceed 35%. The mean coefficient of variation was determined to be 10.6%. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%. The mean coefficient of variation for the whole test period was 1.19%.

The RMS is of the opinion that the reliability of the results is given.

Therefore, the study is considered acceptable.

**Agreed endpoints:**

72 h E<sub>r</sub>C<sub>50</sub> = 83.8 mg p.m/L ,based on nominal concentration

72 h NOE<sub>r</sub>C = 10 mg p.m./, based on nominal concentration

72 h E<sub>b</sub>C<sub>50</sub> = 30.5 mg p. m./L, based on nominal concentration

72 h NOE<sub>b</sub>C = 10 mg p. m./L, based on nominal concentration

**B.9.2.6.17. The toxicity of Trifluoroacetate to the Algae *Selenastrum capricornutum***

<b>Reference:</b>	The Toxicity of Trifluoroacetate to the Algae <i>Selenastrum capricornutum</i> .
<b>Author(s), year:</b>	Groeneveld, A.H.C., de Kok, H.A.M., van den Berg, G., (1992)
<b>Report/Doc. number:</b>	Study No: 56635/52/92, Reference BCS Number: M-247820-01-1
<b>Guideline(s):</b>	OECD Guideline 201 (1984)
<b>GLP:</b>	Yes

It should be noted that the new statistical analysis of the raw data was performed by the Applicant specially for the purpose of the current evaluation assessment and on request by the RMS. That was done in order to get endpoints compliant with the current requirement (EC<sub>10</sub>, EC<sub>20</sub>).

Material and methods:

Test substance:	Sodium trifluoroacetate, Batch No: ACA9135AB, Purity: 99%
Test species:	<i>Selenastrum capricornutum</i>
Number of organisms:	1 x 10 <sup>4</sup> /mL; 4 replicate vessels per test level and 7 replicate vessels per control
Type of test, duration:	Static test, 3 days
<u>Applied concentrations:</u>	
Nominal:	Control (0), 1200, 360, 120, 36, 12, 3.6, 1.2, 0.36 and 0 mg Na-TFA metabolite/L

Test conditions:

Water quality:	APP - Algal medium according to US EPA guideline
Temperature:	23.5-24.5°C (mean, 24.0°C)
pH:	7.3 (0 h), 9.4 (72h) in the control 7.2-7.3(0h), 8.0-9.4 in the tested concentrations
Incubation:	Continuous illumination, 7200 Lux
Test parameters:	The pH was measured at day 0 and 3 in one replicate of each test concentration. At the start of the test the absorption was measured in one replicate per test concentration. At day 1, 2 and 3 the absorption at 680 nm was measured, using a spectrophotometer (Varian DMS 90) and a cuvette with a path length of 5 cm. After 3 days the condition of the algae was examined by microscope and

any abnormalities recorded.

Samples were analysed (HPLC-UV) for the actual concentration of Sodium trifluoroacetate present in the test medium of all treatment levels and controls on Day 0 and Day 3.

#### Findings:

##### Analytical data:

The analytical findings of Sodium trifluoroacetate in the treatment levels found were 97 % to 111 % of nominal. Due to analytical findings all results are based on nominal test concentrations.

##### Morphological effects:

The algae exposed to the highest concentration (1200 mg/L) was clearly affected.

##### Statistic:

The probit analysis of effect concentrations (EC<sub>10</sub>, EC<sub>20</sub> and EC<sub>50</sub>) for yield and growth rate were derived, including confidence limits (95% and 99%). NOECs and LOECs were recalculated if possible (provided the precondition of homogeneity of variance and normal distribution were met) using Williams' Multiple Sequential t-test with significance level  $\alpha = 0.05$ , one-sided smaller. Statistical power of the calculation could be deduced from the minimum detectable difference (%MDD). The statistical analysis were done using software ToxRat Professional (version 2.10) program.

**Table B. 9.2.6.17-1: Effects of Sodium trifluoroacetate on the *Selenastrum capricornutum*.**

Nominal concentration [mg /L]	Yield		Growth rate per day	
	[0-72h] x 10 <sup>6</sup>	% inhibition relative to the control	Average specific growth rate [0- 72h]	% inhibition of growth rate compared to control
			μ	%
0	1.754	-	0.072	-
0.36	1.590*	9.4	0.070	1.9
1.2	1.110*	36.7	0.065*	8.9
3.6	0.877*	50.0	0.062*	13.4
12	0.643*	63.3	0.058*	19.2
36	0.356*	79.7	0.050*	30.4
120	0.152*	91.4	0.039*	46.4
360	0.0740*	95.8	0.029*	59.1
120	0.043*	97.5	0.023*	67.8

\* Statistically significant compared to control (Williams multiple sequential t-test procedure, p<0.05)

Results:

72 h  $E_rC_{10}$  = 2.239 mg/L (95%CI:1.716-2.843 mg met./L)  
 72 h  $E_rC_{20}$  = 10.39 mg met/L (95%CI:8.600-12.208 mg met/L)  
 72 h  $E_rC_{50}$  = 192.48 mg met/L (95%CI: 170.872-217.949 mg met/L)  
 72 h  $E_yC_{10}$  = 0.167 mg met /L (95%CI: 0.108-0.239 mg met./L)  
 72 h  $E_yC_{20}$  = 0.504 mg met /L (95%CI: 0.366-0.661 mg met/L)  
 72 h  $E_yC_{50}$  = 4.19 mg met/L (95%CI:3.526-4.959 mg met/L)  
 72 h  $NOE_yC$  < 0.36 mg met/L  
 72 h  $NOE_rC$  = 0.36 mg met/L

**RMS comments:**

The study was conducted according to the OECD 201 test guideline (1984). In general the study is in line with the stated test guidelines OECD 201 (2006) and all validity criteria are met. The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period. In the study the cell density increased by a factor of 176 for 72 h which is in line with the OECD 201 test guideline. The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control must not exceed 35%. The mean coefficient of variation was determined to be 17.1%. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%. The mean coefficient of variation for the whole test period was 0.7%.

Following deviation from the OECD 201 (2006) test guideline was noted:

-The pH in the control increased more than 1.5 units during the study (measured: 2.1 units (measured pH in the control: 7.3-9.4).

Since all validity was met, this deviation is considered as having no impact of the study.

Hence, the study is considered valid.

**Agreed endpoints:**

72 h  $E_yC_{10}$  = 0.167 mg met./L (95%CI:0.10-0.23 mg met./L)  
 72 h  $E_yC_{20}$  = 0.504 mg met. L (95%CI:0.366-0.661 mg met/L)  
 72 h  $E_yC_{50}$  = 4.19 mg met./L (95%CI:3.526-4.959 mg met/L)  
 72 h  $E_rC_{10}$  = 2.239 mg met./L (95%CI: 1.716-2.843 mg met./L)  
 72 h  $E_rC_{20}$  = 10.39 mg met/L (95%CI:8.600-12.208 mg met/L)  
 72 h  $E_rC_{50}$  = 192.48 mg met/L (95%CI:170.872-217.949 mg met/L)  
 72 h  $NOE_yC$  < 0.36 mg met/L  
 72 h  $NOE_rC$  = 0.36 mg met/L

All results based on the nominal concentration.

**B.9.2.6.18. The toxicity of sodium trifluoroacetate to the algae *Selenastrum capricornutum* at low concentration.**

<b>Reference:</b>	The toxicity of sodium trifluoroacetate to the algae <i>Selenastrum capricornutum</i> at low concentration.
<b>Author(s), year:</b>	Berends A.G, Molenaar, J.A., 1993
<b>Report/Doc. number:</b>	Study No: C047121, Reference BCS Number : M-247818-02-1
<b>Guideline(s):</b>	OECD Guideline 201 (1984)
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Sodium trifluoroacetate, Batch No ACA9135AB, Purity: >99%
Test species:	<i>Selenastrum capricornutum</i>
Number of organisms:	0.64 x 10 <sup>4</sup> /mL; 4 replicate vessels per test level and 4 replicate vessels per control
Type of test, duration:	Static test, 3 days

Applied concentrations:

Nominal:	Control (0), 0.036, 0.12, 0.36, 1.2, mg Na-TFA metabolite/L
----------	---

Test conditions:

Water quality:	Algal medium EPA (1978) - nutrient medium
Temperature:	23.5-24.5°C (mean, 24.0°C)
pH:	7.2 (0 h), 7.6 (72 h) in the control 7.2 (0h), 7.7 in the tested concentrations
Incubation:	Continuous illumination, 6200 Lux
Test parameters:	The pH was measured at day 0 and 3 in one replicate of each test concentration. At day 1, 2 and 3 the absorption at 750 nm was measured using a spectrophotometer (Hitachi U-2000) and a cuvette with a pathlength of 5 cm. At test termination the condition of the algae was examined by microscope and any abnormalities were recorded.  At test initiation four samples (10 ml each) of the stock solution were taken. Also two samples of 40 ml were taken from the highest test concentration at test initiation. At the end of the test one sample of 40 mL was taken from each erlenmeyer of the highest concentration.
Statistic:	The NOEC for biomass was estimated by one side Williams test test (1972).

Findings:

Analytical data: Chemical analysis of the highest test concentration at day 0 and at day 3, and of the stock solution was conducted. The concentration of NaTFA remained constant during the test. The analytical findings of Sodium trifluoroacetate were 100% (mean ) of nominal at highest test concentration.

**B.9.2.6.18-1: Effects of Sodium trifluoroacetate on the *Selenastrum capricornutum*.**

Nominal concentration (mg TFA/L)	Mean cell <sup>1</sup> number per ml ( 0-72h) x10 <sup>6</sup>	Biomass		Growth rate per day	
		Area under the curve (0-72h) x10 <sup>6</sup>	% inhibition relative to the control	Average specific growth rate (0- 72h)	% inhibition of growth rate compared to control
				μ	%
Control	1.25	0.894	-	1.76	-
0.036	1.24	0.885	1	1.76	0.057
0.12	1.26	0.891	0.34	1.76	-0.28
0.36	1.11	0.783	12	1.72	2.3
1.2	0.911 <sup>a</sup>	0.693 <sup>a</sup>	29*	1.65	6.1*

<sup>1</sup> At start 6400 cell/mL

<sup>a</sup> Based on 3 replicates

Growth inhibition values based on nominal concentration obtained with Na-TFA on *Selenastrum capricornutum*.

**Conclusion:**

72 h ErC<sub>50</sub> > 1.20 mg p.m./L

72 h EbC<sub>50</sub> > 1.20 mg p.m./L

NOEbC = 0.12 mg p.m./L

NOErC = 0.12 mg p.m./L

**RMS comments:**

The study was conducted according to the OECD 201 test guideline (1984). In general the study is in line with the stated test guidelines and all validity criteria are met. The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72-hour test period.

In the study the cell density increased by a factor of 195 for 72 h which is in line with the OECD 201 test guideline.

The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control must not exceed 35%. The mean coefficient of variation was determined to be 11.87%. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%. The mean coefficient of variation for the whole test period was 1.25%.

However, there was some deviations from the OECD 201, (2006) guideline:

- The cell density of the test solutions was not determined at day 0. The sensitivity of the spectrophotometer is too low to measure a cell density of  $10^4$  cells/ml (nominal concentration at day 0).

Furthermore measured cell densities at day 0 are not used for the calculations.

Due to an error in the preparation of the calibration line the initial cell density was  $0.64 \times 10^4$  cells/mL instead of  $1.0 \times 10^4$  cells/mL.

Since all validity was met, these deviations are considered as having no impact on the study. Hence, the study is considered valid.

**Agreed endpoints:**

72h  $E_rC_{50} > 1.20$  mg p.m./L, based on nominal concentration

72 h  $E_bC_{50} > 1.20$  mg p.m./L, based on nominal concentration

$NOE_{b,r}C = 0.12$  mg p.m./L, based on nominal concentration

**B.9.2.6.19. A comparison of the toxicity of sodium trifluoroacetate, sodium difluoroacetate, sodiummonofluoroacetate and sodium fluoride to the alga *Scenedesmus supspicatus*.**

<b>Reference:</b>	A comparison for the toxicity of sodium trifluoroacetate, sodium difluoroacetate, sodium monofluoroacetate and sodium fluoride to the alga <i>Scenedesmus supspicatus</i> .
<b>Author(s), year:</b>	Berends, A.G., Keetelaar-Jansen W.A.J., van Dijk N.R.M., 1995
<b>Report/Doc. number:</b>	Study No.: C.SOLS 1.074, Reference BCS Number : M-247825-01-1
<b>Guideline(s):</b>	OECD Guideline 201 (1984)
<b>GLP:</b>	Yes

Material and methods:

Test substance: SODIUM TRIFLUOROACETATE

Test species: *Scenedesmus supspicatus*

Batch No: ACA9135AB

Purity: 99%

Test substance: SODIUM DIFLUOROACETATE

Test species: *Scenedesmus supspicatus*

Batch No: 83278-024

Purity: 98%

Test substance: SODIUM MONOFLUOROACETATE

Test species: *Scenedesmus supspicatus*

Batch No: 9262582

Purity: 97%

Test substance: SODIUM FLUORIDE

Batch No: 6449-0250 8512349 402 (A)

Purity: >99%

The purpose of this study was to compare the toxicity of sodium trifluoroacetate, sodium difluoroacetate, sodium monofluoroacetate and sodium fluoride to the alga *Scenedesmus subspicatus* following OECD Guideline 201 (OECD, 1984). Algae were exposed for three days using erlenmeyers containing 100 ml test solution. Two erlenmeyers were prepared for each combination of test substance and test concentration.

For sodium trifluoroacetate, sodium difluoroacetate and sodium fluoride the test was conducted at nominal concentrations of 0.12; 1.2, 12 and 120 mg/L. Sodium monofluoroacetate was tested at concentrations of 0.00012,

0.0012; 0.012 and 0.12 mg/L. No chemical analyses of the test solutions were conducted because previous algal studies with sodium trifluoroacetate showed a good agreement between nominal and measured concentrations (see the study M; Groeneveld et al., 1992; Berends et al., 1993).

### Results:

The results of the study showed that sodium monofluoroacetate was the most toxic compound of the four test substances which were tested. For sodium trifluoroacetate, sodium difluoroacetate and sodium fluoride no severe inhibition of the biomass integral or growth rate was found during the test. The inhibition percentage was less than 35 % at all concentrations. For sodium monofluoroacetate an 72 EC<sub>50</sub> value for biomass integral and growth rate was estimated with linear interpolation after 3 days of exposure. This resulted in 72 EC<sub>50</sub> values of 0.017 mg/L and 0.075 mg/L for biomass integral and growth rate, respectively. At the highest concentration of sodium monofluoroacetate, citric acid was added to the test solutions at day 3. Between day 3 and 6 an increase in growth of the algae was found.

For sodium trifluoroacetate, cell density of *S. subcapitatus*, measured during the study are given in the Table below:

**Table B.9.2.6.19-1. Measured algal cell densities during the test period after exposure of**

Nominal sodium trifluoroacetate concentration (mg/l)	Cell density x 10 <sup>4</sup> (cells/ml)			
	Day 0	Day 1	Day 2	Day 3
0	1.0	1.8	13.6	55.6
	1.0	2.0	14.6	62.8
	1.0	2.0	19.7	60.6
	1.0	1.8	18.3	55.6
	1.0	2.0	45.9	56.9
0.12	1.0	1.9	16.5	60.8
	1.0	2.0	14.7	65.2
1.2	1.0	1.9	13.8	56.0
	1.0	1.9	13.8	60.5
12	1.0	1.9	14.4	63.5
	1.0	1.6	11.8	44.1
120	1.0	1.8	13.1	54.7
	1.0	1.8	12.8	51.0

\* The cell density of the preculture was measured. Based on this result 456 µl of the preculture was added to all erlenmeyers resulting in an initial cell density of 1.0 x 10<sup>4</sup> cells/ml.

For sodium difluoroacetate cell density of *S. subcapitatus* measured during the study are given in the Table below:

Table B.9.2.6.19-2: Sodium difluoroacetate. Measured algal cell densities during the test period.

Nominal sodium monofluoroacetate concentration (mg/l)	Cell density x 10 <sup>4</sup> (cells/ml)				
	Day 0 *	Day 1	Day 2	Day 3	Day 6
0	1.0	1.8	13.6	55.6	
	1.0	2.0	14.6	62.8	
	1.0	2.0	19.7	60.6	
	1.0	1.8	18.3	55.6	
	1.0	2.0	45.9	66.9	
0.00012	1.0	1.8	14.2	61.8	
	1.0	1.9	12.9	52.2	
0.0012	1.0	2.0	14.2	62.7	
	1.0	1.9	14.4	64.2	
0.012	1.0	1.7	10.6	42.5	
	1.0	1.8	9.9	35.1	
0.12	1.0	1.6	6.0	5.5	66.4**
	1.0	1.4	5.9	4.8	71.8**

\* The cell density of the preculture was measured. Based on this result 456 µl of the preculture was added to all erlenmeyers resulting in an initial cell density of 1.0 x 10<sup>4</sup> cells/ml.

For sodium monofluoroacetate cell density of *S. subcapitatus*, measured during the study are given in the Table below:

Table B.9.2.6.19-3: Measured algal cell densities during the test period after exposure of sodium monofluoroacetate.

Nominal sodium fluoride concentration (mg/l)	Cell density x 10 <sup>4</sup> (cells/ml)			
	Day 0 *	Day 1	Day 2	Day 3
0	1.0	1.8	13.6	55.6
	1.0	2.0	14.6	62.8
	1.0	2.0	19.7	60.6
	1.0	1.8	18.3	55.6
	1.0	2.0	45.9	66.9
0.12	1.0	2.0	13.8	58.8
	1.0	1.9	14.6	64.8
1.2	1.0	1.8	14.1	61.1
	1.0	1.8	14.1	61.5
12	1.0	1.7	13.3	57.9
	1.0	1.9	14.3	63.6
120	1.0	1.9	14.4	63.4
	1.0	2.1	14.8	69.5

\* The cell density of the preculture was measured. Based on this result 456 µl of the preculture was added to all erlenmeyers resulting in an initial cell density of 1.0 x 10<sup>4</sup> cells/ml.

Citric acid was added to the test solution at day 3.

For sodium fluoride cell density of *S. subcapitatus*, measured during the study are given in the Table below:

Table B.9.2.6.19-4: Measured algal cell densities during the test period after exposure of sodium sodium fluoride.

Nominal sodium fluoride concentration (mg/l)	Cell density x 10 <sup>4</sup> (cells/ml)			
	Day 0 *	Day 1	Day 2	Day 3
0	1.0	1.8	13.6	55.6
	1.0	2.0	14.6	62.8
	1.0	2.0	19.7	60.6
	1.0	1.8	18.3	55.6
	1.0	2.0	45.9	66.9
0.12	1.0	2.0	13.8	58.8
	1.0	1.9	14.6	64.8
1.2	1.0	1.8	14.1	61.1
	1.0	1.8	14.1	61.5
12	1.0	1.7	13.3	57.9
	1.0	1.9	14.3	63.6
120	1.0	1.9	14.4	63.4
	1.0	2.1	14.8	69.5

\* The cell density of the preculture was measured. Based on this result 456 µl of the preculture was added to all erlenmeyers resulting in an initial cell density of 1.0 x 10<sup>4</sup> cells/ml.

Table B.9.2.6.16-5: Calculated biomass integral for each Erenmeyer at Day 3after exposure of TFA, DFA, MFA, NAF

Nominal concentration <sup>a</sup> (mg/l)	Test substance			
	TFA	DFA	MFA	NaF
0			40.7 45.4 49.5 45.3 78.9	
0.12 (0.00012)	46.3 46.7	45.8 11.7	44.5 38.3	42.6 46.3
1.2 (0.0012)	41.1 43.4	37.8 41.6	45.0 45.9	43.9 44.1
12 (0.012)	45.6 32.9	38.6 34.2	31.0 26.8	41.5 45.5
120 (0.12)	39.7 37.5	33.7 35.1	7.9 7.1	45.5 49.1

<sup>a</sup> Between brackets the concentration sodium monofluoroacetate is given.

#### Conclusion:

No severe inhibition of total biomass or growth rate was found during the test for sodium trifluoroacetate, sodium difluoroacetate and sodium fluoride. The inhibition was 35% at all tested concentration. Therefore, the value of EC<sub>50</sub> was to be estimated > 120 mg /L.

#### RMS comments:

The study was conducted according to the OECD 201 test guideline (1984). It is also in line with the current OECD 201 guideline (2006), except for the number of replicates in the treatment group.

The following validity criteria should be met according to the OECD 201 guideline (2006):

The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period.

In the study the cell density increased by a factor of 55.6 for 72 h which is in line with the OECD 201 test guideline. The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control must not exceed 35%. The mean coefficient of variation was determined to be 67.03%. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%. The mean coefficient of variation for the whole test period was 1.95%.

The criterion of coefficient of variation of average specific growth rates during the study period was met. The criterion on the mean coefficient variation for section by section specific growth rate was not met.

**RMS is in the opinion that this study is not fully reliable but can be considered as supportive information indicating that the metabolite appears to be clearly less toxic than active substance.**

**Agreed endpoint:**

**72 hEC<sub>50</sub> > 120 mg TFA/L based on nominal concentration**

#### **B.9.2.6.20. The toxicity of sodium trifluoroacetate to algae**

<b>Reference:</b>	The toxicity of sodium trifluoroacetate to algae Third Draft
<b>Author(s), year:</b>	Berends A.G. 1996
<b>Report/Doc. number:</b>	Reference BCS Number : M-247822-01-1
<b>Guideline(s):</b>	n.a.
<b>GLP:</b>	n.a.

#### Material and methods:

	Sodium trifluoroacetate
Batch No:	ACA9135AB
Purity:	99%

The present study is a review of algal laboratory studies which were conducted with sodium trifluoroacetate (NaTFA), including Report No: M-247818-02-1 (C047121), Report No: M-247820-01-1 (C047124) and Report No: M-247825-01-1 (C047129).

The toxicity of sodium trifluoroacetate was tested in the laboratory on 11 different algal species cultivated on different alga media (OECD, EPA). Based on the studies results the *Selenastrum capricornutum* was the most sensitive species to exposure of sodium trifluoroacetate.

**B.9.2.6.20-1. The toxicity test with alga an TFA and sodium trifluoroacetate.**

Species <sup>A</sup>	EC <sub>50</sub>		NOEC <sup>B</sup>		LOEC <sup>B</sup>	
	Biomass integral	Growth rate	Biomass integral	Growth rate	Biomass integral	Growth rate
<i>S. capricornutum</i> (2)	4.8	160	< 0.36		0.36 (11)	
<i>S. capricornutum</i> (3)			0.12 (0.34)		0.36 (12)	
<i>S. capricornutum</i> (4)	0.7 <sup>C</sup>	14 <sup>C</sup>				
<i>S. capricornutum</i> (5)	1.5	27	0.30 (11)		1.0 (11)	
<i>S. capricornutum</i> (6)	1.5	7.7	0.15 <sup>D</sup> (10)	1.2 <sup>D</sup> (10)		
<i>C. vulgaris</i> (10)	> 1200	> 1200	1200 (1.6)			
<i>S. subspicatus</i> (13)	> 120	> 120				
<i>C. reinhardtii</i> (16)	> 120	> 120	120 (-2.4)			
<i>D. tertiolecta</i> (17)	> 124	> 124	124 (5.1) <sup>E</sup>			
<i>E. gracilis</i> (18)	> 112	> 112	112 (0.0)			
<i>P. tricornutum</i> (19)	> 117	> 117	117 (1.4)			
<i>N. pelliculosa</i> (20)	1200	2400	600 (16)	600 (5)	1200 (53)	
<i>S. costatum</i> (21)	> 2400	> 2400	2400 (10)	2400 (-1)		1200 (17)
<i>A. flos-aquae</i> (22)	2400	> 2400	600 (13)	600 (0)	1200 (29)	1200 (3)
<i>M. aeruginosa</i> (23)	> 117	> 117	117 (6.8)			

<sup>A</sup> Between brackets the relevant table number of this report is given  
<sup>B</sup> Between brackets the inhibition percentage of the biomass integral or growth rate is given  
<sup>C</sup> Due to the large ratio between test concentrations (10) and the low number of replicates (only 2 erlenmeyers per concentration) the value is only a rough estimate  
<sup>D</sup> EC<sub>10</sub> value (determined with log/probit method)  
<sup>E</sup> The inhibition of the biomass integral was statistically significant ( $\alpha=0.018$ )

The tests reported for *Pseudokirchneriella subcapitata*, in addition to M-247818-02-1 (C047121) and M-247820-01-1 (C047124) was only a preliminary test using 2 replicates per concentration. In another test the design was also limited to 2 replicates per concentration and in addition there was a large ratio (10) between the test concentrations. In a third test the growth rate of control algae decreased during the test due to a high initial cell density ( $4.9 \times 10^4$  cells/mL).

For the other algal species the ErC<sub>50</sub> was reported to be between >112 to >2400 mg/L.

The results of a semi-field study in stream mesocosm (BoT.L and Standley L.J: Potential effects of trifluoroacetate of freshwater algal communities and primary productivity, 1995) were also reported in the present study. Algal species was monitored only on one date.

The samples, taken for algal determination species, revealed that diatoms, blue-green alga and Chlorophyta were present in the mesocosm. However no quantitative data were reported.

The results of the study showed that a short term exposure to relatively high concentration of TFA (up to 200 mg/L) had no severe effect on the primary productivity. The long term exposure (several months) to a mean NaTFA concentration of 31-32 µg/L had no effect on the algal primary production in the mesocosm stream.

In several cases statistical significant effects on primary productivity were found between between control and exposed mesocosm or between different concentration of sodium trifluoroacetate. However, this effects were not reproducible.

**RMS comments:**

In opinion of RMS the brief summary on studies included in present report provided very limited information and can be considered as supplemental information only.

**B.9.2.6.21. *Pseudokirchneriella subcapitata* growth inhibition test with BCS-CU62474**

<b>Reference:</b>	<i>Pseudokirchneriella subcapitata</i> growth inhibition test with BCS-CU62474
<b>Author(s), year:</b>	Bruns, E., 2012
<b>Report/Doc. number:</b>	Study No.: E 323 4353, Report ID: EBFOP017 Reference BCS No: M-444217-01-1
<b>Guideline(s):</b>	OECD 201 (March 2006)
<b>GLP:</b>	Yes

Material and methods:

Test substance:	BCS-CU62474, Batch No.: NLL 8865-4-1, Purity: 99.4% (analysed)
Test species:	<i>Pseudokirchneriella subcapitata</i>
Number of organisms:	10,000 cells/mL, 6 replicate vessels per test level and 6 replicate vessels per control
Type of test, duration:	Static test, 72 hours

Applied concentrations:

Nominal:	Control (0), 100 mg pure metabolite/L
Test conditions:	
Water quality:	Nutrient medium according to OECD 201 guideline
Temperature:	21.4-22.4°C (mean, 21.8°C)
pH:	8.1 (0 h), 7.9-8.4 (72 h)
Incubation:	Continuous illumination, 7380-8020 Lux (mean, 7749Lux)
Test parameters:	The pH was measured at each observation time in all test levels and the control. Cell numbers per volume (as a surrogate for biomass per volume) were estimated photometrically. For this purpose, small samples of treated, inoculated test medium were placed in 5 cm cuvettes on day 1, day 2, and day 3 of the exposure

period (without replacing after measurement). The extinctions were determined at a wave length of 578 nm using a single-beam-photometer.

To detect possible alterations in algae cells that might influence extinction measurements, such as unusual cell size, samples from each flask were examined under a microscope at a magnification of 400 times.

Samples were analysed (HPLC-UV) for the actual concentration of present in the test medium of all treatment levels and controls on Day 0 and Day 3.

#### Findings:

Analytical data: The analytical finding of BCS-CU62474 in the treatment level found on day 0 was 103 % of nominal. On day 3 analytical finding of 99.4 % of nominal was found. All results are based on nominal test concentrations of the metabolite.

Statistic: The following statistic analysis were conducted  
 Relation of Yield on concentration at 72h  
 -Shapiro-Wilk's Test on Normal Distribution  
 -Welch-t test for Inhomogeneous Variances  
 Relation of Growth Rate on concentration at 72h  
 -Shapiro-Wilk's Test on Normal Distribution  
 -STUDENT-t test for Homogeneous Variances

#### Software:

-Microsoft Excel spreadsheets  
 -Statistical Software ToxRat Professional version 2.10.05  
 -ToxRat Validation Report, valid for ToxRat Version 2.10

#### Findings:

Morphological effects: No morphological change in algae was observed in any test concentration.

**Table B.9.2.6.21-1: Effects of BCS-CU62474 on the *Pseudokirchneriella subcapitata*. Growth.**

Nominal concentration (mg p.m./L)	Cell number after 72 h per mL	Growth rate per day	
		$\mu$	%
Control	807 000	1.463	-
100	913 000	1.504	-2.8 <sup>ns</sup>

-Increase in growth relative to the control

ns not significant compared to control (Welch-t test)

**Table B.9.2.6.2.21-2: Effects of BCS-CU62474 on the *Pseudokirchneriella subcapitata*. Yield**

Nominal concentration (mg p.m./L)	Yield (0- 72h)	% inhibition of growth rate compared to control
Control	797000	-
100	903000	-13.4 <sup>ns</sup>

-% inhibition: increase in growth relative to the control

ns not statistically significant compared to control (Welch-t test, )

Growth inhibition values based on nominal concentration obtained with BCS-CU62474 on *Pseudokirchneriella subcapitata* were as follows:

Test parameter	Results
E <sub>r</sub> C <sub>50</sub>	>100 mg metabolite/L
E <sub>y</sub> C <sub>50</sub>	>100 mg metabolite/L
NOE <sub>r</sub> C NOE <sub>y</sub> C	>100 mg metabolite/L

#### **RMS comments:**

The study was conducted according to the OECD 201 test guideline (2006). In general the study is in line with the stated test guidelines and all validity criteria are met. The biomass in the control cultures should have increased exponentially by a factor of at least 16 within the 72 hour test period. In the study the cell density increased by a factor of 80.7 which is in line with the OECD 201 test guideline. The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control must not exceed 35%. The mean coefficient of variation was determined to be 17.6%. The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 7%. The mean coefficient of variation for the whole test period was 0.9%.

The RMS is of opinion the reliability of the results is given.

#### **Agreed endpoints:**

E<sub>r</sub>C<sub>50</sub> /E<sub>y</sub>C<sub>50</sub> > 100 mg metabolite /L (based on nominal concentration)

NOE<sub>r</sub>C/NOE<sub>y</sub>C >100 mg metabolite /L (based on nominal concentration)

**B.9.2.6.22. Effects of 14 C-Thiadone, a metabolite of FOE5043, to the Green Algae*****Selenastrum carpicornutum*.**


---

<b>Reference:</b>	Toxicity of 14 C-Thiadone, a metabolite of FOE5043, to the Green Algae <i>Selenastrum carpicornutum</i> .
<b>Author(s), year:</b>	Hall A. T., Lam, C.V., 1999
<b>Report/Doc. number:</b>	Report No: 108823, Reference BCS Number: M-009214-01-1
<b>Guideline(s):</b>	EPA 123-2 (EPA 540/9-86-134)
<b>GLP:</b>	Yes

---

Material and methods:

Test substance:	Thiadone, Vial No: K 778. Purity:99, 4% <sup>14</sup> C-Thiadone, Vial No: C-784, Purity 97.7%
Test species:	<i>Pseudokirchneriella subcapitata</i> (formerly <i>S. carpicornutum</i> )
Number of organisms:	10,000 cells/mL; 3 replicate vessels per test level and 3 replicate vessels per control
Type of test, duration:	Static test, 4 days

Applied concentrations:

Nominal:	Control (0), 0.06, 0.20, 0.61, 1.92, and 6.0 mg pure metabolite/L
Mean measured:	Control: (0), 0.06, 0.22, 0.66, 2.10, 6.46 mg pure metabolite/L
Solvent:	DMF, Dimethylformamide

Test conditions:

Water quality:	APP Nutrient Medium according to EPA guideline
Temperature:	23.1-23.8 °C
pH:	7.5 (0 h), 7.6 -8.4 (72h)
Incubation:	Continuous illumination, approximately 4306 Lux,
Test parameters:	Each day, density was determined in three replicates at each test concentration using a light microscope and an Improved Neubauer hemocytometer. A calibrated datalogger and a thermocouple were used to monitor the test system temperature each hour in the environmental chamber. In addition, manual temperature readings were recorded daily using a calibrated thermometer.

The thermometer and thermocouple were immersed in a centrally located, 250-ml flask of water in the environmental chamber. The conductivity and pH were measured on Day 0 and Day 4. Samples of test solutions, including control and solvent control, were taken on Day 0 and Day 4 to measure actual thiadone exposure concentrations. Samples were analysed (HPLC-UV) for the actual concentration of Thiadone present in the test medium of all treatment levels and controls on Day 0 and Day 4.

Findings:

Statistic:

The cell density, or standing crop, was determined daily by direct cell counts. The growth rate was analyzed by comparing the change in cell density from Day 0 to Day 4. The cumulative biomass, or area under the growth curve, was determined by plotting the daily cell density from Day 0 to Day 4. Day 4 growth data as cell density, growth rate and area under the growth curve were analyzed using the following statistical tests:

- 1) Student's t-test to determine if the controls were poolable;
- 2) Shapiro-Wilks test for normality and Levene's test for homogeneity of variance;
- 3) ANOVA followed by the Dunnett's Test;
- 4) Fitting the Cumulative Normal Model Using Nonlinear (weighted) regression analysis was used to estimate the EC<sub>50</sub>. All data were analyzed without transformations when the Shapiro-Wilks test and homogeneity of variance tests indicated normality and homogeneity of variance ( $\alpha = 0.05$ ). Growth data were analyzed by a one-way analysis of variance (ANOVA) followed by the Dunnett's Test. The Dunnett's Statistical analyses were conducted using PC based computer programs (SAS ver. 6.12).<sup>13</sup>.

Analytical data:

The mean measured concentrations of <sup>14</sup>C-Thiadone were 0.06, 0.22, 0.66, 2.10 and 6.46 mg met./L which represents 100 to 110% of the nominal test concentrations.

Morphological effects:

No morphological change in algae was observed in any test concentration.

**Table B.9.2.6.22-1: Effects of Thiadone on Pseudokirchneriella subcapitata.**

Mean measured concentration (mg p.m./L)	Mean cell number per mL (0-72h) $\times 10^4$	Inhibition relative to the control % <sup>1</sup>	Mean cell number per mL (0-96h) $\times 10^4$	Inhibition relative to the control %
Pooled Control	108.8	-	189.4	-
0.06	112.03	2.88	181.9	4.0
0.22	110.6	1.62	175.2	7.5
0.66	102.1	6.15	176.8	6.7
2.10	85.1	21.78	157.3	16.9
6.46	34.4	68.38	92.3	51.3*

<sup>1</sup> the statistical analysis were reported

Growth inhibition values based on mean measured concentration obtained with <sup>14</sup>C Thiadone on Pseudokirchneriella subcapitata.

Test parameter	Results
<b>(72 hours)</b>	
72 h E <sub>r</sub> C <sub>50</sub>	15 mg p.m./L (95% CI: 10.7-21.0 mg met/L)
72 h NOE <sub>r</sub> C	2.10 mg p.m./L
72 h E <sub>b</sub> C <sub>50</sub>	4.1 mg p.m./L (95% CI: 3.2-5.2 mg met./L)
72 h NOE <sub>b</sub> C	0.66 mg p.m./L
<b>(96 hours)</b>	
96 h E <sub>r</sub> C <sub>50</sub>	33.4 mg p.m./L (95% CI: 12.7-87.7 mg met./L)
96 h NOE <sub>r</sub> C	2.10 mg p.m./L
96 h E <sub>b</sub> C <sub>50</sub>	4.7 mg p.m./L (95% CI: 3.8-5.8 mg p.m./L)
96 h NOE <sub>b</sub> C	0.22 mg p.m./L

**RMS comments:**

The study was conducted according to the OECD 201 test guideline (1984).

- In the study the cell density increased by a factor of 108 for 72 h and 184.6 for 96 hours, which is in line with the OECD 201 test guideline (cell number should increased by 16 fold).

- The mean coefficient of variation of average specific growth rates during the whole test period in replicate control cultures calculated for period of 72 and 96 hours were within limits set by validity criteria (1.79% and 2.14%, respectively).

-The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the control were determined to be 66% for 72 h and 76.3 for 96 hours (should not exceed 35% according to test guideline).

The third validity criterion was not met. For this reason the study is considered not fully reliable.

**RMS is in the opinion that this study should be considered as supportive information indicating that the metabolite appears to be clearly less toxic than active substance.**

**Agreed endpoints:**

72 h E<sub>r</sub>C<sub>50</sub>= 15 mg p.m./L (95% CI: 10.7-21.0 mg met/L) based on mean measured concentration

72 h NOE<sub>r</sub>C=2.10 mg p.m./L, based on mean measured concentration

72 h E<sub>b</sub>C<sub>50</sub> = 4.1 mg p.m./L (95%CI: 3.2-5.2 mg met./L), based on mean measured concentration

72 h NOE<sub>b</sub>C=0.66 mg p.m./L, based on mean measured concentration

#### B.9.2.7. Effects on aquatic macrophytes

For the first approval of the active substance flufenacet one laboratory study with aquatic macrophyte – *Lemna gibba* was submitted. This study was reevaluated in this RAR and was replaced by the new studies for *Lemna gibba*.

For the renewal of active substance, one new laboratory study for *Lemna sp.* was performed.

In addition to *Lemna* studies, a second aquatic macrophyte has to be tested according to the new data requirement for active substance (Commission Regulation (EU) 283/2013).

Therefore, laboratory studies with macrophytes *Myriophyllum spicatum* were submitted. This study was considered not valid.

Studies investigating the toxicity to *Lemna gibba* were performed for all metabolites of the residue definition for the risk assessment in surface water (FOE-oxalate, FOE-methylsulfide, FOE-Methylsulfone, TFA and FOE-Trifluoroethanesulfonic acid). For FOE-sulfonic acid the study was considered not fully reliable but an acceptable indication of low concern.

The study summaries are given below:

**Table B.9.2.7-1: Effect data of flufenacet to aquatic *Lemna gibba*.**

Organism	Test substance	Endpoint (type of the test)	Value (mg a.s./L)	Reference
<b>Aquatic macrophyte</b>				
<i>Lemna gibba</i> Duckweed	Flufenacet	7-d E <sub>r</sub> C <sub>50</sub> (frond no) NOE <sub>r</sub> C 7-d E <sub>r</sub> C <sub>50</sub> (frond area) NOE <sub>r</sub> C 7 -d E <sub>y</sub> C <sub>50</sub> (frond no) NOE <sub>y</sub> C 7 -d E <sub>y</sub> C <sub>50</sub> (frond area) NOEC	0.0161 nom 0.000658 nom <b>0.0139 nom</b> 0.000658 nom 0.00763 nom 0.00658 0.00682 0.000658	Bruns (2013) M-451198-011
<i>Lemna gibba</i> Duckweed	Flufenacet	Peak exposure: one or two 24-h-peaks; total test duration 14d	No inhibition >50% up to 0.126 mg a.s./L peak E <sub>r</sub> C <sub>50</sub> >0.126 mg/L nom	Bruns (2013) M452567011*

Aquatic macrophyte				
<i>Lemna gibba</i> Duckweed	FOE oxalate	7-d E <sub>r</sub> C <sub>50</sub> ( frond no) NOE <sub>r</sub> C 7-d E <sub>r</sub> C <sub>50</sub> (frond area) NOE <sub>r</sub> C	> <b>100 nom</b> 50 nom >100 nom >100 nom	Bruns E. (2009) M-358823-01-1
<i>Lemna gibba</i> Duckweed	FOE methylsulfide	7- d E <sub>r</sub> C <sub>50</sub> ( frond no) NOE <sub>r</sub> C 7-d E <sub>r</sub> C <sub>50</sub> (frond area) NOE <sub>r</sub> C 7-d E <sub>y</sub> C <sub>50</sub> (frond no) NOE <sub>y</sub> C 7-d E <sub>y</sub> C <sub>50</sub> (frond area ) NOEC	125.30 nom 29.60 nom <b>106.0 nom</b> 13.2 nom 65.02 nom 13.20 nom 61.97 nom 29.60 nom	Bruns E. (2010) M-393709-011
<i>Lemna gibba</i> Duckweed	FOE methylsulfone	7 day E <sub>r</sub> C <sub>50</sub> (frond no) NOE <sub>r</sub> C 7day E <sub>r</sub> C <sub>50</sub> (frond area) NOE <sub>r</sub> C	> <b>10 nom</b> >10 nom >10 nom ≥10 nom	Bruns E. (2010) M-369703-011
<i>Lemna gibba</i> Duckweed	FOE sulfonic acid	14 d E <sub>r</sub> C <sub>50</sub> (frond no) 14 d NOE <sub>r</sub> C (frond no)	>79.5 mm >79.5 mm	Dorgerloh, M. (1995) M-004929-01-1 <sup>1</sup>
<i>Lemna gibba</i> Duckweed	TFA	7d E <sub>r</sub> C <sub>50</sub> ( frond no) 7d NOEC 7d E <sub>y</sub> C <sub>50</sub> ( frond no) NOEC	<b>1990 nom</b> 300 nom 768.6 nom 600 nom	Smyth et al. (1993) M-247900-011
<i>Lemna gibba</i> , <i>Myriophyllum</i> <i>spicatum</i> <i>Myriophyllum</i> <i>sibiricum</i>	TFA	7d EC <sub>50</sub>  14 EC <sub>50</sub>  14 EC <sub>50</sub>	618.3 nom (wet mass) 222.1 nom (root length) 357.1 no (wet mass)	Hanson & Solomon, (2004) M-455787-011 <sup>a</sup>
<i>Lemna gibba</i> Duckweed	FOE 5043- Thiadone	7 d E <sub>r</sub> C <sub>50</sub> (frond no) NOE <sub>r</sub> C 7 d E <sub>r</sub> C <sub>50</sub> (frond area) NOE <sub>r</sub> C 7d E <sub>y</sub> C <sub>50</sub> (frond no) NOE <sub>y</sub> C 7d E <sub>y</sub> C <sub>50</sub> (frond area) NOEC	20.80 nom <1.25 nom <b>18.32 nom</b> 5 nom 9.86 nom <1.25 nom 8.68 nom <1.25 nom	Bruns E. (2010) M-393718-013
<i>Lemna gibba</i> Duckweed	FOE- Trifluoroethane sulfonic acid	7 d E <sub>r</sub> C <sub>50</sub> (frond no) NOE <sub>r</sub> C 7 d E <sub>r</sub> C <sub>50</sub> (frond area) NOE <sub>r</sub> C 7 d E <sub>y</sub> C <sub>50</sub> (frond no) NOE <sub>y</sub> C 7 d E <sub>y</sub> C <sub>50</sub> (frond area) NOE <sub>y</sub> C	> <b>10 nom</b> >10 nom >10 nom >10 nom >10 nom >10 nom >10 nom >10 nom	Weyers (2013) M-445884-011

nom...nominal test concentration mm.....mean measured concentration

<sup>1</sup>The study is not fully reliable but can be used as supportive information indicating that metabolites are clearly less toxic than active substance.

<sup>a</sup> The study used as only as supplementary information.

\* The study is not considered in the current risk assessment. The study is valid it may be used in the refined risk assessment for macrophytes only if:

- Further evidence is provided that rooted macrophytes are not more sensitive to flufenacet than *Lemna* sp.
- The peak exposure design of the study covers the peaks observed in the FOCUS scenarios.

**B.9.2.7.1. Effects of active substance flufenacet on aquatic plant duckweed (*Lemna gibba*).**

<b>Reference:</b>	Acute toxicity of FOE 5043 (technical) to <i>Lemna gibba</i> G3.
<b>Author(s), year:</b>	Hughes J.S., Alexander M.M, 1994
<b>Report/Doc. number:</b>	Study No: 105198, Reference BCS No: M-002418-02-1 Amended: 1998-09-01- Dorgerloh, 1998
<b>Guideline(s):</b>	U.S.-EPA-FIFRA § 123-2, Tier 2, Non-target Aquatic Plant Toxicity, OECD-Draft
<b>GLP:</b>	Yes

**RMS comments:**

In the original study: Huges J.S, Alexander M.M., (1995) the 14 d EC<sub>50</sub> value for *Lemna* sp. was determined. The new calculations of 7 day ErC<sub>50</sub> and NOEC values for *Lemna* sp., based on the raw data from original report were performed in the report - Dorgerloh M., 1998.

The results from the original study (Huges J.S, Alexander M.M., 1995) and the recalculations of the endpoints are summarized in the study report below:

**B.9.2.7.1.1. Acute toxicity of FOE 5043 (technical) to *Lemna gibba* G3.**

<b>Reference:</b>	Acute toxicity of FOE 5043 (technical) to <i>Lemna gibba</i> G3.
<b>Author(s), year:</b>	Dorgerloh M., 1998 (Hughes J.S., Alexander M.M, 1994, Study No: 105198, Reference BCS No: M-002418-02-1)
<b>Report/Doc. number:</b>	Report No: DOM 98091, Reference BCS No: M-086479-01-1
<b>Guideline(s):</b>	OECD <i>Lemna</i> inhibition test (Draft of June, 1998)
<b>GLP:</b>	Yes

<u>Test substance:</u>	FOE 5043 Technical, (BFOE 5043 Technical), Batch No.:2030032, Purity: 97.5%
Test species:	<i>Lemna gibba</i> G3
Source:	Horticulture Crops Quality Laboratory, U.S. Department of Agriculture, Beltsville, MD U.S.A
Growth medium:	The medium 20 x AAP according to OECD guideline
Growth chamber	The inoculum of <i>L. gibba</i> used to begin the test was taken from 10-day old stock cultures.

Number of organism: Three replicates per controls and treatments, 3 uniform healthy looking plants with 4 fronds each per replicate.

Type of test, duration: Static, 14 days

Applied concentrations:

Nominal: 0 (control), Solvent control (0), 0.626, 1.25, 2.50, 5, 10, 20, and 100 µg a.s./L

Measured (mean): 0 (control), Solvent control (0) 0.440, 0.849, 1.83, 4.01, 7.97, 17.3 µg a.s./L

Solvent control: DMF, 0.20 ml/ L

Test conditions:

Water quality: The medium M-Hoagland's, with pH=7.5 ±0.1

Temperature: 23.85°C to 25.65°C

pH: At start 7.74-7.98

Test end: 8.74-9.56

Light regime: Continuous illumination of 4198 - 5813 lumens/m<sup>2</sup>

Test parameters: The instantaneous temperature were manually read and recorded each day, while an automated system recorded temperature continuously.

Analytical data: Samples were analysed for the actual concentration on day 0 and on day 14 of the exposure period. At the test termination, the contents of the replicate flasks were combined and pH was recorded.

The test solutions were analysed by HPLC technique.

Observation: Frond counts were made using a lighted magnifying lens on test days 2, 4, 7, 9, 11 and 14. In order to eliminate subjective decisions on frond maturity, every frond visibly projecting beyond the edge of the parent frond was counted.

Statistic: To determine the EC<sub>25</sub> and EC<sub>50</sub> values and associated 95% confidence limits, weighted least squares nonlinear regression of the log of test concentration against the day 14 frond counts was performed in the original study

(Study: Hughes J.S., Alexander M.M, 1994).

The NOEC was determined from an analysis of variance (ANOVA) and Dunnett's test. Statistical analyses were performed using SAS Software (SAS Institute, Gary, NC). The level of significance was at  $\alpha = 0.05$ .

New calculation of 7 d E<sub>r</sub>C<sub>50</sub> based on nominal test concentration was performed using Probit analysis. Shapiro-Wilk, Barlett test were used to statistical calculations. The NOEC was determined from Dunnett's test (Study: Dorgerloh M.1998).

The recalculation of the 7 day  $E_rC_{50}$ , 7 d a  $E_rC_{50}$  and NOEC based on mean measured concentration of flufenacet were submitted by Applicant in the additional statistic report.

#### Findings:

Analytical measurements: The measured concentrations yielded from 75.6% to 89.1% of the nominal concentrations on day 0. Similar recoveries (70.4 to 89.0%) were obtained in the four highest test concentrations on day 14, while the measured values in the two lowest test concentrations yielded only 51.8% and 57.8% of the nominal concentrations at test termination. Analysis of the secondary stock solution used to begin the test indicated 96.9% recovery. No detectable amount of FOE 5043 technical was found in the no-treatment control or the solvent control on day 0 or day 14. The results are expressed based upon the mean measured test concentrations.

**Table B. 9.2.7.1.1-1 Mean number of fronds and % inhibition relative to pooled control 14 days period, and with % inhibition relative to control <sup>1</sup>.**

Mean measured (nominal) concentration ( $\mu\text{g a.s./L}$ )	FronD number	
	Mean frond number on 14 day	% Inhibition on 14 day
Control	744	-
Solvent control	649	-
Pooled controls	697	697
0.440 (0.626)	644	7.67
0.849 (1.25)	547**	21.9
1.83 (2.50)	425**	39.7
4.01 (5.00)	250**	65.2
7.97 (10)	103**	86.7
17.3 (20)	57**	93.4

\* Statistically different compared to pooled controls (Dunnets test,  $\alpha=0.05$ )

<sup>1</sup> Results from original report Hughes J.S., Alexander M.M, 1994

**Table B. 9.2.7.1.1-2: Mean number of fronds <sup>2</sup>, growth rate and % inhibition relative to pooled control for 7 days period.**

Nominal concentration (µg a.s./L)	Mean frond number on 7 day	Growth rate per day	
		µ (0-7 day)	% inhibition compared to control
Control	171	0.380	-
Solvent control	154	0.365	-
Pooled controls	163	0.372	-
0.626	154	0.365	2
1.25	122	0.331	11*
2.50	110	0.317	14.9*
5.00	105	0.310	16.7*
10	83	0.276	25.7*
20	49	0.201	46.0*

\* Statistically significant compared to pooled controls (Dunnets test,  $\alpha=0.05$ )

<sup>2</sup> recalculation by Dorgerloh M.1998

Observed visual effect: No visual effects for plants were observed during the study

### **Results:**

14 Day Frond number       $EC_{50} = 2.43 \mu\text{g s.a./L}$  (95% CI: 1.89-3.14  $\mu\text{g s.a. /L}$ )  
                                      based on mean measured concentration  
                                       $EC_{25} = 1.01 \mu\text{g s.a./L}$  (95% CI: 0.693-1.48  $\mu\text{g s.a. /L}$ )  
                                      based on mean measured concentration  
                                       $NOEC = 0.440 \mu\text{g s.a. /L}$   
                                      based on mean measured concentration

7 Day Frond number:       $E_rC_{50} = 31.8 \mu\text{g s.a./L}$  (95% CI: 16.0-154  $\mu\text{g s.a. /L}$ )  
                                      based on nominal concentration  
                                       $E_rC_{25} = 6.95 \mu\text{g s.a./L}$  (95% CI: 4.22-13.0  $\mu\text{g s.a. /L}$ )  
                                      based on nominal concentration  
                                       $NOEC = 0.626 \mu\text{g s.a./L}$   
                                      based on nominal concentration

**RMS comments:**

The original study (Hughes J.S., Alexander M.M, 1994) was conducted according to the U.S.-EPA-FIFRA § 123-2, Tier 1.

In this study, only frond number was determined on days 2, 4, 7, 9, 11, and 14. A second endpoint like frond dry weight of frond area, which is mandatory according to OECD 221 (2006) has not been determined.

Inhibition percentages were calculated by using absolute fronds counts in the treatment compared to control.

To fulfill the validity criteria stated in the OECD 221 guideline, the new calculations of 7 day growth rate for *Lemna* sp. based on frond counts value from the original report were performed in the additional study (Dorgerloh, 1995).

The study fulfills the validity criteria given in the current valid OECD 221 (2006) test guideline.

-The doubling time of the frond number in the control was less than 2.5 days (being 1.86 day) corresponding to approx. a seven-fold increase in seven days and an average specific growth rate of 0.275 per day.

-The mean growth rate in the control was determined to be 0.380 after 7 days.

-The factor of frond number, measured in the control between 0 and 7 days, was 13.6 (pooled control).

The recalculated value of 7 day  $E_rC_{50}$  = 31.8 µg s.a./L as well as the 7 day NOEC value of 0.626 µg s.a./L were expressed in the nominal concentrations in the study by Dorgerloh 1995, although, in the original report, the mean measured concentrations of active substance were performed. For this reason the RMS asked the Applicant for the new recalculations of the 7 d  $E_rC_{50}$  and 7 d NOEC values, based on the mean, measured concentrations and geomean measured concentration of flufenacet.

The new recalculated values by Tox Rat program were submitted by Applicant and were as follows:

7 d  $EC_{10}$  (frond number) = 1.455 µg s.a./L (95% CI: 0.808-2.121 µg s.a./L), based on mean measured concentration.

7 d  $EC_{20}$  (frond number) = 3.910 µg s.a./L (95% CI: 2.083-4.983 µg s.a./L), based on mean measured concentration.

7 d  $EC_{50}$  (frond number) = **25.920 µg s.a./L** (95% CI: 18.653-42.740 µg s.a./L), based on mean measured concentration.

NOEC = 0.440 µg s.a./L, based on mean measured concentration.

7 d NOEC (frond number) = 0.4330 µg s.a./L, based on mean measured concentration.

7 d  $EC_{10}$  (frond number) = 1.442 µg s.a./L (95% CI: 0.796-2.107 µg s.a./L), based on geomean measured concentration.

7 d  $EC_{20}$  (frond number) = 3.884 µg s.a./L (95% CI: 2.807-4.957 µg s.a./L), based on geomean measured concentration.

7 d  $EC_{50}$  (frond number) = **25.875 µg s.a./L** (18.558-42.834 µg s.a./L), based on geomean measured concentration.

In opinion of RMS the study is considered acceptable, however its results may be considered only as indicative, confirming the results obtained in another study – that by [Bruns; 2013]. That is due to the fact that the current guidelines require the reporting of two parameters – in addition to the reported here frond number/counts, also frond area or dry weight should be determined and presented.

**B.9.2.7.1.3: *Lemna gibba* G3 - Growth inhibition test with flufenacet (technical substance) under static conditions.**


---

<b>Reference:</b>	<i>Lemna gibba</i> G3 - Growth inhibition test with flufenacet (technical substance) under static conditions.
<b>Author(s), year:</b>	Bruns.E, 2013
<b>Report/Doc. number:</b>	Study No: E 412 4412-8, Report No: EBFON004, Reference BCS No: M-451198-01-1
<b>Guideline(s):</b>	OECD Guideline 221, (March 2006)
<b>GLP:</b>	Yes

---

Material and methods:

Test substance:	FOE5043 (flufenacet tech./AE F133402), Batch No: 07969-00, Purity: 97.49% (analysed)
Test species:	<i>Lemna gibba</i> , strain G3,
Source:	Horticulture Crops Quality Laboratory, U.S. Department of Agriculture, Beltsville, MD 20705, U.S.A.
Number of organism:	3 replicate vessels per test level and 3 replicate vessels per control. 12 fronds per vessel
Type of test, duration:	Static, 7 days
Growth chamber :	Glass dishes were used with a diameter of 10 cm and a height of 6 cm (total volume of approx. 470 mL). These test vessels were covered with lids of glass to permit gas exchange and illumination under sterile conditions to the greatest possible extent.
Applied concentrations:	
Nominal:	0 (control), 0.658, 1.50, 3.40, 7.73, 17.6 and 39.9 µg a.s./L
Solvent:	None

Test conditions:

Water quality:	The medium 20 x AAP according to the guideline OECD, pH= 7.5 ± 0.1.
Temperature:	24.6-25°C (mean, 24.8°C)
pH:	7.5-8.1
Light regime:	Continuous light. 8890-9270 Lux (mean, 9270 Lux)

Test parameters:

Temperature:	The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature was recorded hourly.
--------------	--

pH:	The pH was measured in all freshly prepared and all aged test levels and the control
Biomass quantification:	<p>Counting of fronds and determination of total frond area was carried out using the LemnaTec Scanalyzer machine, validated for such measurements.</p> <p>According to the study plan a 7-10 day old pre-culture has to be used for the study. The respective documentation is missing for this study.</p>
Observation:	<p>Within a Lemna growth inhibition test, the effects are measured and documented by photo-analysis (LemnaTec Scanalyzer). This photo documentation was used to check the Lemna plants used within this study visually. The visual check of the photo-documentation revealed that healthy and suitable plants were used for the performance of the test. The validity criteria for the growth of the control were fulfilled and exponential growth was observed. Visual observations were made on study days 1, 2, 5, 7.</p> <p>Plant frond numbers and total frond area of plants were determined on days 1, 2, 5, 7 for this study. Growth and growth inhibition were calculated.</p> <p>The concentration, which inhibited the growth of this species by 50 percent (<math>EC_{50}</math>), was determined where possible.</p>
Statistics:	<p>The <math>EC_{50}</math> values (yield and growth rate) and their 95% confidence limits were calculated by Probit analyses. For the determination of the NOEC values significant difference at the test concentrations compared to the control values were tested by the Williams Multiple Sequential t-test.</p> <p>The software used to perform the statistical analyses was ToxRat Professional.</p>
Analytical data:	<p>Samples were analysed for the actual concentration of flufenacet present in all freshly prepared test levels on day 0 and in all aged test levels on day 7 of the exposure period. Aliquots for freshly prepared test levels for day 0 analyses were sampled from the prepared volume of each test treatment level. For sampling of aged test media, and after removing of plant material from the test vessels on day 7 the contents of all replicate vessels were combined, and the pH was measured. The combined test solutions were then submitted for HPLC-MS/MS analysis.</p>

## Findings:

Analytical measurements:

The analytical findings of flufenacet in the treatment levels found after 0 hours were 92% to 106 % of nominal and after 7 days 92 % to 106% of nominal were found. Due to the analytical results, all results are based on nominal test concentrations.

Data on final frond number, final total frond area and growth rate based on final and total frond area are summarized in the tables that follow:

**Table B. 9.2.7.1.3-1: Mean growth rate (based on fronds number and total frond area).**

Nominal concentration (µg a.s./L)	Frond number			Total frond area		
	Final frond number	Average growth rate	% inhibition of average growth rate of fronds number	Final total frond area	Average growth rate	% inhibition of average growth rate of total frond area
				Mean (mm <sup>2</sup> )		
control	212.3	0.410	--	1726.0	0.378	-
0.658	220.7	0.416	-1.4	1711.3	0.385	-1.9
1.50	161.0	0.370*	9.8	1284.7	0.353*	6.6
3.40	172.7	0.381*	7.2	1376.0	0.341*	9.8
7.73	135.7	0.346*	15.7	1037.0	0.300*	20.7
17.6	36.0	0.155*	62.3	280.0	0.122*	67.7
39.9	23.7	0.097*	76.3	198.0	0.075*	80.2

\* Statistically significant difference from control, (Williams t-test,  $\alpha = 0.05$ , one-sided.)  
 Negative values indicate an increase in growth relative to that of the control <sup>a</sup> Starting number of fronds (day 0): 12 fronds/test vessel

**Table B. 9.2.7.1.3-2: Mean yield (based on fronds number and total frond area).**

Nominal Concentration (µg a.s./L)	Mean yield			
	Frond number		Total frond area	
	After 7 day	% inhibition relative to the control	After 7 days	% inhibition relative to the control
control	200.3	-	1,604	-
0.658	208.7	-4.2	1,596	0.5
1.50	149.0*	25.6	1,176.7*	26.6
3.40	160.7*	19.8	1,249.3*	22.1
7.73	123.7*	38.3	910.0*	43.2
17.6	24*	88.0	160.3*	90.0
39.9	11.7*	94.2	80.7*	95.0

\* Statistically significant difference from control, (Williams t-test,  $\alpha = 0.05$ , one-sided)

Negative values indicate an increase in growth relative to that of the control <sup>a</sup> Starting number of fronds (day 0): 12 fronds/test vessel

Observed visual effect:

No morphological change in *Lemna gibba* was observed at any test concentration.

Results:

Growth inhibition values based on nominal concentration with flufenacet on *Lemna gibba*, were as follows:

0-7 d Frond number	:	Growth rate: $E_rC_{50} = 16.1 \mu\text{g s.a./L}$ (95%CI: 10.41-25.75 $\mu\text{g s.a./L}$ ) Growth rate: $E_rC_{20} = 6.1 \mu\text{g s.a./L}$ (95% CI: 2.57-8.81 $\mu\text{g s.a./L}$ ) Growth rate: $E_rC_{10} = 3.91 \mu\text{g s.a./L}$ (95%CI: 1.17-6.33 $\mu\text{g s.a./L}$ ) $NOE_rC = 0.658 \mu\text{g s.a./L}$
0-7 d Total frond area:		Growth rate $E_rC_{50} = 13.9 \mu\text{g s.a./L}$ (95% CI: 9.71 – 20.0 $\mu\text{g s.a./L}$ ) Growth rate $E_rC_{20} = 6.04 \mu\text{g s.a./L}$ (95%CI: 2.58-8.81 $\mu\text{g s.a./L}$ ) Growth rate $E_rC_{10} = 3.91 \mu\text{g s.a./L}$ (95%CI: 1.16-6.33 $\mu\text{g s.a./L}$ ) $NOE_rC = 0.658 \mu\text{g s.a./L}$
0-7 d Frond number	:	Growth rate : Yield: $E_yC_{50} = 7.638 \mu\text{g s.a./L}$ (95%CI: 2.75-21.85 $\mu\text{g s.a./L}$ ) Yield: $E_yC_{20} = 2.95 \mu\text{g s.a./L}$ (95% CI: 0.05-5.738 $\mu\text{g s.a./L}$ ) Yield: $E_yC_{10} = 1.792 \mu\text{g s.a./L}$ ( 95%CI: 0.04-4.0 $\mu\text{g s.a./L}$ ) $NOE_yC = 0.658 \mu\text{g s.a./L}$

0-7 d Total Frond Area: Growth rate: Yield  $E_yC_{50} = 6.824 \mu\text{g s.a./L}$  (95% CI: 2.77-17.36  $\mu\text{g s.a./L}$ )  
 Yield  $E_yC_{20} = 2.531 \mu\text{g s.a./L}$  (95% CI: 0.116-4.879  $\mu\text{g s.a./L}$ )  
 $E_yC_{10} = 1.507 \mu\text{g s.a./L}$  (95%: 0.017-3.36  $\mu\text{g s.a./L}$ )  
 $NOE_yC = 0.658 \mu\text{g s.a./L}$

#### Conclusion:

The most sensitive response variable in this study was total frond area of plants resulting in a (0-7 day)  $E_rC_{50}$  of 13.9  $\mu\text{g a.s./L}$  and  $E_yC_{50} = 6.824 \mu\text{g s.a./L}$ , based on nominal concentration.

The  $NOE_rC/NOE_yC$  values were estimated to be 0.658  $\mu\text{g a.s./L}$ , based on statistical data analysis of the total frond area of plants and frond number.

#### **RMS comments:**

The study was conducted according to the OECD 221 (2006) test guideline. The study is in the line with test guideline and all validity criteria are met.

The doubling time of the frond number in the control was less than 2.5 days (being 1.7 days) corresponding to approx. a seven-fold increase in seven days and an average specific growth rate of 0.275 per day. The mean growth rate in the control was determined to be 0.410 after 7 days.

The factor of frond number, measured in the control between 0 and 7 days, was 17.7.

The RMS is of the opinion that the reliability of the results is given.

Therefore, the study was considered acceptable.

#### **Agreed endpoints:**

0-7 d Frond number	Growth rate: $E_rC_{50} = 16.1 \mu\text{g s.a./L}$ (95% CI: 10.41-25.75 $\mu\text{g s.a./L}$ ) Growth rate: $E_rC_{20} = 6.1 \mu\text{g s.a./L}$ (95% CI: 2.57-8.81 $\mu\text{g s.a./L}$ ) Growth rate: $E_rC_{10} = 3.91 \mu\text{g s.a./L}$ (95% CI: 1.17-6.33 $\mu\text{g s.a./L}$ ) $NOE_rC = 0.658 \mu\text{g s.a./L}$
0-7 d Total frond area:	Growth rate $E_rC_{50} = 13.9 \mu\text{g s.a./L}$ (95% CI: 9.71 – 20. $\mu\text{g s.a./L}$ ) Growth rate $E_rC_{20} = 6.04 \mu\text{g s.a./L}$ (95% CI: 2.58-8.81 $\mu\text{g s.a./L}$ ) Growth rate $E_rC_{10} = 3.91 \mu\text{g s.a./L}$ (95% CI: 1.16-6.33 $\mu\text{g s.a./L}$ ) $NOE_rC = 0.658 \mu\text{g s.a./L}$
0-7 d Frond number:	Growth rate : Yield: $E_yC_{50} = 7.638 \mu\text{g s.a./L}$ (95% CI: 2.75-21.85 $\mu\text{g s.a./L}$ ) Yield: $E_yC_{20} = 2.95 \mu\text{g s.a./L}$ (95% CI: 0.058-5.738 $\mu\text{g s.a./L}$ ) Yield: $E_yC_{10} = 1.792 \mu\text{g s.a./L}$ (95% CI: 0.04-4.0 $\mu\text{g s.a./L}$ ) $NOE_yC = 0.658 \mu\text{g s.a./L}$
0-7 d Total Frond Area:	Growth rate: Yield $E_yC_{50} = 6.824 \mu\text{g s.a./L}$ (95% CI: 2.77-17.36 $\mu\text{g s.a./L}$ )

Yield  $E_y C_{20} = 2.531 \mu\text{g s.a./L}$  (95% CI: 0.116-4.879  $\mu\text{g s.a./L}$ )

$E_y C_{10} = 1.507 \mu\text{g s.a./L}$  (95% CI: 0.017-3.36  $\mu\text{g s.a./L}$ )

$NOE_y C = 0.658 \mu\text{g s.a./L}$

All result is based on nominal concentration.

#### B.9.2.7.1.4: *Lemna gibba* G3 - Growth inhibition test with flufenacet tech. under peak exposure conditions.

<b>Reference:</b>	<i>Lemna gibba</i> G3 - Growth inhibition test with flufenacet tech. under peak exposure conditions.
<b>Author(s), year</b>	Bruns, E., 2013
<b>Report/Doc. number:</b>	Study No: E 412 4411-7, Report No: EBFOL234, Reference BCS No: M-452567-01-1
<b>Guideline(s):</b>	OECD Guideline 221, (2006)
<b>GLP:</b>	Yes

#### Material and methods:

Test substance:	Flufenacet tech. (AE F133402), Batch No: NK61BX0367, Purity: 97.5 % w/w (analysed)
Test species:	<i>Lemna gibba</i> , strain G3
Source:	Horticulture Crops Quality Laboratory, U.S. Department of Agriculture, Beltsville, MD 20705, U.S.A.
Growth medium:	The medium 20 x AAP according to OECD guideline
Number of replicate:	3 replicate vessels per test level and 3 replicate vessels per control (= replicate A, B, C) for the concentrations: 12, 21.6, 39, 70, and 126 $\mu\text{g/L}$ . 3 replicates vessels per test level and 3 replicates vessels per control : 21.6, 39.0 and 70.0 $\mu\text{g/L}$ (=replicate D, E, F). 12 fronds per vessel.
Type of test, duration:	Static, 2 time 7 days
Growth chamber:	Glass dishes were used with a diameter of 10 cm and a height of 6 cm (total volume of approx. 470 mL). These test vessels were covered with lids of glass to permit gas exchange and illumination under sterile conditions to the greatest possible extent.

---

Innoculum:	To ensure that the plants used as inoculum are exponentially growing, an inoculum pre-culture will be prepared 7 – 10 days before the start of the test and cultivated under the same conditions as in the main test.
Biomass quantification:	Counting of fronds and determination of total frond area was carried out using the LemnaTec Scanalyzer machine.
<u>Applied concentrations:</u>	
Nominal peak concentration:	12, 21.6, 39, 70, and 126 µg a.s./L Control (0), Solvent control (0)
Solvent	DMF
<u>Test conditions:</u>	
Water quality:	The medium 20 x AAP according to OECD test guideline, pH= 7.5 ± 0.1
Temperature:	24.8-25.2 °C (0-7 Day) and 24.8-25.4°C (7-14 Day)
pH:	Peak 1: 7.6-7.7 (Day 0), 7.6-7.8 (Day 1) Peak 1: 7.5 (Day 1, untreated media), 8.8-9 (Day 7, untreated media aged) (all replicates: A, B, C, D, E, F) Peak 2: 7.6-7.9 (Day 7), 8.4-8.6 (Day 8, replicates A-C, untreated media), Peak 2: 8.8-8.9 – (Day 14, replicate, A-C, untreated media aged) Peak 2: 8.4-8.6 (Day 8, replicates D-F) 8.9-9.0 ( Day 14, replicates D-F, untreated media, aged)
Light regime:	Continuous light: 8810-8742 Lux, (mean, 8742 Lux)
<u>Test parameters:</u>	
Temperature:	The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature was recorded hourly.
pH:	The pH was measured on the day 1, 7, 8, 14 in all test vessels and in the control.
Light regime:	The light was measured at least once during the test.
Biomass quantification:	Counting of fronds and determination of total frond area was carried out using the LemnaTec Scanalyzer machine, validated for such measurements.
Innoculum	To ensure that the plants used as inoculum are exponentially growing, an inoculum pre-culture will be prepared 7 – 10 days before the start of the test and cultivated under the same conditions as in the main test.
<u>Methods:</u>	
For this study exponentially growing cultures of <i>Lemna gibba</i> G3 were used. The test started with a 24 h exposure period (peak 1).	

After this exposure period, the plants were washed and transferred into untreated growth medium. After the first week either a second peak of 24 hours (peak 2) was applied for the plants were transferred into untreated medium.

At the start of the second week 12 fronds per investigated replicate (A, B, C) were transferred either into untreated test media or the respective peak concentration. After 24 hours, the plants exposed to peak 2 were transferred into untreated media for additional six days.

Peak concentrations of 12.0, 21.6, 39.0, 70.0 and 126 µg a.s./L were tested.

In addition, the single peak exposure scenario was investigated using the concentrations 21.6, 39.0 and 70.0 µg /L.

In case of the single peak exposure scenario plants were exposed for 24 h followed by a 13 day period in untreated test media.

Fronde numbers and total frond area of plants were recorded after 0, 2, 5, 7, 9, 12, and 14 days.

The test design is presented in the Figure 9.2.7.14-1.1 and Figure 9.2.1.4-2 below:

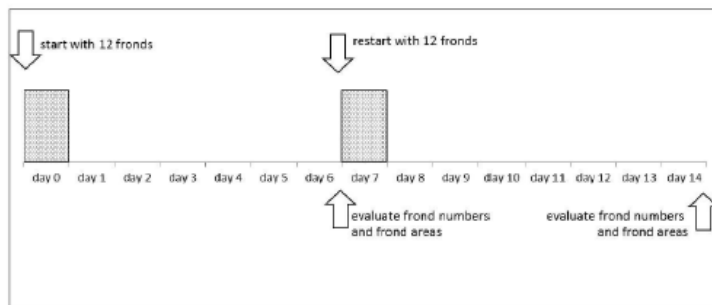


Figure 9.2.7.14-1. 1. Two peaks exposure scenario.

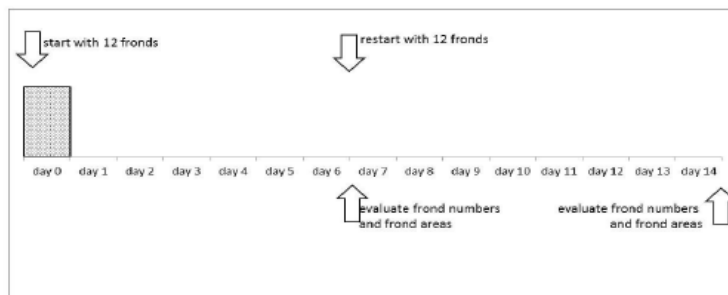


Figure 9.2.7.14-2. 1. 2. One peak exposure scenario.

**Table B.9.2.7.1.4-1: Nominal concentration of peaks of flufenacet in 1 peak and 2 peaks exposure scenarios.**

Nominal concentration	2 peak exposure scenario	1 peak exposure scenario
Control and solvent control	Replicates A,B,C	Replicates D,E,F
12	Replicates A,B,C	Not tested
21.6,39.0,70 µg/L	Replicates A,B,C	Replicates D,E,F
126 µg/L	Replicates A,B,C	Not tested

**Statistics:**

ECx values and confidence intervals were calculated for the stated exposure period (using ToxRAT Profesional Program.

The LOEC determinations from the appropriate parameter (inhibition) were done, using the ANOVA procedure (p = 0.05, one sided) and properly selected multiple t-tests of a commercial program.

the STUDENT t-test was used.

Calculations were carried out using MicrosoftExcel® spreadsheets.

All further statistical evaluations were done using the commercial program ToxRat Professional.

**Analytical data:**

Samples were analysed for the actual concentration of flufenacet tech. present in all freshly prepared test levels at the day 0, on day 1, on day 7 and on day 8 and in all aged test levels after 14 days of the exposure period.

Aliquots for freshly prepared test levels for start-day-sample analyses were sampled from the prepared volume of each test treatment level.

For sampling of aged test media, after removing of plant material from the test vessels after the 14 day test period, the contents of all replicate vessels were combined, and the pH was determined. The combined test solutions were then submitted for analyses by HPCM/MS technique.

**Analytical measurements:**

The analytical findings of flufenacet detected in all freshly prepared test levels on day 0 ranged between 102 and 105 % of nominal. In aged test solutions on day one analytical result ranged between 100 and 105 % of nominal. For the second peak on day seven, the analytical findings ranged between 99.0 and 105 % of nominal peak concentrations. In the aged media on day 8 the chemical analysis revealed recoveries between 96.0 and 108 %. Therefore, the study results are presented based on nominal peak concentrations. Analytical results are presented in the Table B. 9.2.7.14-2, presented below:

**Table B.9.2.7.1.4-2: Measured concentrations of flufenacet . Week 1 (Day 0 to Day 7), Week 2 ( Day7 to Day 14, two peaks exposure scenario) and Week 2 (Day 7-14, one peak exposure scenario).**

Nominal concentration of flufenacet (µg s.a./L)	Measured concentration of flufenacet (µg s.a./L) Week 1 (0-7d)*		Recoveries based on nominal concentration (%)		Measured concentration of flufenacet (µg s.a./L) week 2 (7-14d) <sup>1</sup>		Recoveries based on nominal concentration (%)		Measured concentration of flufenacet (µg s.a./L) week 2 (7-14d) <sup>2</sup>	
Day	Day 0	Day1	Day0	Day1	Day 7	Day 8	Day 7	Day 8	Day 7 <sup>A</sup>	Day 8 <sup>A</sup>
Control	<1.10	<1.10	-	-	<1.10	<1.10			<1.10	<1.10
Solvent control	<1.10	<1.10	-	-	<1.10	<1.10			-	<1.10
12	12.3	12.4	102	104	12.6	11.5	105	96.0	-	<1.10
21.6	22.8	22.5	105	104	22.2	22.1	103	102	-	<1.10
39.0	40.7	41.1	104	105	40.1	41.5	103	107	-	<1.10
70.0	72.4	69.9	103	100	72.6	75.7	104	108	-	<1.10
126	129	129	103	102	125	132	99	105	-	<1.10

\* for (replicates A, B, C, D, E, F)

<sup>1</sup> two peaks exposure scenario<sup>2</sup> one peaks exposure scenario<sup>A</sup> untreated media

Data on mean frond number growth rate and % inhibition of average growth rate of fronds number are summarized in the tables below:

**FROND NUMBER****Table B.9.2.7.1.4-3: Mean frond number on Days 0-7 and growth rate, following of one peak exposure, in duration 24 h, and 6 days recovery in untreated media.**

Flufenacet Nominal concentration (µg a.s./L)	Mean frond number <sup>1</sup>					Average growth rate	
	Day 0	Day 2	Day 5	day 7	Doubling time (d)	(0-7d) µ	% inhibition of average growth rate of fronds number
Control	12	32.3	111	239.7	1.6	0.428	-
Solvent control	12	34.0	115.8	240.2	1.7	0.427	-
12	12	30.3	109.7	227.3	1.7	0.420	1.7
21.6	12	31.2	114.7	236.2	1.7	0.425	0.5
39.0	12	28.2	98.8	202.7	1.7	0.403*	5.7
70	12	29.3	98.3	198.7	1.8	0.401*	6.3
126	12	27	93.7	195.7	1.9	0.398*	6.8

<sup>1</sup> Replicates: A, B, C, 12 fronds at day 0 for each replicate

\* Statistically significant compared to pooled control (Williams Multiple Sequential t- test , p≤0.05)

**Table B.9.2.7.1.4-4: Mean frond number on Days 7-14 and growth rate, following to two peaks exposure on Days 0 and 7, in duration of each 24 hours, and 6 days recovery in untreated media after each peaks.**

Flufenacet nominal concentration (µg a.s./L)	Mean frond number <sup>1</sup>					Average growth rate (7-14d)	
	Day 7	Day 9	Day 12	Day 14	Doubling time (d)	µ	% inhibition of average growth rate of fronds number
Control	12	30.3	107.3	234	1.6	0.424	-
Solvent control	12	27.3	98.7	215.3	1.7	0.412	-
12	12	31.7	109.3	227.0	1.7	0.416	0.4
21.6	12	32.7	111	204.3	1.7	0.404	3.3
39.0	12	28.0	101	197	1.7	0.400	4.4
70	12	27.7	93.3	187	1.8	0.392*	6.2
126	12	25.3	91.3	159.3	1.9	0.369*	11.8

Negative value shows growth stimulation

<sup>1</sup> replicates: A,B,C, 12 fronds at day 0 for each replicate

\* Statistically significant compared to pooled control (Williams Multiple Sequential t- test, p≤0.05)

**Table B.9.2.7.1.4-5: Mean frond number on Days 7-14 and growth rate on day 14, following to one peak of exposure in duration 24 h, on Day 0 and 13 days recovery in untreated media.**

Flufenacet nominal concentration (µg a.s./L)	Mean frond number <sup>1</sup>					Average growth rate (7-14d)	
	Day 7	Day 9	Day 12	Day 14	Doubling time (d)	µ	% inhibition of average growth rate of fronds number
Control	12	31.7	112.7	238.7	1.6	0.427	-
Solvent control	12	30.0	109.7	235.3	1.6	0.424	-
21.6	12	35.0	132.7	276	1.6	0.447	-5
39.0	12	28.3	99.3	216.3	1.7	0.413	3
70	12	29.3	104.3	228.7	1.2	0.420	1.2

Negative value shows growth stimulation,

<sup>1</sup> replicates D, E, F, 12 fronds at day 0 for each replicate

**TOTAL FROND AREA****Table B.9.2.7.1.4-6: Mean total frond area and % inhibition of the growth rate, following of exposure of one peak in duration 24 h, and 6 days recovery in untreated media.**

Flufenacet Nominal concentration (µg a.s./L)	Mean total frond area (mm <sup>2</sup> )				Average growth rate (0-7d)	
	Day 0	Day 2	Day 5	Day 7	µ	% inhibition of average growth rate of frond area (0-7d)
Control	101	251.7	839	1782.2	0.410	-
Solvent control	107.7	266.7	879	1935.2	0.404	-
12	102.7	238	809.7	1658	0.398	2.3
21.6	110.2	235.8	840	1694.5	0.390*	4.1
39.0	102.5	205.8	734	1436.8	0.377*	7.5
70	106.7	197.7	716.3	1422.3	0.370*	9.1
126	102.7	180	670.3	1321.3	0.365*	10.3

\* Statistically significant compared to pooled control (Williams Multiple Sequential t- test,  $p \leq 0.05$ )

**Table B.9.2.7.1.4-7: Mean total frond area and growth rate, following to two peaks exposure on Days 0 and 7, in duration of each 24 hours, and 6 days recovery in untreated media after each peak.**

Flufenacet Nominal concentration (µg a.s./L)	Mean total frond area (mm <sup>2</sup> )				Average Growth rate (7-14 d)	
	Day 7	Day 9	Day 12	14	µ	% inhibition of average growth rate of frond area
Control	91.7	216	800	1660.7	0.413	
Solvent control	90.7	220.7	781	1517.7	0.402	
12	96.7	234.0	829	1620.7	0.402	-1.7
21.6	104	220	814.7	1455.7	0.376*	9.8
39.0	96	187.3	700	1329.7	0.375*	17.6
70	93	176.7	660	1203.3	0.366*	25.9
126	87.3	156.3	585.3	1072	0.357*	34.3

Negative value shows growth stimulation

\* Statistically significant compared to pooled control (Williams Multiple Sequential t- test,  $p \leq 0.05$ )

**Table B.9.2.7.1.4-8: Mean total frond area and growth rates, following of one peak in duration 24 hours on Day 0, and 13 days recovery in untreated media.**

Flufenacet Nominal concentration (µg a.s./L)	Mean total frond area (mm <sup>2</sup> )					
	Day 7	Day 9	Day 12	Day 14	Average growth rate (7-14days) µ	% inhibition of average growth rate of fronds area
Control	98.7	240.3	856	1726.3	0.409	-
Solvent control	92.3	235.3	838	1705.7	0.416	-
21.6	101.3	260.3	962	2015.0	0.426	-3.3
39.0	89	212.3	772.7	1580.3	0.411	0.3
70	90.3	211.7	804.3	1716.3	0.420	-2.0

Replicates D,E,F

Negative value shows growth stimulation

**Table B. 9.2.7.1.4.-9: The endpoints obtained from the one peak and two peaks exposure scenarios.GROWTH**

End point	Time period	Mean growth rate	
		Effects of frond number (µg a.s./L)	Effects of frond area of plants (µg a.s./L)
EC <sub>10</sub>	0-7 one peak	>126 (95% CI: 87.5-937239.6)	100.9 (95% CI: 64.6-278.7)
LOEC	0-7 one peak	39	21.6
NOEC	0-7 one peak	21.6	12
EC <sub>10</sub>	7-14 two peaks	106.1 (95% CI: 8.21 - 155.7)	<b>70.3</b> <b>(95% CI: 12.2 - 170518.5)<sup>1</sup></b> <b>(95% CI: 43-130.8)<sup>1</sup></b>
LOEC	7-14 two peaks	70	21.6
NOEC	7-14 two peaks	39	<b>12</b>
EC <sub>10</sub>	7-14 One peak	nd	nd
LOEC	7-14 One peak	>70	>70
NOEC	7-14 One peak	>70	>70

n.d. = not determined due to mathematical reasons or inappropriate data

<sup>1</sup> Because of the wide confidence Interval (95%: 12.2 - 170518.5), the Applicant recalculated the EC<sub>10</sub> based on individual replicates instead of the mean values. The EC<sub>10</sub> value was not changed after recalculations.

Observed visual effect:

No morphological change in Lemna gibba was observed at any test concentration.

## YIELD

**Table B.9.2.7.1.4-10: Mean yield and % inhibition for frond number, following to one peak on Day 0 in duration 24 h and 6 days recovery in untreated media.**

Nominal concentration of flufenacet (µg a.s./L)	Mean frond number	
	Yield Day 7	% Inhibition of yield (7 d)
Control	227.7	-
Solvent control	228.2	-
Pooled control	227.9	
12	215.3	5.5
21.6	224.2	1.6
39.0	190.7	16.3*
70	186.7	18.1*
126	183.7	19.4*

<sup>1</sup> replicates: A,B,C

\*Statistically significant compared to pooled control (Williams multiple sequential test, p≤0.05)

**Table B.9.2.7.1.4-11: Mean yield and % inhibition for mean total frond area, following to one peak on Day 0 in duration 24 h, and 6 days recovery in untreated media.**

Nominal concentration (µg a.s./L)	Mean total frond number <sup>1</sup>	
	Yield Day 7	% Inhibition of yield (7 d)
Control	1,681.2	-
Solvent control	1,727.5	-
Pooled control	1,704.3	-
12	1,555.3	8.7
21.6	1,584.3	7.0
39.0	1,334.3	21.7**
70	1,315.7	22.8**
126	1,218.7	28.5**

<sup>1</sup> replicates: A,B,C

\*\* Statistically significant compared to pooled control (Welch – test with Bonferroni –Holm adjustment)

**Table B.9.2.7.1.4-12: Yield and % inhibition of mean frond number, following to two peaks exposure on Days 0 and 7, in duration of each 24 hours, and 6 days recovery in untreated media after each peaks.**

Nominal concentration (µg a.s./L)	Mean frond number <sup>1</sup>	
	Yield (14 Day)	% Inhibition of yield (14 d)
Control		-
Solvent control		-
Pooled control	212.7	
12	210.7	0.9
21.6	192.3	9.6
39.0	185.0	13.0*
70	175.0	17.7*
126	147.3	30.7*

<sup>1</sup> replicates: A,B,C

Negative value shows growth stimulation

\* Statistically significant compared to pooled control (Williams multiple sequential t-test procedure, p≤0.05)

**Table B.9.2.7.1.4-13: Yield and % inhibition of total frond area , following to two peaks exposure on Days 0 and 7, in duration of each 24 hours, and 6 days recovery in untreated media after each peaks.**

Nominal concentration of flufenacet (µg a.s./L)	Total frond area <sup>1</sup>	
	14 Day	% Inhibition of yield
Control	1569	-
Solvent control	1427	-
Pooled control	1,498	-
12	1524.0	-1.7
21.6	1351.7	9.8
39.0	1233.7	17.6*
70	1110.3	25.9*
126	984.7	34.3*

<sup>1</sup> replicates: A, B, C

Negative value shows growth stimulation

\* Statistically significant compared to pooled control (Williams multiple sequential t-test procedure, p≤0.05)

**Table B.9.2.7.1.4-14: Yield and % inhibition of frond number, following of one peak in duration 24 h exposure, on Day 0, and 13 days recovery in untreated media.**

Nominal concentration of flufenacet (µg a.s./L)	Total frond number <sup>1</sup>	
	14 Day	% Inhibition of yield (0-14 d)
Control	226.7	
Solvent control	223.3	
Pooled control	225	
21.6	264	-17.3
39.0	204.3	9.2
70	216.7	3.7

<sup>1</sup> replicates: D, E F,

Negative value shows growth stimulation

**Table B.9.2.7.1.4-15: Yield and % inhibition of total frond area following of one peak in duration 24 h exposure, on Day 0, and 13 days recovery in untreated media.**

Nominal concentration (µg a.s./L)	Total frond area <sup>1</sup>	
	14 Day	% Inhibition of yield
Control	1,627.7	-
Solvent control	1,613.3	-
Pooled control	1,620.5	
21.6	1,913.7	-18.1
39.0	1,491.3	8.0
70	1,626.0	-0.3

Negative value shows growth stimulation

<sup>1</sup> replicates: D, E, F

Negative value shows growth stimulation

Observed visual effect:

No morphological change in Lemna gibba was observed at any test concentration.

**Table B. 9.2.7.1.4-16: The endpoints obtained from the one peak and two peaks exposure scenarios. Yield.**

End point	Time period	Yield	
		Effects on frond number (µg a.s./L)	Effects of frond area of plants (µg a.s./L)
EC <sub>10</sub>	0-7 one peak	32 nd	n.d.
LOEC	0-7 one peak	39	39
NOEC	0-7 one peak	21.6	21.6

End point	Time period	Yield	
		Effects on frond number (µg a.s./L)	Effects of frond area of plants (µg a.s./L)
EC <sub>10</sub>	7-14 two peaks	31.5 (12.9-45.9)	24.2 (7.30-38.0)
LOEC	7-14 two peaks	39.0	39.0
NOEC	7-14 two peaks	21.6	21.6
EC <sub>10</sub>	7-14 One peak	>70	>70
LOEC	7-14 One peak	>70	>70
NOEC	7-14 One peak	>70	>70

\*Probit analysis

n.d. not determined due to mathematical reasons or inappropriate data

#### Summary of the effects on Growth rate and Yied based on frond number and frond area.

**Table B.9.2.7.1.4-17: EC<sub>50</sub>, LOEC and NOEC for mean growth rate and yield estimated during the stusy.**

Test design	Time period	Doses µg a.s./L	Mean growth rate		Yield		
			% inhibition (frond number)	% inhibition (total frond area)	% inhibition (frond number)	% inhibition (total frond area)	
TWO PEAKS EXPOSURE SCENARIO ( replicates A, B, C)							
( 1-peak) (24 h)	Week 1 (0-7 d)	12	1.7	2.3	5.5	8.7	
		21.6	0.5	4.1	1.6	7.0	
		39	5.7	7.5	16.3	21.7	
		70	6.3	9.1	18.1	22.8	
		126	6.8	10.3	19.4	28.5	
	Results after the first peak	EC <sub>10</sub> >126 µg a.s./L		EC <sub>10</sub> =100.9 µg a.s./L		EC <sub>10</sub> =32 µg a.s./L	EC <sub>10</sub> =16.4 µg a.s./L
		NOEC=21.6 µg a.s./L		NOEC=12.0 µg a.s. /L		NOEC=21.6 µg a.s./L	NOEC=21.6 µg a.s./L
LOEC=39.0 µg a.s./L		LOEC=21.6 µg s.a./L		LOEC=39.0 µg a.s./L	LOEC=39.0 µg s.a/L		
(2 peaks, each 24 h)	Week 2 (7-14 d)	12	0.4	-1.7*	0.9	-1.7*	
		21.6	3.3	9.8	9.6	9.8	
		39	4.4	17.6	13.3	17.6	
		70	6.2	25.9	17.7	25.9	

Test design	Time period	Doses µg a.s./L	Mean growth rate		Yield	
			% inhibition (frond number)	% inhibition (total frond area)	% inhibition (frond number)	% inhibition (total frond area)
		126	11.8	34.3	30.7	34.3
	Results after the second peak  ( 7-14 days)	EC <sub>10</sub> =106.1 µg a.s./L	EC <sub>10</sub> <sup>-1</sup>	EC <sub>10</sub> = 31.5 µg a.s./L	EC <sub>10</sub> = 24.2 µg a.s./L	
		NOEC=39.0 µg a.s./L	NOEC=12.0 µg a.s./L	NOEC= 21.6 µgs.a./L	NOEC=21.6 µg a.s./L	
		LOEC=70 µg a.s./L	LOEC=21.6 µg a.s./L	LOEC= 39 µg a.s./L	LOEC= 39 µg a.s./L	
ONE PEAK EXPOSURE SCENARIO ( replicates D, E, F)						
1 peak (24 h)	Week 2	21.6	-5.0*	-3.3*	-17.3	-18.1*
		39	3.0	0.3	9.2	8.0
		70	1.2	-2.0*	3.7	-0.3*
	Results after the first peak ( 7-14 days)	EC <sub>10</sub> n.d NOEC >70 µg a.s./L LOEC >70 µg a.s./L	EC <sub>10</sub> n.d NOEC >70 µg a.s./L LOEC >70 µg a.s./L	EC <sub>10</sub> n.d NOEC >70 µg a.s./L LOEC >70 µg a.s./L	EC <sub>10</sub> n.d NOEC >70 µg a.s./L LOEC >70 µg a.s./L	

\* Negative value shows growth inhibition

n.d . not determined due to mathematical reasons or inappropriate data

EC<sub>50</sub> Calculations were not performed as in no case the observed growth inhibition exceeded the 50% niveau.

1 The 7-14 two peaks EC<sub>10</sub> value proposed by the Applicant for the effects on frond area ( 70.3 µg/L) underestimate the biological effects since 26% effects are observed at 70.3 µg/L. Given the very high uncertainty of the estimation, EC<sub>10</sub> should be considered as not reliable.

### Conclusion RMS:

Based on the study results with two peaks of up 126 µg/L (the first on Day 0, the second on Day 7), each lasting 24 hours, with 6 day growth period, it can be concluded that the most sensitive endpoints were estimated for mean growth rate based on mean total frond area of plants and mean yield based on total frond area of plants.

Those levels were: the highest inhibition rates of growth rate -11.8% for frond number and 34.3% for frond area – which were observed after two peaks of 126 µg a.s./L.

In the same time it should be note that two short peaks of up 12 µg/L, each lasting 24h, with 7 day interval did not result in significant effects on the growth of *Lemna gibba*. Therefore, the NOEC of 12 µg/L was determined for two peaks exposure experiment.

A single one-day peak of up 70 µg a.s./L did not result in adverse effects on the growth of *Lemna gibba* within the 13-day period following the peak exposure.

On the basis of the obtained results the Applicant determined the overall value of E<sub>r</sub>C<sub>50</sub> > 126 µg/L .

That value, was determined on the basis of the highest observed inhibition effect – 34% occurring after the second peak, for the highest concentration of the test substance – 126 µg/L, used in the study.

**RMS comments:**

The study fulfils the validity criteria given in OECD test guideline 221 (2006).

The doubling time of the frond number in the control was less than 2.5 days (being 1.6 days for the first week (0-7 day) as well as for the second week (7-14 day, two peaks exposure scenario) corresponding to approx. a seven-fold increase in seven days and an average specific growth rate of 0.275 per day.

The mean growth rate in the control was determined to be 0.428 (based on frond number) and 0.410 (based on frond area) after 7 days and between 7-14 days the mean growth rate 0.424 (based on frond area) and 0.413 based on frond number ( two peak exposure scenario).

The factor of frond number, measured in the control between 0 and 7 days was 20, between 7 and 14 days 19.5 (two peaks exposure) and between 7 and 14 -19.9 days (one peak scenario).

Some deviation from the recommendation given in the current OECD 221 guideline were recorded:

- On day 7, the pH had risen to pH 7.9 in the controls within less than three hours after adding the test item.

The RMS is of the opinion that the reliability of the results is given. Therefore, the study is considered valid.

However, RMS decided not to use it in the risk assessment due to the fact that it was not clearly demonstrated in the standard laboratory studies that Lemna sp. was the most sensitive species of aquatic macrophytes. It shall be also indicated that the study's design enabled mainly to derive  $E_rC_{10}$  and NOEC values, what limits its utility.

According to the EFSA guidance (2013), such study should be designed for the estimation of an  $E_rC_{50}$  under peak exposure condition which is to be compared to the  $E_rC_{50}$  under constant exposure.

On the basis of the obtained results the overall value of  $E_rC_{50}$  is  $> 126 \mu\text{g/L}$ . That value, was determined on the basis of the highest observed inhibition effect – 34% occurring after the second peak, for the highest concentration of the test substance –  $126 \mu\text{g/L}$ , used in the study.

Finally it has to be pointed out that one of the main goals of the experiment was

to demonstrate the exposure pattern comprises relatively high, short lasting and relatively distant concentrations of Flufenacet – the exposure pattern typical for streams in R scenarios.

The limitation factor here was the number of the Flufenacet peaks to which the test plants were exposed – two, each lasting 24 hours. As there are some doubts with regard to Lemna sp. being the most sensitive aquatic macrophyte species, the conclusions drawn in this area also cannot be considered definitive.

RMS is in the opinion that study is valid and can be used for the refined risk assessment of macrophytes if:

- The new study performed with Macrophytes indicates that rooted macrophytes are not more sensitive to flufenacet than Lemna sp.
- The peak exposure design of the study covers the peaks observed in the FOCUS scenarios.

**Therefore, the study will not be used in the current risk assessment.**

**Agreed endpoint:**

$E_rC_{50} > 126 \mu\text{g a.s./L}$

**B.9.2.7.1.5: Toxicity of Flufenacet (FOE 5043) to the aquatic macrophyte, *Myriophyllum spicatum*.**

<b>Reference:</b>	Toxicity of Flufenacet (FOE 5043) to the aquatic macrophyte, <i>Myriophyllum spicatum</i> .
<b>Author(s), year:</b>	Banman, C.S., Alexander, T.M., Lam, C.V.; 2011
<b>Report/Doc. number:</b>	Study No: EBFOL116, Reference BCS No: M-408819-01-1
<b>Guideline(s):</b>	OECD 221 guideline
<b>GLP:</b>	Yes
Test substance:	Flufenacet Technical, Batch No: K664078, Purity 97.5%
Test species:	<i>Myriophyllum spicatum</i>
Source:	Environmental Sciences Laboratory of DuPont Company, Newark, Delaware, USA
Number of replicate:	2 (Surrogate), 4 (Control, Solvent Control and 0.76 µg a.s./L), 3 (2.4, 7.8 and 25 µg a.s./L), and 2 (80 µg a.s./L) with 4 plants per replicate
Type of test, duration:	Static, 14 days
<u>Applied concentrations:</u>	
Nominal:	Control (0), Solvent Control, 0.76, 2.4, 7.8, 25, and 80 µg a.s./L
Measured (mean):	Control (0), Solvent Control (0), 0.55, 1.8, 5.7, 18.8, and 59.6 µg a.s./L
Solvent control:	Dimethylformamide (DMF), 0.1 ml/L
<u>Test conditions:</u>	
Water quality:	Hard processed water (Spring water blended with reverse osmosis water)
Sediment	Artificial sediment used for the culturing <i>Myriophyllum</i> was a modification of the OECD 218, sediment for the testing of Chironomids. The modified sediment was comprised of peat moss (504 g), clay (2017 g), sand, and coral which were autoclaved prior to mixing in order to reduce algal contamination in the test system. The final sediment pH was 6.6. Instead of adding distilled water to wet the sediment, 20 XAAP media (5942 ml) was used to provide shoots with a fertiliser source
Temperature:	19.62-21.93°C
pH:	Test start: 7.8-8.1 Test end: 8.9-9.5
Conductivity:	Test start: 476-592 µmhos/cm Test end: 407-476 µmhos/cm

Light regime: 16 hours light, 8 hours dark. 8020-8910 Lux (mean, 8515 Lux)

Methods: Prior to the definitive exposure, a preliminary rangefind study was conducted consisting of a 7 day acclimation period followed by a 14 day exposure. Nominal concentrations for the preliminary test were control, solvent control, 0.5, 2.0, 8.0 and 64  $\mu$ g a.s./L. A significant reduction in growth occurred at the highest treatment level for the endpoints of shoot length yield and wet weight yield. Percent inhibition from the pooled control group ranged from -10.2% to 57.4% for shoot length yield and from -4.6% to 76.8% for wet weight yield. Data from the rangefind study was to set the definitive test concentrations. The definitive study consisted of a pre-exposure (establishment) and exposure phase. The pre-exposure phase lasted for seven days. The exposure phase lasted for 14 days. Seven days prior to the start of the definitive exposure, shoots were cut from healthy cultures at a length of 7 cm. The leaves were removed from the bottom 2 cm of each shoot. Shoots were then planted 2 cm deep into 650-ml glass crystallization dishes containing 550 grams of artificial sediment. The surface of the sediment was then covered with a layer (approximately 100 ml) of crushed coral that had been previously autoclaved. Four shoots were planted into each crystallization dish. The dishes containing the planted macrophytes were placed into 4-L beakers (25 cm tall, 15.5 cm diameter). The four plants in the crystallization dish held in the 4-L beakers make up a single replicate. During the seven day pre-exposure phase each replicate vessel contained 3.5-L of dilution water. The start of the exposure period was marked by the addition of stock solution to each exposure vessel, with the exception of the control vessels which received no stock solution and the solvent control vessels which received DMF only. The stock mixed into the test beakers using a disposable pipette for approximately one minute. Following 14 days of exposure, all plants were removed from the test system. Length of the main shoot and all side shoots were measured, wet weights were measured, and following drying of plants for at least 72 hours, dry weight measurements were collected.

Test parameters: Temperature was measured hourly via a calibrated probe and daily manual records via a calibrated thermometer.

pH was measured at Days -7, 0, 7 and 14. Wet and dry weight as well as shoot length was measured on Day 0 and Day 14.

Analytical data: On Days 0, 7 and 14 samples for analytical verification were taken and were analysed using LC-MS/MS technique.

Statistic: For normality Shapiro-Wilks test and for homogeneity of variance Levene's test were performed.

To determine EC<sub>50</sub> values and associated 95% confidence limits, the logistic model or Bruce/Versteg cumulative normal model using nonlinear (weighted) regression analysis was performed.

The NOEC was determined from an analysis of variance (ANOVA) and Dunnett's test. Statistical analyses were performed using SAS Software (SAS Institute, Gary, NC). The level of significance was at  $\alpha = 0.05$ . In addition the Applicant performed the additional calculations of 14 day-ErC<sub>50</sub>, E<sub>b</sub>C<sub>50</sub> and NOEC (based on total shoot length) using the Tox Rat program.

#### Findings:

Analytical measurements: The measured concentrations yielded from 84% to 94% of the nominal concentrations on day 0, 74-76% on the day 7, 58-61 % on the day 14.

Mean measured concentration during the study ranged from 73 to 76% of nominal. Hence, the results are expressed based upon the mean measured test concentrations.

Data on mean yield for plant shoots, wet and dry weight are summarized in the tables that follow:

**Table B.9.2.7.1.5-1: Mean yield for plant shoots, wet and dry weights.**

Mean measured concentration [a.s./L]	Length (Day 14) <sup>1</sup>		Wet weight (Day 14) <sup>2</sup>		Dry weight (Day 14) <sup>3</sup>	
	cm	Inhibition %	g	inhibition	g	inhibition
Control	14.40	-	0.4039	-	0.0956	-
Solvent control	17.19	-	0.6544	-	0.1085	-
Pooled control	15.80	-	0.5291	-	0.1020	-
0.55	18.25	-15.5	0.8330	-57	0.1461	-43.1
1.8	18.84	-6.6	0.8515	-60.9	0.1673	-64.1
5.7	15.08	4.5	0.7090	-34	0.1059	-3.8

Mean measured concentration [a.s./L]	Length (Day 14) <sup>1</sup>		Wet weight (Day 14) <sup>2</sup>		Dry weight (Day 14) <sup>3</sup>	
	cm	Inhibition %	g	inhibition	g	inhibition
18.8	11.4	29.5	0.5804	-9.7	0.1187	-16.3
59.6	2.46	84.4*	0.1021	80.7**	0.0442	56.7**

\*Statistically significant difference from control (Dunnett's one-tailed test;  $p \leq 0.05$ ).

\*\*Empirically determined adverse effect due to the nature of data EC<sub>50</sub> value was not calculated.

% Inhibition =  $100 - ((\text{Treatment group parameter mean} / \text{pooled control parameter mean}) * 100)$ .

<sup>1</sup> Based on a mean shoot length of 9.04 cm at the start of the test (Day 0)

<sup>2</sup> Based on a mean wet weight of 0.3867 g at the start of the test (Day 0)

<sup>3</sup> Based on a mean dry weight of 0.0968 g at the start of the test (Day 0)

**Table B.9.2.7.1.5-2: Growth rates for plant shoots, wet and dry weights.**

Mean measured Flufenacet concentration a.s./l	Length (Day 14) <sup>1</sup>		Wet weight (Day 14) <sup>2</sup>		Dry weight (Day 14) <sup>3</sup>	
	cm	Inhibition %	g	Inhibition %	g	inhibition
Control	0.061	-	0.043	-	0.046	-
Solvent control	0.073	-	0.064	-	0.048	-
Pooled control	0.067	-	0.053	-	0.047	-
0.55	0.074	0.00	0.074	0.00	0.048	0.10
1.8	0.074	0.00	0.081	0.00	0.070	0.10
5.7	0.066	0.61	0.066	0.00	0.046	2.04
18.8	0.055	17.73	0.061	0.00	0.055	0.10
59.6	0.016	76.16	0.012	78.34	0.022	52.38

<sup>1</sup> Based on a mean shoot length of 9.04 cm at the start of the test (Day 0)

<sup>2</sup> Based on a mean wet weight of 0.3867 g at the start of the test (Day 0)

<sup>3</sup> Based on a mean dry weight of 0.0968 g at the start of the test (Day 0)

Observed visual effect:

Plants in the control vessels and all treatment groups appeared normal throughout the study. However, at study termination roots and shoots appeared normal in controls and all treatment groups with one exception. In the control group, five plants were observed to have underdeveloped roots and eight plants were observed to have shoots with light red colored tips. However, growth data for all plants was included in the data analysis.

Conclusion:

Inhibition values based on mean measured concentration of flufenacet and total shoot length yield, total plant wet weight yield, total plant dry weight yield were as follows:

14 d Day Growth Rate (shoot length)  $E_rC_{50} = 36.10 \mu\text{g a.s./L}$ ,  $NOEC = 18.80 \mu\text{g a.s./L}$

14 d Day Growth Rate (plant dry weight)  $E_rC_{50} = 58.30 \mu\text{g a.s./L}$

14 d Day Growth Rate (plant wet weight)  $E_rC_{50} = 48.53 \mu\text{g a.s./L}$ ,  $NOEC = 59.600 \mu\text{g a.s./L}$

14 d Day Yield (shoot length yield)  $E_yC_{50} = 26.2$  (15.7 to 43.7)  $\mu\text{g a.s./L}$ ,  $NOEC = 18.8 \mu\text{g a.s./L}$

14 d Day Yield (plant wet weight yield)  $E_yC_{50} = 18.8$ -59.6  $\mu\text{g a.s./L}$ , empirically determined  $NOEC = 18.8 \mu\text{g a.s./L}$

#### **RMS comments:**

The study was conducted according to the OECD test guideline 221 (Lemna growth inhibition test).

According to validity criteria given in the test guideline OECD 239 (Water-Sediment Myriophyllum Spicatum toxicity test, 2014) for the study is considered valid if the following points are met:

- The mean total shoot length and mean shoot fresh weight in control plants must at least double during the exposure phase of the test.
- Control plants must not show any visual symptoms of chlorosis and should be visibly free from contamination by other organisms such as algae and/or bacterial films on the plants, at the surface of the sediment and in the test medium.
- The mean coefficient of variation for yield based on measurements of shoot fresh weight (i.e. from test initiation to test termination) in the control cultures must not exceed 35% between replicates.

The results obtained from control and solvent control were presented below:

**Mean shoot length and mean total shoot fresh weight in the control and solvent control is presented below:**

Mean total shoot length (cm)		Mean total shoot fresh weight (g)	
		(g)	
Control			
Day 0	Day 14	Day 0	Day 14
9.04	23.4	0.3867	0.4039
Solvent control			
9.04	26.2	0.3867	0.6554

In this study the mean total shoot length increased about 2.6 and 2.9 times in the control and in the solvent control, respectively.

However, the mean shoot fresh weight in control and in solvent control did not double during the exposure phase of the test.

The mean CV variation for yield based on measurements of shoot fresh weight (i.e. from test initiation to test termination) in the control cultures are above the trigger value of 35% and were 86 and 67.6 % in control and in the

solvent control, respectively.

No symptoms of chlorosis as well as no any visible contamination by other organisms were noted. However, it should be indicated that at study termination in the control group, five plants were observed to have underdeveloped roots and eight plants were observed to have shoots with light red colored tips.

In opinion of RMS, the validity criteria were not met, therefore the study is considered not valid.

**For that reason RMS proposes to consider that issue a data gap to be solved on the MS level. At the same time RMS is of the opinion that the setting of that data gap should be agreed by the Member States during the peer-review.**

#### Metabolites of flufenacet:

##### **B.9.2.7.1.6. *Lemna gibba* G3 Growth inhibition test with flufenacet-oxalate under static conditions.**

<b>Reference:</b>	<i>Lemna gibba</i> G3 Growth inhibition test with flufenacet-oxalate under static conditions.
<b>Author(s), year:</b>	Burns E., 2009
<b>Report/Doc. number:</b>	Study No: E 412 3757-9, Report No: EBFOL138, Reference BCS No:M-359515-02-1
<b>Guideline(s):</b>	OECD Guideline 221, (March 23, 2006)
<b>GLP:</b>	Yes
<b>Test substance:</b>	Flufenacet-oxalate (BCS-AB16305): Batch No: BCS-AB16305-01-01, Purity: 95.3% w/w (analysed)
<b>Test species:</b>	<i>Lemna gibba</i> G3
<b>Source:</b>	Horticulture Crops Quality Laboratory, U.S. Department of Agriculture, Beltsville, MD 20705, U.S.A.
<b>Growth medium:</b>	The medium 20 x AAP, according to OECD guideline
<b>Growth chamber:</b>	Glass dishes were used with a diameter of 10 cm and a height of 6 cm (total volume of approx. 470 mL). These test vessels were covered with lids of glass to permit gas exchange and illumination under sterile conditions.
<b>Inoculum:</b>	To ensure that the plants used as inoculum were exponentially growing, an inoculum pre-culture was prepared 7 – 10 days before the start of the test and were cultivated under the same conditions as in the main test.

---

Number of organism:	3 replicate vessels per test level and 3 replicate vessels per control. 12 fronds per vessels
Type of test, duration:	Static, 7 days
<u>Applied concentrations:</u>	
Nominal	1.56, 3.13, 6.25, 12.5, 25.0, 50.0 and 100 mg met./L
<u>Test conditions:</u>	
Water quality:	The medium 20 x AAP medium according to the guideline with pH= 7.5±0.1.
Temperature:	22.7°C-24.1°C (average, 23.4°C)
pH:	7.5-8.7
Light regime:	Continuous light. Mean 7210-8900 Lux ( mean, 8090 Lux)
Test parameters:	The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature was recorded hourly. The pH was measured in all freshly prepared and all aged test levels and the control.
Biomass quantification:	Counting of fronds and determination of total frond area was carried out using the LemnaTec Scanalyzer machine, validated for such measurements.
Analytical data:	Samples were analysed for the actual concentration of flufenacet-oxybate present in all freshly prepared test levels on day 0 and in all aged test levels on day 7 of the exposure period. Aliquots for freshly prepared test levels for day 0 analyses were sampled from the prepared volume of each test treatment level. For sampling of aged test media, after removing of plant material from the test vessels on day 7, the contents of all replicate vessels combined and pH was measured. All test concentrations and control replicates were analysed HPLC-MS/MS analysis.
Observation:	Visual observations were made on study days 3, 5, and 7. Plant frond numbers and total frond area of plants were determined on days 0, 2, 5 and 7.
Statistics:	The EC <sub>50</sub> values (growth rate) and their 95% confidence limits were calculated by Probit analyses. For the determination of the NOErC values significant difference at the test concentrations compared to the control values were tested by the Williams Multiple Sequential t-test. The software used to perform the statistical analyses was ToxRat Professional.

Findings:

Analytical measurements: The analytical findings of flufenacet-oxalate determined in all test levels on day 0 ranged between 100 and 106 % (average 104 %), on day 7 the analysed concentrations ranged between 103 and 132 % (average 110 %) of nominal concentrations.

Test concentrations were based on nominal concentration.

Data on final frond number, final total frond area and growth rate based on final number and total frond area are summarized in the tables that follow.

**Table B.9.2.7.1.6-1: Mean growth rate (based on fronds number and total frond area).**

Nominal concentration [mg p.m./L]	Frond number			Total frond area		
	Final frond Number at 7 day	Average growth of frond number (0-7d)	% inhibition of average growth rate of fronds number	Final total frond area at 7 day	Average growth rate	% inhibition of average growth rate of total frond area
	mean [mm <sup>2</sup> ]			Mean [mm <sup>2</sup> ]	[0-7d]	
control	134	0.344		458	0.356	
1.56	125	0.334	2.92	456	0.373	-4.94
3.13	130	0.340	1.15	462	0.369	-3.66
6.25	124	0.333	3.29	464	0.368	-3.35
12.5	131	0.341	0.88	497	0.364	-2.46
25	116	0.324	5.88	401	0.333	6.34
50	120	0.328	4.65	405	0.323	9.23
100	114	0.322*	6.48	395	0.323	7.59

\*Negative values mean growth stimulation

Results:

Growth inhibition values based on nominal concentration with Flufenacet-oxalate on Lemna gibba G3 were as follows:

0-7 d frond number growth rate:  $EC_{50} > 100$  mg p. m. /L,  $NOEC = 50$  mg metabolite/L

0-7 d total frond area growth rate:  $EC_{50} > 100$  mg p. m./L,  $NOEC \geq 100$  mg metabolite /L

**Comments RMS:**

The study was conducted according to the OECD 221 (2006) test guideline.

The study is in the line with test guideline OECD 221 (2006) and all validity criteria are met.

The doubling time of the frond number in the control was less than 2.5 days (being 2 days) corresponding to approx. a seven-fold increase in seven days and an average specific growth rate of 0.275 per day. The mean growth rate in the control was determined to be 0.344 after 7 days. The factor of frond number, measured in the control between 0 and 7 days, was 11.2.

The RMS is of the opinion that the reliability of the results is given.

Therefore, the study was considered acceptable.

**Agreed endpoints:**

7d E<sub>r</sub>C<sub>50</sub> > 100 mg Flufenacet-oxalate/L, based on frond number

NOE<sub>r</sub>C = 50 mg Flufenacet-oxalate /L, based on frond number

7d E<sub>r</sub>C<sub>50</sub> > 100 mg Flufenacet-oxalate /L, based on frond area

NOE<sub>r</sub>C ≥ 100 mg Flufenacet-oxalate /L, based on frond area

All results are based on nominal concentration

**B.9.2.7.1.7. FOE 5043 sulfonic acid –Toxicity (14 days) to Lemna gibba G3.**

<b>Reference:</b>	FOE 5043 sulfonic acid -Toxicity (14 days) to Lemna gibba G3.
<b>Author(s), year:</b>	Dorgerloh M., 1995
<b>Report/Doc. number:</b>	Study No: E412 0997-2, Report No: DOM 95072, Reference BCS No: M-004929-01-1.
<b>Guideline(s):</b>	U.S. EPA-FIFRA Pesticide Assessment Guideline § 123-2
<b>GLP:</b>	Yes

Material and methods:

Test substance:	FOE 5043 sulfonic acid Batch No: (WAK 6222-3), Purity (as sodium salt): 93.6% corr. to free acid: 86.7%
Test species:	Lemna gibba G3
Source:	Horticulture Crops Quality Laboratory, U.S. Department of Agriculture, Beltsville, U.S.A

---

Inoculum:	The inoculum of <i>Lemna gibba</i> G3 used to begin the test was taken from a less than 14-day old sterile stock culture.
Growth chamber:	Glass dishes for testing had a diameter of 10 cm and a high of 6 cm. The total volume was 400 ml. These test vessels were covered with lids of glass to permit gas exchange and illumination under sterile conditions.
Number of organism:	3 replicate, with five plants, consisting of three fronds each (for a total of 15 fronds) for control and all test concentrations.
Type of test, duration:	Static, 14 day

Applied concentrations:

Nominal:	2.93, 5.85, 11.7, 23.40, 46.8, 93 and 60 mg FOE 5043-sulfonic acid sodium salt/L
Measured (mean):	2.24, 4.40, 8.99, 18.2, 38.7 and 75.9 mg FOE 5043-sulfonic acid/L

Test conditions:

Water quality:	Hoagland E Medium without sucrose, yeast extract, Bactotryptone and EDTA, according to EPA.
Temperature:	22°C to 25.3 °C
pH:	5.3-5.4
Light regime:	Continuous illumination of 4483Lux

Test parameters:

	The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of water as in the test vessels. Temperature was recorded hourly. The pH was measured at the end of test.
Analytical measurement:	Samples were analysed for the actual concentration of test substance present in the test medium at each treatment level and in the no-treatment control on day 0 and day 14.
Observation:	Frond counts were made using a lighted magnifying lens on study days 2, 5, 7, 9, 12 and 14. In order to eliminate subjective decisions on frond maturity, every frond visible projecting beyond the edge of the parent frond was counted.
<u>Statistic:</u>	Growth data expressed as frond counts on day 14 were used to conduct the following statistical analyses:

- Chi-square test to determine the normality of the data set and,
- Bartlett's test for homogeneity of variances

Data which did not fit a normal distribution were analyzed by the non-parametric Kruskal-Wallis' test and Dunn's Multiple Comparison test to determine if there was a significant difference between the control and treatment groups. This growth data, without previous data transformation were analysed according to the following statistical methods:

- the Dunnett's test;
- the Bonferroni's t-test,
- the Tukey's test and,
- the Williams 'test.

All statistical analyses were conducted using a PC based computer program with conclusions of statistical significance based on a 95 percent confidence level ( $\alpha = 0.05$ ). In addition, the frond count values at test termination for each test concentration were calculated using percent inhibition relative to that in the control.

The value of  $EC_{25}$  and  $EC_{50}$  was not determined due to the lack of the effect  $>50\%$ .

#### Findings:

##### Analytical measurements:

The measured concentrations ranged from 87 % to 92 % of nominal on day 0 and from 71 % to 88 % of nominal on day 14. No detectable amount (at a limit of quantification higher than 0.01 mg/l) of FOE 5043-Sulfonic acid sodium salt was found in the control neither on day 0 nor on day 14. The results of this study are expressed based upon the mean measured test concentrations of FOE 5043-Sulfonic acid.

Data on final frond number on Day 14 and % inhibition was summarized in the B.9.2.7.1.7-1 below:

**Table B.9.2.7.1.7-1: Final fronds number and % inhibition of growth.**

Mean measured concentration [mg p.m. /L]	Mean frond number	
	Frond Number at 14 day	% inhibition of relative to control
Control	217	
2.24	248	-14 <sup>ns</sup>
4.40	220	-2 <sup>ns</sup>
8.99	183	16 <sup>ns</sup>
18.2	220	-1 <sup>ns</sup>
38.7	191	12 <sup>ns</sup>
75.9	202	7 <sup>ns</sup>

\*Negative values mean growth stimulation

Observed visual effect: There were no morphological changes  
(e.g. chlorosis, necrosis, plant disintegration) during this study.

Conclusion:

Growth inhibition values based on mean measured concentration of FOE-Sulfonic acid on Lemna gibba G3 were as follow:

14 d Growth frond number      14 d EC<sub>50</sub> > 79.5 mg p.m./L, NOEC > 79.5 mg p.m./L

**RMS comments:**

The study was conducted according to U.S. EPA- Guideline § 123-2 and was accepted during the first inclusion to of flufenacet to Annex 1.

According to the current OECD 221 (2006) test guideline the following criteria should be met in the study:

-The doubling time of the frond number in the control should be less than 2.5 days corresponding to approx. a seven-fold increase in seven days and an average specific growth rate of 0.275 per day.

In this study the doubling time of frond number in the control was 3.631 day and the mean growth rate in the control was 0.190 after 7 days.

In addition, the following deviations from recommendation given in the OECD 221 (2006) test guideline were recorded :

-pH value was measured only at the end of test

- only one parameter was tested during the study - frond number

The validity criteria were not met.

**Therefore, the study is not fully reliable but can be used as supportive information indicating that metabolites are clearly less toxic than active substance.**

**Agreed endpoints:**

14 d  $E_rC_{50} > 79.5$  mg p.m./L, (frond number) based on mean measured concentration

14 d  $NOE_rC > 79.5$  mg p.m./L, (frond number) based on mean measured concentration

**B.9.2.7.1.8. Lemna gibba G3 - Growth inhibition test with Flufenacet-methylsulfone (BCS-CO62475) under static conditions.**

<b>Reference:</b>	Lemna gibba G3 - Growth inhibition test with Flufenacet-methylsulfone (BCS-CO62475) under static conditions.
<b>Author(s), year:</b>	Bruns E., 2010
<b>Report/Doc. number:</b>	Study Number: E 412 3824-4, Report No: EBFOL145, Reference BCS No: M-369703-01-1
<b>Guideline(s):</b>	OECD Guideline 221 (March 23, 2006)
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet-methylsulfone (BCS-CO62475, Batch code: BCS-CO62475-01-01, Purity: 97.67 % w/w (analysed))
Test species:	Lemna gibba G3
Source:	Horticulture Crops Quality Laboratory, U.S. Department of Agriculture, Beltsville, MD 20705, U.S.A.
Growth medium:	The medium 20 x AAP according to OECD guideline
Growth chamber:	Glass dishes were used with a diameter of 10 cm and a height of 6 cm (total volume of approx. 470 mL). These test vessels were covered with lids of glass to permit gas exchange and illumination under sterile conditions to the greatest possible extent.
Inoculum:	To ensure that the plants used as inoculum were exponentially growing, an inoculum pre-culture was prepared 7 – 10 days before the start of the test and were cultivated under the same conditions as in the main test.
Number of organism:	Six replicate vessels per test level and 6 replicate vessels per control. 12 fronds
Type of test, duration:	Static, 7 days

Applied concentrations:

Nominal 0 (control), 100 mg metabolite/L

Test conditions:

Water quality: 20 x AAP medium according to the OECD guideline with pH 7.5±0.1. After adding test item to the beakers, within less than three hours the pH had risen to pH 7.9 to 8.0 on day 0 in control and treatment

Temperature: 23.1°C to 25.5°C (mean: 24.4°C)

pH: 7.5-8.9

Light regime: Continuous light. 7690-8880 Lux (mean, 8260 Lux)

Test parameters: The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature was recorded hourly.

The pH was measured in all freshly prepared and all aged test levels and the control.

Biomass quantification:. Counting of fronds and determination of total frond area was carried out using an optical recording system for such measurements.

Analytical data: Samples were analyzed for the actual concentration of flufenacet-methylsulfone present in all freshly prepared test levels on day 0 and in all aged test levels on day 7 of the exposure period.

All test concentrations and control replicates were analysed HPLC-UV analysis.

Observation: Visual observations were made on study days 3, 5, and 7.

Plant frond numbers and total frond area of plants were determined days 0, 2, 5 and 7.

Statistic: Since the highest tested concentration of 100 mg metabolite /L caused no growth inhibition effects, statistical evaluation were not applicable.

Findings:

Analytical measurements: The chemical analysis of Flufenacet-methylsulfone revealed recoveries of 102% of the nominal concentration on day 0 and 99% of the nominal concentration on day 7. Test concentrations were based on nominal concentration.

Data on final frond number, final total frond area and growth rate based on final number and total frond area are summarized in the table below:

**Table B.9.2.7.1.8-1: Final fronds number and final total frond area and % inhibition of their average growth rate.**

Nominal concentration [mg p.m./L]	Frond number			Total frond area		
	Final frond Number [0-7d]	Average growth rate of frond number	% inhibition of average growth rate of fronds number	Final total frond area [0-7d]	Average growth rate	% inhibition of average growth rate of total frond area
				Mean [mm <sup>2</sup> ]		
Control	181	0.388	-	759	0.423	-
10	160	0.369	4.81	633	0.394	6.84

Observed visual effects: No visual signs of toxicity caused by the test item.

Conclusion:

Growth inhibition values based on nominal concentration with Flufenacet-methylsulfone on Lemna gibba G3 were as follows:

0-7 d Frond number growth rate:  $E_rC_{50} > 10$  mg p.m./L  
 $NOE_rC \geq 10$  mg p.m./L

0-7 d Total Frond Area growth rate:  $E_rC_{50} > 10$  mg p.m./L  
 $NOE_rC \geq 10$  mg p. m./L

**RMS comments :**

The study was conducted according to the current OECD 221 (2006) test guideline and the study fulfils the all validity criteria stated in that guideline.

The doubling time of the frond number in the control was less than 2.5 days (being 1.8 day) corresponding to approx. a seven-fold increase in seven days and an average specific growth rate of 0.275 per day.

The mean growth rate in the control was determined to be 0.338 after 7 days.

The factor of frond number, measured in the control between 0 and 7 days, was 15.1.

The RMS is of the opinion that the reliability of the results is given. Therefore, the study was considered acceptable.

**Agreed endpoints:**

0-7 d Frond number growth rate:  $E_rC_{50} > 10$  mg Flufenacet-methylsulfone/L  
 $NOE_rC \geq 10$  mg Flufenacet-methylsulfone/L

0-7 d Total Frond Area growth rate:  $E_rC_{50} > 10$  mg Flufenacet-methylsulfone/L  
 $NOE_rC \geq 10$  mg Flufenacet-methylsulfone/L

All results based on based on nominal test concentration.

**B.9.2.7.1.9. Lemna gibba G3 - Growth inhibition test with flufenacet-methylsulfide under static conditions.**

<b>Reference:</b>	Lemna gibba G3 - Growth inhibition test with flufenacet-methylsulfide under static conditions.
<b>Author(s), year:</b>	Bruns E., 2010
<b>Report/Doc. number:</b>	Study No:EBFOL143, Reference BCS No: M-393709-01-1
<b>Guideline(s):</b>	OECD Guideline 221, (March 23, 2006)
<b>GLP:</b>	Yes

It should be noted that the new statistical analysis of the raw data was performed by the Applicant specially for the purpose of the current evaluation assessment and on request by the RMS. That was done in order to get endpoints compliant with the current requirement (EC<sub>10</sub>, EC<sub>20</sub>, EC<sub>50</sub> NOEC for growth rate and yield).

Material and methods:

Test substance:	Flufenacet-methylsulfide (BCS-CP38571), Batch No.: SES 11158-2-4, Purity: 98.0 % w/w (analysed)
Test species:	Lemna gibba G3
Source:	Horticulture Crops Quality Laboratory, U.S. Department of Agriculture Beltsville, MD 20705, U.S.A.
Growth medium:	20X AAP nutrient medium according to OECD guideline
Growth chamber:	Glass dishes were used with a diameter of 10 cm and a height of 6 cm (total volume of approx. 470 mL). These test vessels were covered with lids of glass to permit gas exchange and illumination under sterile conditions to the greatest possible extent.
Inoculum:	To ensure that the plants used as inoculum were exponentially growing, an inoculum pre-culture was prepared 7 – 10 days before the start of the test and were cultivated under the same conditions as in the main test.
Number of organism:	3 replicate vessels per test level and 3 replicate vessels per control.
Type of test, duration:	Static, 7 days

Applied concentrations:

Nominal:	0 (control), 8.78, 13.2, 19.8, 12.5, 29.6, 44.4, 66.7 and 100 mg p.m./L
Test conditions:	
Water quality:	20 x AAP nutrient medium according to the OECD test guideline, pH=7.5±0.1

---

Temperature:	23.9° to 24.4.0°C
pH:	7.5-8.9
Light regime:	Continuous light. 7690-8350 Lux (mean 8010 Lux)
Test parameters:	<p>The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature was recorded hourly.</p> <p>The pH was measured on the day 0 and 7 in all test level and the control.</p>
Biomass quantification:	Counting of fronds and determination of total frond area was carried out using the LemnaTec Scanalyzer machine, validated for such measurements.
Analytical data:	<p>Samples were analysed for the actual concentration of present in all freshly prepared test levels on day 0 and in all aged test levels on day 7 of the exposure period. Aliquots for freshly prepared test levels for day 0 analyses were sampled from the prepared volume of each test treatment level. For sampling of aged test media, and after removing of plant material from the test vessels on day 7 the contents of all replicate vessels were combined, and the pH was measured. The combined test solutions were then submitted for HPLC-UV analysis.</p>
Observation:	<p>Visual observations were made on study days 2, 5, and 7.</p> <p>Plant frond numbers and total frond area of plants were determined on days for this study.</p>
Statistics:	<p>The EC<sub>50</sub> values (yield and growth rate) and their 95% confidence limits were calculated by Probit analyses. For the determination of the NOEC values significant difference at the test concentrations compared to the control values were tested by the Williams Multiple Sequential t-test.</p> <p>The software used to perform the statistical analyses was ToxRat Professional.</p>
Analytical data:	<p>Samples were analysed for the actual concentration of Flufenacet-methylsulfid present in all freshly prepared test levels on day 0 and in all aged test levels on day 7 of the exposure period. Aliquots for freshly prepared test levels for day 0 analyses were sampled from the prepared volume of each test treatment level. For sampling of aged test media, and after removing of plant material from the test vessels on day 7 the contents of all replicate vessels were combined, and the pH was measured. The combined test solutions were then submitted for HPLC-UV analysis.</p>

Findings:

Analytical measurements: The analytical findings of flufenacet-methylsulfide determined in all test levels on day 0 ranged between 101 and 103 % and on day 7 the analysed concentrations ranged between 102 and 109 % of nominal concentrations.

All results are based on nominal values of the test item.

Data on final frond number, final total frond area and growth rate based on final number and total frond area are summarized in the table below:

**Table B. 9.2.7.1.9-1-: Final fronds number % inhibition of growth. Final fronds number and final total frond area and % inhibition of their average growth rate.**

Nominal concentration (mg p.m./L)	Final Frond number			Final frond area		
	at 7 day	Average growth rate [0-7d]	% inhibition of average growth rate of fronds number	at 7 day	Average growth rate [0-7d]	% inhibition of average growth rate of total frond area
				Mean [mm <sup>2</sup> ]		
control	127	0.335		836	0.338	
8.78	114	0.321	4.26	766	0.339	-0.221
13.2	120	0.329	1.99	815	0.337	0.416
19.8	104	0.309	7.92	710	0.321	5.05**
29.6	110	0.316	5.68	735	0.319	5.73
44.4	89	0.286	14.9*	605	0.290	14.3**
66.7	69	0.250	25.6*	429	0.256	24.3**
100	47	0.192	42.7*	258	0.172	49.2**

\*Statistically significant compared to control (Williams t-test , $p \leq 0.05$ )

\*\*Statistically significant compared to control (Welch–test Bonferroni t-Test)

Observed visual effect: No visual signs of toxicity caused by the test item.

**Table B.9.2.7.1.9-2: Mean yield (based on fronds number and total frond area).**

Nominal Concentration (µg met./L)	Mean yield			
	Frond number		Total frond area	
	After 7 day	% inhibition relative to the control	After 7 days	% inhibition relative to the control
control	115.3	-	758.0	-
8.78	101.7	11.8	694.7	8.4
13.2	108.0	6.4	737.7	2.7
19.8	92.3	19.9*	635.0	16.2
29.6	98.3	14.7*	656.0	13.4
44.4	77.0	33.2*	525.7	30.7*
66.7	57.0	50.6*	357.3	52.9*
100	34.7	69.9*	181.0	76.1*

\* Statistically significant difference from control, (Williams t-test,  $\alpha = 0.05$ , one-sided.)

**Conclusion:**

Growth inhibition values based on nominal concentration with on *Lemna gibba* G3 were as follows:

0-7 d Frond number growth rate:  $ErC_{50} = 125.30$  mg p.m./L (95% CI: 108.39-15)  
 $ErC_{20} = 52.90$  mg p.m./L (95% CI: 46.35-58.65)  
 $ErC_{10} = 33.71$  mg p.m./L (95% CI: 26.56-39.53)

$NOE_rC = 29.60$  mg p.m./L

0-7 d Yield frond number growth rate  $EyC_{50} = 65.02$  mg p.m./L (95% CI: 51.52-91.25)  
 $EyC_{20} = 26.81$  mg p.m./L (95% CI: 15.65-35.32)  
 $EyC_{10} = 16.87$  mg p.m./L (95% CI: 7.32-24.62)  
 $NOE_yC = 13.20$  mg p.m./L

0-7 d Growth rate frond area:  $ErC_{50} = 106$  mg p.m./L (95% CI: 95.1-122)  
 $ErC_{20} = 54.69$  mg p.m./L (95% CI: 51.29-57.76)  
 $ErC_{10} = 38.76$  mg p.m./L (95% CI: 34.89-42.16)  
 $NOE_rC = 13.2$  mg p.m./L

0-7 d Yield frond area  $EyC_{50} = 61.97$  mg p.m./L (95% CI: 52.14-78.46)  
 $EyC_{20} = 31.75$  mg p.m./L (95% CI: 21.62-39.24)  
 $EyC_{10} = 22.38$  mg p.m./L (95% CI: 12.71-29.74)  
 $NOE_yC = 29.60$  mg p.m./L

**Comments RMS:**

The study was conducted according to the current OECD 221 (2006) test guideline and the study fulfils the all validity criteria stated in that guideline.

The doubling time of the frond number in the control was less than 2.5 days (being 2.1 day) corresponding to approx. a seven-fold increase in seven days and an average specific growth rate of 0.275 per day.

The mean growth rate in the control was determined to be 0.335 after 7 days.

The factor of frond number, measured in the control between 0 and 7 days, was 10.6

The RMS is of the opinion that the reliability of the results is given.

Therefore, the study was considered acceptable.

**Agreed endpoints:**

0-7 d	Frond number growth rate:	$E_rC_{50} = 125.30 \text{ mg p.m./L (95\% CI: 108. 39-15)}$ $E_rC_{20} = 52.904 \text{ mg p.m./L (95\%CI: 46.35-58.65)}$ $E_rC_{10} = 33.71 \text{ mg p.m./L ( 95 \%CI: 26.56-39.53)}$ $NOE_rC = 29.60 \text{ mg p. m/L}$
0-7 d	Yield Frond number	$E_yC_{50} = 65.020 \text{ mg p.m./L (95\% CI:51.52-91.25)}$ $E_yC_{20} = 26.81 \text{ mg p.m./L (95\% CI: 15.65-35.32)}$ $E_yC_{10} = 16.87 \text{ mg p.m./L (95\% CI:7.32-24.62)}$ $NOE_yC = 13.20 \text{ mg p.m./L}$
0-7 d	Growth rate frond area	$E_rC_{50} = 106 \text{ mg p.m./L (95\% CI: 95.1-122 )}$ $E_rC_{20} = 54.69 \text{ mg p.m./L (95\% CI: 512-57.76)}$ $E_rC_{10} = 38.76 \text{ mg p.m./L ( 95\%: CI: 34.89-42.16)}$ $NOE_rC = 13.2 \text{ mg p.m./L}$
0-7 d	Yield frond area	$E_yC_{50} = 61.97 \text{ mg p.m./L (95\%CI: 52.14-78.46)}$ $E_yC_{20} = 31.75 \text{ mg p.m./L (95 \%CI: 21.62-39.24)}$ $E_yC_{10} = 22.38 \text{ mg p.m./L (95\%CI: 12.71-29.74)}$ $NOE_yC = 29.60 \text{ mg p.m./L}$

All results based on the nominal concentration.

**B.9.2.7.1.10. Sodium Trifluoroacetate: Toxicity to the duckweed (*Lemna gibba*).**


---

<b>Reference:</b>	Sodium Trifluoroacetate: Toxicity to the duckweed ( <i>Lemna gibba</i> ).
<b>Author(s):</b>	Smith, D.V., Thompson, R.S., Gillings, E., 1993
<b>Report/Doc. number:</b>	BL4872/B, M-247900-01-1
<b>Guideline(s):</b>	ASTM (1991). E1415-91 : BCS eference NO; M-247900-01-1
<b>GLP:</b>	Yes

---

It should be noted that the new statistiactal analysis of the raw data was performed by the Applicant specially for the pupurpose of the current evaluation assessment and on request by the RMS. That was done in order to get endpoints compliant with the current requirement (EC<sub>10</sub>, EC<sub>20</sub>, EC<sub>50</sub> NOEC for growth rate and yield).

<u>Test substance:</u>	Sodium trifluoroacetate. Batch No:W907. Purity: 99 % (analysed)  The test substance was mixed with radiolabelled trifluoro[2- <sup>14</sup> C]acetic acid before use with the reference CFQ7300 and was assigned the Brixham test substance number X188.  The specific activity was 54 mCi/mmol (2.0 GBq/mmol) and the radiochemical purity was 99.6%.
Test species:	<i>Lemna gibba</i> G3
Source:	University of Waterloo, Canada.
Growth medium:	M-Hoagland Medium
Test vessel:	The test vessels were borosilicate glass crystallising dishes of 400 ml nominal capacity with loose-fitting lids. Each vessel contained 160 ml of test solution.
Number of organism:	3 replicate per control and each concentration tested with 3 plants with four fronds (total 12 fronds) each.
Type of test, duration:	Static, static, 7 day
Applied concentrations:	
Nominal:	0 (control), 19, 38, 75, 150, 300, 600, 1200, 2400 mg /L

Test conditions:

Water quality:	M-Hoagland Medium
Temperature:	24.7°C to 25.1°C (daily measurement) 25 to 25.8°C (hourly measurement)
pH:	4.6-4.7 (at start) and 5-5.6 (at end)
Light regime:	9220 Lux on test day 1.

Test parameters:

The temperature of the incubator was measured daily by thermometer and at hourly intervals using an automatic recording system. The light intensity was measured once during the study. The pH of each test solution was measured at the start of the test.

Analytical data:

Samples of each test solution were taken at the start and the end of the test to determine the concentration of the test substance by radiochemical analysis (liquid scintillation counting).

The dry plant tissues from each replicate vessel were analysed for <sup>14</sup>C residues, by liquid scintillation (LSC) following sample oxidation. The fresh weight and dry weight were determined to provide a fresh weight/dry weight ratio.

Observation:

On days 2, 5 and 7 the number of plants and the number of fronds were counted and recorded for each test vessel. All fronds, which visibly projected beyond the edge of the parent frond, were counted. Any other symptoms of toxicity were recorded. At the end of the test (7 days) the duckweed from each vessel was rinsed briefly in distilled water and dried to constant weight at 60°C. The dry weight of the tissue was determined.

## Statistics:

The EC<sub>50</sub> values (yield and growth rate) and their 95% confidence limits were calculated by Probit analyses. For the determination of the NOEC values significant difference at the test concentrations compared to the control values were tested by the Williams Multiple Sequential t-test.

The software used to perform the statistical analyses was ToxRat Professional.

Findings:

## Analytical measurements:

The measured concentrations at the start of the test ranged from 102 to 113% of the nominal values. At each nominal concentration, the mean measured values at the finish were the same as those at the start of the test. Therefore the results of the study based on nominal concentration.

Data on final frond number, dry weight and % inhibition are summarized in the

tables that below:

Observed visual effect:	From day 5 at concentrations 600, 1200 and 2400 mg/L were observed following toxicity symptoms pale, misshapen frond with decreased root growth, compared to control.
Tissue residues:	Fresh/dry weight ratio:19.0 The BCF values ranged from 1.0 to 1.6 indicating only light bioconcentration above ambient water concentration.

The results are included in the Table below:

**Table B. B.9.2.7.1.10-1: Mean growth and yield (based on frond numbers).**

Nominal TFA- Na concentration (mg met/L)	Frond number at day 7 <sup>a</sup>	Mean growth rate		Mean yield	
		0-7 (1/d)	% inhibition relative to the control	0-7 d	% inhibition relative to the control
Control	100.0	0.303	--	88.0	--
19	(107) <sup>b</sup>	n.d. <sup>b</sup>	n.d. <sup>b</sup>	(95.0) <sup>b</sup>	(-0.08) <sup>b</sup>
38	(110) <sup>b</sup>	n.d. <sup>b</sup>	n.d. <sup>b</sup>	(98.0) <sup>b</sup>	(-0.11) <sup>b</sup>
75	144.0	0.354	-17.0	132.0	-50.0
150	146.7	0.357	-18.0	134.7	-53.0
300	121.0	0.330	-9.0	109.0	-23.9
600	68.0	0.247*	18.5	56.0	36.4
1200	47.3	0.196*	35.3	35.3	59.8*
2400	41.0	0.175*	42.2	29.0	67.0*

\* Statistically significant difference from control, Williams t-test,  $\alpha = 0.05$ , one-sided

Negative values indicate an increase in growth relative to that of the control

n.d. not determined

<sup>a</sup>Starting number of fronds (day 0): 12 fronds/test vessel

<sup>b</sup> the two lowest concentrations were excluded from the analysis, as the template is fixed to 7 treatment levels and could not be changed. As no effects were observed in the five lowest concentrations this has no or only minor effects on the calculation. RMS, having performed the thorough analysis of the available data set, found the Applicant's approach acceptable.

**Results:**

Growth inhibition values based on nominal concentration with on Lemna gibba G3 were as follows:

Parameter tested	Results
7 d Yield (frond number):	$E_yC_{10}$ =242.8 mg p.m./L (95%CI: 133.477-441.757) $E_yC_{20}$ =360.6 mg p.m./L (95%CI:205.11-633.799) $E_yC_{50}$ =768.6 mg p.m./L (385-1515.14) LOEC=1200 mg p.m./L NOE <sub>y</sub> C:=600 mg p.m./L
7 d Growth rate (frond number):	$E_rC_{10}$ =315.2 mg p.m./L (95%:258.61-384.17) $E_rC_{20}$ =593.3 mg p.m./L (95%: 487.08-722.83) $E_rC_{50}$ =1990.0 mg p.m./L (95%: 1496.79-2609.18) LOEC=600 mg p.m./L NOE <sub>r</sub> C=300 mg p.m./L

**Comments RMS:**

The study was conducted according to the ASTM test guideline (1991).

According to the current OECD 221 (2006) test guideline the following criteria should be met in the study.

-The doubling time of the frond number in the control should be less than 2.5 days (being: 2.33 day) corresponding to approx. a seven-fold increase in seven days and an average specific growth rate of 0.275 per day.

The mean growth rate in the control was determined to be 0.303 after 7 days.

-The factor of frond number, measured in the control between 0 and 7 days should be >7 (being 8.3).

The RMS is of the opinion that the reliability of the study results is given.

Therefore, the study is considered acceptable.

**Agreed endpoints:**

Growth rate (frond number):

7 d  $E_rC_{10}$  = 315.2 mg p.m./L

7 d  $E_rC_{20}$  = 593.3 mg p.m./L

7 d  $EC_{50}$  = 1990.0 mg p.m./L

LOEC = 600 mg p.m./L

NOE<sub>r</sub>C = 300 mg p.m./L

7 d  $E_yC_{10}$  = 242.8 mg p.m./L

7 d  $E_yC_{20}$  = 360.6 mg p.m./L

7 d  $E_yC_{50}$  = 768.6 mg p.m./L

NOE<sub>y</sub>C = 600 mg p.m./L

All results based on the nominal concentration.

**B.9.2.7.1.11. Lemna gibba G3 – growth inhibition test with BCS-CU62474****(potassium salt of trifluoroethanesulfonic acid, metabolite of flufenacet) under static conditions.**


---

<b>Reference:</b>	Lemna gibba G3 – growth inhibition test with BCS-CU62474 (potassium salt of trifluoroethanesulfonic acid, metabolite of flufenacet) under static conditions.
<b>Author(s), year:</b>	Weyers A., 2013
<b>Report/Doc. number:</b>	Study No: E412 4413-9, Reference BCS No: M-445884-01-1
<b>Guideline(s):</b>	OECD Guideline 221 (March 23, 2006)
<b>GLPP:</b>	Yes

---

Material and methods:

Test substance:	Potassium salt of trifluoroethanesulfonic acid (BCS-CU62474), Batch No.: BCS CU62474-01-01; Purity: 94.7 %
Test species:	Lemna gibba G3
Source:	Horticulture Crops Quality Laboratory, U.S. Department of Agriculture, Beltsville, MD 20705, U.S.A.
Growth medium:	20 x AAP nutrient medium, according to OECD guideline
Growth chamber:	Glass dishes were used with a diameter of 10 cm and a height of 6 cm (total volume of approx. 470 mL). These test vessels were covered with lids of glass to permit gas exchange and illumination under sterile conditions to the greatest possible extent.
Inoculum:	To ensure that the plants used as inoculum were exponentially growing, an inoculum pre-culture was prepared –10 days before the start of the test and were cultivated under the same conditions as in the main test.
Number of organism:	6 replicate vessels per test level and 6 replicate vessels per control. 12 fronds per vessel
Type of test, duration:	Static, 7 days
Applied concentrations:	
Nominal	0 (control), 10.0 mg metabolite/L
<u>Test conditions:</u>	
Water quality:	20 x AAP medium according to the guideline OECD with pH= 7.5±0.1. After adding test item to the beakers, within less than three hours the pH had risen to pH 7.9 to 8.0 on day 0 in control and treatment.
Temperature:	26.6°C to 24.8°C,

---

pH:	8 -9.1
Light regime:	Continuous light. Nominally 6,500-10,000 Lux ( $\pm$ 15% variation from the mean).
<u>Test parameters:</u>	<p>The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature was recorded hourly.</p> <p>The pH was measured in all freshly prepared and all aged test levels and the control.</p>
Biomass quantification:.	Counting of fronds and determination of total frond area was carried out using the LemnaTec Scanalyzer machine, validated for such measurements.
Analytical data:	<p>Samples were analysed for the actual concentration of flufenacet present in all freshly prepared test levels on day 0 and in all aged test levels on day 7 of the exposure period. Aliquots for freshly prepared test levels for day 0 analyses were sampled from the prepared volume of each test treatment level. For sampling of aged test media, and after removing of plant material from the test vessels on day 7 the contents of all replicate vessels were combined and the pH was measured. The combined test solutions were then submitted for HPLC-UV analysis.</p>
Observation:	<p>Visual observations were made on study days 2, 5, and 7.</p> <p>Plant frond numbers and total frond area of plants were determined on days for this study.</p>
Statistics:	<p>The EC<sub>50</sub> values (yield and growth rate) and their 95% confidence limits were calculated by Probit analyses. For the determination of the NOEC values significant difference at the test concentrations compared to the control values were tested by the Williams Multiple Sequential t-test.</p> <p>The software used to perform the statistical analyses was ToxRat Professional.</p>
Analytical data:	<p>Samples were analysed for the actual concentration of BCS-CU62474 -present in all freshly prepared test levels on day 0 and in all aged test levels on day 7 of the exposure period. Aliquots for freshly prepared test levels for day 0 analyses were sampled from the prepared volume of each test treatment level. For sampling of aged test media, and after removing of plant material from the test vessels on day 7 the contents of all replicate vessels were combined, and the pH was measured. The combined test solutions were then submitted for HPLC-UV analysis.</p>

Findings:Analytical measurements:

The analytical finding of BCS-CU62474 found on day 0 was 88 % of nominal and 104 % of nominal on day 7. All results are based on nominal values 10 mg p.m./L of the test item. Data on final frond number, final total frond area and growth rate based on final number and total frond area are summarized in the tables that follow.

**Table B.9.2.7.1.11-1: Final fronds number and final total frond area and % inhibition of their average growth rate.**

Nominal concentration (mg p.m. /L)	Frond number			Total frond area		
	Final frond Number 7d	Average growth rate of frond number	% inhibition of average growth rate of fronds number	Final total frond area 7d	Average growth rate	% inhibition of average growth rate of total frond area
				Mean (mm <sup>2</sup> )		
control	229.8	0.422	-	1571.3	0.413	
10	265.2	0.441	-4.6	1838	0.408	1.3

Observed visual effect:

No visual signs of toxicity caused by the test item.

Conclusion:

Growth inhibition values based on nominal concentration with BCS-CU62474 on Lemna gibba G3 were as follows:

0-7 d Day Frond Number growth rate  $E_rC_{50} > 10 \text{ mg p.m./L}$   
 $NOE_rC \geq 10 \text{ mg p.m./L}$

0-7 d Day Frond Number Yield  $E_yC_{50} > 10 \text{ mg p.m./L}$   
 $NOE_yC \geq 10 \text{ mg p.m./L}$

0-7 d Total Frond Area growth rate  $E_rC_{50} > 10 \text{ mg p.m./L}$   
 $NOE_rC \geq 10 \text{ mg p.m./L}$

0-7 d Day Total Frond area Yield  $E_yC_{50} > 10 \text{ mg p.m./L}$   
 $NOE_yC \geq 10 \text{ mg p.m./L}$

**Comments RMS:**

The study fulfils the validity criteria given in the current test guideline OECD 221 (2006).

The doubling time of the frond number in the control was less than 2.5 days (being 1.6 day) corresponding to approx. a seven-fold increase in seven days and an average growth rate of 0.275 per day.

The mean growth rate in the control was determined to be 0.442 after 7 days.

The factor of frond number, measured in the control between 0 and 7 days, was 19.2.

The following deviation from the test guideline was noted:

- pH value at test start was slightly higher than is stated in the guideline OECD 221 (2006)

The RMS is of the opinion that the reliability of the results is given.

**Agreed endpoints:**

0-7 d	Day Frond Count growth rate	$E_rC_{50} > 10 \text{ mg p.m./L}$ $NOE_rC \geq 10 \text{ mg p.m./L}$
-------	-----------------------------	--

0-7 d	Total Frond Area growth rate	$E_rC_{50} > 10 \text{ mg p.m./L}$ $NOE_rC \geq 10 \text{ mg p.m./L}$
-------	------------------------------	--

0-7 d	Day Frond Count Yield	$E_yC_{50} > 10 \text{ mg p.m./L}$ $NOE_yC \geq 10 \text{ mg p.m./L}$
-------	-----------------------	--

0-7 d	Day Total Frond area Yield	$E_yC_{50} > 10 \text{ mg p.m./L}$ $NOE_yC \geq 10 \text{ mg p.m./L}$
-------	----------------------------	--

All results based on the nominal test concentration.

**B.9.2.7.1.12. Lemna gibba G3 - Growth inhibition test with Flufenacet-thiadone under static conditions.**

<b>Reference:</b>	Lemna gibba G3 - Growth inhibition test with Flufenacet-thiadone under static conditions.
<b>Author(s), year:</b>	Bruns, E.; 2010
<b>Report/Doc. number:</b>	Study No: E 412 4413-9, Report No: EBFOL144 Reference BCS No: M-393718-01-3
<b>Guideline(s),</b>	OECD Guideline 221, (March 23, 2006)
<b>GLP:</b>	Yes

It should be noted that the new statistiactal analysis of the raw data was performed by the Applicant specially for the purpose of the current evaluation assessment and on request by the RMS. That was done in order to get endpoints compliant with the current requirement (EC<sub>10</sub>, EC<sub>20</sub>, EC<sub>50</sub> NOEC for growth rate and yield).

Material and methods:

Test substance:	Flufenacet-thiadone (AE 1258593), Batch No.: SES 10558-3-5, Tox No.: 09021-00, Purity: 98.6 % w/w (analysed)
Test species:	Lemna gibba G3
Source:	Horticulture Crops Quality Laboratory, U.S. Department of Agriculture, Beltsville, MD 20705, U.S.A.
Growth medium:	20 x AAP nutrient medium according to OECD test guideline
Growth chamber:	Glass dishes were used with a diameter of 10 cm and a height of 6 cm (total volume of approx. 470 mL). These test vessels were covered with lids of glass to permit gas exchange and illumination under sterile conditions to the greatest possible extent.
Inoculum:	To ensure that the plants used as inoculum were exponentially growing, an inoculum pre-culture was prepared 7 – 10 days before the start of the test and were cultivated under the same conditions as in the main test.
Number of organism:	Three replicate vessels per test level and 3 replicate vessels per control. 12 fronds per replicate
Type of test, duration:	Static, 7 days

Applied concentrations:

Nominal: 0 (control), 1.25, 2.50, 5.00, 10.0, 20.0, 40.0 and 80.0 mg p.m./L

Test conditions:

Water quality: 20 x AAP nutrient medium, according to the OECD test guideline with pH= 7.5± 0.1

Temperature: 23.7° to 26.0°C

pH: 7.4-8.8

Light regime: Continuous light. 7960-8820 Lux (mean 8390 Lux )

Test parameters:

The temperature was determined by a continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature was recorded hourly. The pH was measured on the day 0 and 7 in all test level and the control.

Biomass quantification: Counting of fronds and determination of total frond area was carried out using the LemnaTec Scanalyzer machine, validated for such measurements.

Analytical data: Samples were analysed for the actual concentration of present in all freshly prepared test levels on day 0 and in all aged test levels on day 7 of the exposure period. Aliquots for freshly prepared test levels for day 0 analyses were sampled from the prepared volume of each test treatment level. For sampling of aged test media, and after removing of plant material from the test vessels on day 7 the contents of all replicate vessels were combined, and the pH was measured. The combined test solutions were then submitted for HPLC-UV analysis.

Observation: Visual observations were made on study days 2, 5, and 7.  
Plant frond numbers and total frond area of plants were determined on days for this study.

Statistics: The EC<sub>50</sub> values (yield and growth rate) and their 95% confidence limits were calculated by Probit analyses. For the determination of the NOEC values significant difference at the test concentrations compared to the control values were tested by the Williams Multiple Sequential t-test.

The software used to perform the statistical analyses was ToxRat Professional.

## Analytical data:

Samples were analysed for the actual concentration of present in all freshly prepared test levels on day 0 and in all aged test levels on day 7 of the exposure period. Aliquots for freshly prepared test levels for day 0 analyses were sampled from the prepared volume of each test treatment level. For sampling of aged test media, and after removing of plant material from the test vessels on day 7 the contents of all replicate vessels were combined, and the pH was measured. The combined test solutions were then submitted for HPLC-UV analysis.

Findings:

## Analytical measurements:

The analytical findings of determined in all test levels on day 0 ranged between 100 and 104 %, on day 7 the analysed concentrations ranged between 104 and 107 % (average 105 %) of nominal concentrations. Therefore, all results are based on nominal values of the test item.

Data on final frond number, final total frond area and growth rate based on final number and total frond area are summarized in the tables that follow.

**Table B.9.2.7.1.12-1. Final fronds number and final total frond area and % inhibition of their average growth rate.**

Nominal Concentration [mg p.m. /L]	Frond number			Total frond area		
	Final frond number	Average growth rate	% inhibition of average growth rate of fronds number	Final total frond area	Average growth rate	% inhibition of average growth rate of total frond area
				Mean [mm <sup>2</sup> ]		
control	131	0.342	-	873	0.340	-
1.25	111	0.318**	6.87	756	0.333	1.90
2.50	115	0.322**	5.77	777	0.320	5.81
5.00	110	0.293*	14.2	613	0.302	11.1
10.00	94	0.274**	19.7	480	0.272	20.0*
20.00	82	0.192**	43.8	233	0.165	51.4*
40.00.	46	0.08**	76.6	106	0.04	88.2*
80	21	0.0**	100	76	0.006	120.9*

\*Statistically significant compared to control (one side smaller, Williams Multiple Sequential t-test Procedure  $\alpha=0.05$ )

\*\* Statistically significant compared to control (Welch-t test for Inhomogeneous Variances with Bonferroni Adjustment,  $\alpha=0.05$ )

**Table B.9.2.7.1.12-2: Mean yield (based on fronds number and total frond area).**

Nominal Concentration (µg met/L)	Mean yield			
	Frond number		Total frond area	
	After 7 day	% inhibition relative to the control	After 7 days	% inhibition relative to the control
control	119.3	-	791.7	-
1.25	99.3	16.8*	682.7	13.8*
2.50	102.7	14.0*	694.7	12.3*
5.00	82.3	31.0*	539.0	31.9*
10.00	70.00	41.3*	408.7	48.4*
20.00	34.0	71.5*	159.7	79.8*
40.00	9.0	92.5*	25.7	96.8*
80	0.0	100*	17.0	102.1*

\* Statistically significant difference from control, (Williams t-test,  $\alpha = 0.05$ , one-sided.)

Observed visual effect:

No visual signs of toxicity caused by the test item.

#### Conclusion:

Growth inhibition values based on nominal concentration with on Lemna gibba G3 were as follows:

0-7 d Frond number growth rate:	ErC <sub>50</sub> = 20. 80 mg p.m./L ( 95% CI:18.46-23.44) ErC <sub>20</sub> =9.34 mg p.m./L (95% CI:7.76-11.32) ErC <sub>10</sub> =6.44 mg p.m/L (95% CI: 4.80-7.95) NOE <sub>r</sub> C < 1.25 mg p.m./L
0-7 d Total frond area growth rate:	ErC <sub>50</sub> =18.32 mg p.m./L (95% CI: 16.96-19.78) ErC <sub>20</sub> = 9.60mg p.m./L (95% CI: 8.40-10.68) ErC <sub>10</sub> = 6.84 mg p.m./L (95% CI: 65.71-7.88) NOE <sub>r</sub> C=5 mg p.m./L
0-7 Yield Frond number	EyC <sub>50</sub> = 9.86 mg p.m./L (95%CI:8.15-11.93 ) EyC <sub>20</sub> =3.39 mg p.m/L (95% CI: 2.37-4.38) EyC <sub>10</sub> =1.94 mg p.m/L (95% CI: 1.19-2.71) NOE <sub>y</sub> C<1.25mg p.m./L
0-7 - Yield Total frond area	EyC <sub>50</sub> =8.68 mg p.m./L (95% CI: 7.46-10.09)

$EyC_{20} = 3.42 \text{ mg p.m./L}$  (95% CI: 2.59-4.19)

$EyC_{10} = 2.10 \text{ mg p.m./L}$  (95% CI: 1.44-2.74)

$NOE_yC < 1.25 \text{ mg p met/L}$

#### **RMS comments :**

The study was conducted according to the current OECD 221 (2006) test guideline and the study fulfils the all validity criteria stated in that guideline.

The doubling time of the frond number in the control was less than 2.5 days (being 2.0 day) corresponding to approx. a seven-fold increase in seven days and an average specific growth rate of 0.275 per day.

The mean growth rate in the control was determined to be 0.342 after 7 days.

The factor of frond number, measured in the control between 0 and 7 days, was 10.9.

The RMS is of the opinion that the reliability of the results is given.

Therefore, the study was considered acceptable.

#### **Agreed endpoints:**

0-7 d Frond number growth rate:	$ErC_{50} = 20.80 \text{ mg p.m./L}$ (95% CI:18.46-23.44)
	$ErC_{20} = 9.34 \text{ mg p.m./L}$ (95% CI:7.76-11.32)
	$ErC_{10} = 6.44 \text{ mg p.m./L}$ (95% CI: 4.80-7.95)
	$NOE_rC < 1.25 \text{ mg p.m./L}$
0-7 d Total frond area growth rate:	$ErC_{50} = 18.32 \text{ mg p.m./L}$ (95% CI: 16.96-19.78)
	$ErC_{20} = 9.60 \text{ mg met/L}$ (95% CI: 8.40-10.68)
	$ErC_{10} = 6.84 \text{ mg met/L}$ (95% CI:65.71-7.88)
	$NOE_rC = 5 \text{ mg p.m./L}$
0-7 Yield Frond number	$EyC_{50} = 9.86 \text{ mg p.m./L}$ (95% CI:8.15-11.93 )
	$EyC_{20} = 3.39 \text{ mg p.m./L}$ (95% CI:2.37-4.38)
	$EyC_{10} = 1.94 \text{ mg p.m./L}$ (95% CI:1.19-2.71)
	$NOE_yC < 1.25 \text{ mg p.m./L}$
0-7 - Yiel Total frond area	$EyC_{50} = 8.68 \text{ mg p.m./L}$ (95% CI: 7.46-10.09)
	$EyC_{20} = 3.42 \text{ mg p.m./L}$ (95% CI: 2.59-4.19)
	$EyC_{10} = 2.10 \text{ mg p.m./L}$ (95% CI: 1.44-2.74)
	$NOE_yC < 1.25 \text{ mg met/L}$
All results based on the mean measured concentration	

**B.9.2.8. Further testing on aquatic organism**

Reference:	The fate and biological effects of Flufenacet WG 60 in aquatic indoor organism.
Author(s), year:	Foekema E.M., Jak R.G, 1999
Report/Doc. number:	Report No TNO-MEP, Reference no. M 329959 01-1
Guideline(s):	OECD Guidance Document „Freshwater Lentic Field Tests“ (July 1996, Draft) and the Guidance Document on Testing Procedures for Pesticides in Freshwater Mesocosm (SETAC-Europe Workshop, Monks Wood, UK, (July 1991).
<b>GLP:</b>	Yes

The results of the existing laboratory studies revealed that green algae and aquatic macrophytes are the most sensitive species regarding possible adverse effects of Flufenacet.

Applicant provided the re-evaluation of microcosm study based on the recommendations of de Jong et al. 2008 to demonstrate the suitability of the indoor microcosm study by Foekema & Jak (1999) as being higher tier for the risk assessment of Flufenacet with a special focus especially on algae and aquatic macrophytes. RMS provided an independent re-evaluation based on the original study Foekema & Jak (1999) submitted already during peer review of approval of active substance.

The summary of the evaluation is presented below:

#### 1. Study design

The microcosm study “The fate and biological effects of Flufenacet WG 60 in aquatic indoor microcosms” was based on the OECD Guidance Document „Freshwater Lentic Field Tests“ (July 1996, Draft) and the Guidance Document on Testing Procedures for Pesticides in Freshwater Mesocosm (SETAC-Europe Workshop, Monks Wood, UK, (July 1991).

#### 2. Experimental design

The indoor microcosm test was performed to investigate the effect of a concentration series of flufenacet FOE 5043 WG 60 (Batch-No. 04317/0405/0380, TOX-No. 5020-00; expiration date 22 October 1999, purity: 61.5 %) on an aquatic biocoenosis. The test substance was applied just under the water surface as a stock solution in water.

The concentration series was: 0.75 µg a.s./L, 1.5 µg a.s./L, 3 µg a.s./L, 6 µg a.s./L, 12 µg a.s./L and 24 µg a.s./L. All test concentrations were duplicated, with the exception of the highest one, which was not replicated. Untreated reference systems were triplicated. The microcosm were sited in an experimental room at TNO laboratory in Netherlands.

The study started on 20 May 1999 and final to 12 August 1999. The test period was 84 days.

Each microcosm consisted of polyethylene container with diameter of approximately 100 cm and height of 80 cm. Each container was illuminated for 14 hours per day with 7.3+10.klx (7300-10000 lux). Each fourteen of this containers were used in this study. In order to simulate natural mixing regime, the water column was gently aerted throughout the study period. The microcosm contained a 10 cm deep layer of sediment (silty sand, 1.3% organic content) covered by 50 cm water column. Four weeks before application of the test substance the microcosm was filled with natural sediment and water.

Effects on phytoplankton (species composition and abundance, chlorophyll-a), periphyton (chlorophyll-a), zooplankton (species composition and abundance), % cover of macrophytes, general water quality parameters (P-PO<sub>4</sub>, N-NH<sub>4</sub>, N-(NO<sub>3</sub>+NO<sub>2</sub>), and community metabolism parameters (temperature, pH, dissolved oxygen (DO) and electrical conductivity (EC<sub>25</sub>) were evaluated during the study.

Sampling was performed according to the schedule presented in the Table B.9.2.8-1.

**Table B.9.2.8-1. Sampling in the microcosms.**

Day	Water	Macrophytes	Lemna	Periphyton	Plankton	Macrofauna	Chemical analysis
0	w1, w2	Me			Ch, sc		
-21	w1	Me			Ch		
-14	w1, w2	Me			Ch, sc		
-7	w1	Me			Ch		
0	w1, w2	Me, Mb	Lb	Pb	Ch, sc		Ca
7	w1	Me			Ch		Ca
14	w1, w2	Me, Mb	Lb		Ch, sc		Ca
21	w1	Me			Ch		Ca
28	w1, w2	Me, Mb	Lb	Pb	Ch, sc		Ca
28-29	ox						
35	w1	Me			Ch		
42	w1, w2	Me, Mb	Lb	Pb	Ch, sc		Ca
49	w1	Me			Ch		
56	w1, w2	Me	Lb	Pb	Ch, sc		
56-57	ox						
63	w1	Me			Ch		
70	w1, w2	Me	Lb	Pb	Ch, sc		
77	w1	Me			Ch		
83-84	ox						
84	w1, w2	Me, Mb	Lb	Pb	Ch, sc		Ca
85						Mf	

w1 In situ measurement of water temperature, oxygen concentration, pH and conductivity

w2 Samples collected for determination of nutrient concentrations (PO<sub>4</sub>, NO<sub>3</sub>, NH<sub>4</sub>)

ox Diurnal oxygen concentration cycle

Me Macrophyte coverage

Mb Macrophyte biomass sampling

Lb Determination of effect on Lemna

Pb Determination of periphyton biomass (chlorophyll-a)

Ch Determination of chlorophyll-a concentration in water

Sc Samples collected for composition of phytoplankton and zooplankton

Ca Samples collected for chemical analyses of test substance

Mf Macrofauna sampling

Methods of sampling:*Phytoplankton*

Phytoplankton biomass was expressed as chlorophyll-a concentration and was determined weekly. The chlorophyll development was measured by means of a BBE-Moldaenke 1-Hz-Kuvetten-Fluorimeter, in a water sample that was taken on a depth of ca. 30 cm, less than 30 minutes prior to the analysis. The phytoplankton species composition was determined every other week in a water sample of approximately 80 ml collected from the centre of every test container at a depth of approximately 30 cm. Almost immediately after collection, the sample was conserved with 1 ml Lugol-solution and stored in the dark at room temperature. Identification and counting of the species were achieved by using a microscope with a 400 x magnification and an Utermohl counting-cuvet, containing a sub-sample of 5 ml.

*Zooplankton*

Zooplankton was sampled on a two weekly basis by using a PVC core provided with a ball-valve at the end. In each microcosm 10 vertical cores were sampled (2\*centre, 2\*North, 2\*East, 2\*South, 2\*West) at a depth of 0 - 41.5 cm with a total water volume of 2litre (10\* 0.2 L). The collected water was flushed through a 55 µm plankton net and the zooplankton captured was conserved with formaldehyde. Sub-samples were counted and determined in duplicate using a microscope with 100 x magnification.

*Periphyton*

The development of the periphyton was followed by determining the biomass (as chlorophyll-a) on glass slides (76 x 26 mm) that served as substrate, incubated in the microcosms. Three glass slides were placed in a perspex holder that was placed vertically 10 cm below the water surface at the side of the microcosm tank.

With each sampling event the slides were removed for analysis, and replaced by new ones. Before introduction into the microcosms the glass slides had been cleaned with acetone and demineralised water. The incubated glass slides were collected separately in small glass jars. These jars were then shaken until the glass slide broke into such small pieces that all would be covered by the 10 ml ethanol solution (80%) that was added to the jar afterwards. The sample was then placed for 1 minute in an ultra-sonic bath and stored for at least 12 hours in a refrigerator, to allow extraction of the chlorophyll. The sample was placed in a water bath of 60-75°C for 5 minutes, cooled down and centrifuged. The absorbance of the supernatant was then measured spectrophotometrically at wavelengths of 750, 663 and 435 nm. Measurements at 750 and 663 nm were repeated after the samples had been acidified. From the results of this analysis chlorophyll-a concentrations were calculated.

The study protocol stipulated an incubation period of 2 weeks. However, during the first sampling on day 0 no substantial periphyton development was observed. Hence, the incubation period was extended to 4 weeks. To achieve this the periphyton substrates were not sampled at day 14 but new additional substrates were placed in each

microcosm. From day 28 onwards substrates were sampled every two weeks and replaced by new ones. Each substrate had then been incubated for 4 weeks.

*Macrophates:*

*Lemna gibba*

The duckweed (*Lemna gibba*) that was used for the study was collected one week before introduction into the microcosms from a local ditch in an area free of industrial or agricultural activities. After collection it was placed in a tank with water from lake and illuminated with the same light/dark conditions as used in the actual study. Duckweed was introduced two weeks before the application of the test compound. The 15-20 fronts were placed in a floating ring with a diameter of 15 cm that was fixed just out of the centre of each microcosm. Every two weeks, the fronts were counted, and the density was reduced to the initial number. The removed fronts were dried for at least 24 hours at approximately 100°C and used for the determination of the mean dry weight per front.

*Potamogeton pectinatus*

*Potamogeton pectinatus* was collected from the same location as the duckweed. Directly after collection the plants were sorted and healthy plants selected. Stems were cut to a length between 15 and 20 cm. Four of these stems were placed together in a polyethylene ring (diameter 5 mm, height 3 mm) that was placed around the base.

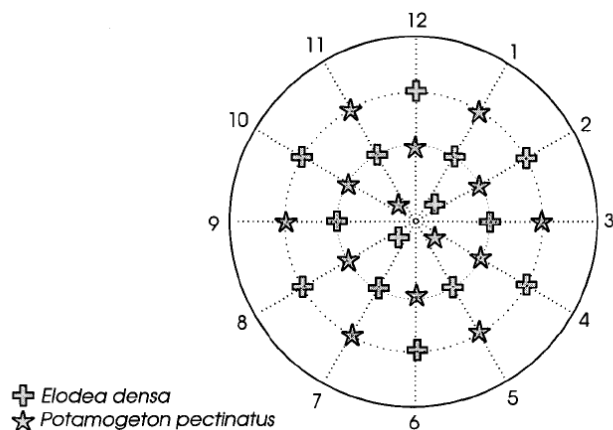
The groups of plants were then planted in the microcosm sediment in two rings Figure 9.2.8-1.

On day 0 the two groups of *P. pectinatus* that had been planted in the centre of the microcosms were sampled. On days 14, 28, 42 and 84 two other groups of *P. pectinatus* were sampled from each microcosm: on each sampling date, one group of plants was collected from the outside and one from the inside ring. The groups to be sampled from each ring were chosen, using a computer generated random number table. After collection, sediment was washed off the plants with tap water and attached animals were removed. The plants were then dried for at least 24 hours at ca. 100°C after which the dry weight of each group was determined. The plants that remained after the final sampling on day 84 were collected together and treated as one group following the procedure described above.

*Elodea densa*

*Elodea densa* was acquired from a commercial distributor of aquatic plants. The plants were delivered as 30 cm long stems one day before they were planted in the microcosms. The material was stored overnight at temperatures between 4 and 7°C. Before introduction in the microcosms the shoots were removed and stems were cut to a length of 15 cm. Four of these stems were bound together with a small 'tie-wrap' and the groups were planted.

Sampling and handling procedures were similar as for *P. pectinatus*, with this exception that the groups remaining after the sampling on day 84 were treated as individual groups.



**Figure 9.2.8-1. Plankton position of macrophytes ( *Elodea* and *Potamogeton pectinatus*) in the microcosm.**

### Macrofauna

Macrofauna was sampled from the microcosms at the end of the test. The water of each microcosm was pumped through a 1 mm sieve and the remaining material, including the macrofauna, was collected and preserved in formaldehyde. Once the water had been removed, the internal side of the microcosm walls was scraped with a rubber blade, causing the macrofaunal organisms that were attached to the walls to fall to the sediment surface. Finally the top layer (approximately 2 cm depth) of the sediment was collected. This sediment was washed through a 1 mm sieve and the remaining material was preserved with formaldehyde. A dye (1% solution of 'rose bengal B'), which gives organic material a bright colour was added later. At least 24 hours after the addition of the dye to the samples, the macrofauna was collected. To do this the samples were washed through 3 sieves of 10, 5 and 1 mm respectively. Each sieve residue was completely searched by eye for macrofauna that was determined to the genus level. For the interpretation of the data, all the macrofauna collected from one microcosm was pooled together.

### 3. Representation of a realistic freshwater community

The microcosm was designed to investigate a representative aquatic biocenosis. In this study the aquatic biocenosis were represented by: phytoplankton (3 dominated groups and the other less abundant), zooplankton (8 species), periphyton (expressed as chlorophyll -a), aquatic macrophytes (3 species) and macrofauna.

The microcosm consisted of natural sediment and water was the natural source of zooplankton and phytoplankton organism. *Lemna gibba* and periphyton substrate was introduced two weeks before application and *Patamogena pectinatus* and *Elodea densa* were introduced in the test system four weeks before application.

Parameters of used water was presented in the below:

**Table B.9.2.8-2. Physical parameters of microcosm water.**

<b>Physical characteristics</b>	
Water temperature	13.2 °C
Oxygen concentration	10.4 mg/l
pH	8.29
Conductivity	788 µs/cm
<b>Nutrients</b>	
Ammonium-N	0.03 mg/l
Nitrite-N	0.015 mg/l
Nitrate-N	1.9 mg/l
Phosphate-P	0.03 mg/l
Silicate	0.17 mg/l
<b>Phytoplankton</b>	
Flagelates 3-10 µm	896 cells/ml
Flagelates < 3 µm	480 cells/ml
<i>Crucigenia</i> sp.	1792 cells/ml
<i>Scenedesmus</i> sp.	96 cells/ml
Tintinids	32 cells/ml
<b>Zooplankton</b>	
<i>Bosmina</i> sp.	163 ind/l
<i>Daphnia longispina</i>	1 ind/l
Copepods (exc. nauplii)	15 ind/l
Copepod nauplii	15 ind/l
Rotifers	64 ind/l

#### 4. Exposure regime

The test substance was applied just under the water surface as a stock solution in water. The nominal concentration series was: 0.75 µg a.s./L, 1.5 µg a.s./L, 3 µg a.s./L, 6 µg a.s./L, 12 µg a.s./L and 24 µg a.s./L. All test concentrations were duplicated, with the exception of the highest one, which was not replicated. Control systems were triplicated. Four hours after the application of the test substance, water samples were taken from each microcosm for the analysis of the actual concentration of the active ingredient. The results of these analyses showed actual concentrations that were between 120 and 150% of the nominal concentration. This relatively high measured initial concentration is most probably the result of an incomplete mixing of the test substance with the total water column of the microcosm at the moment that the sample was taken. At the next sampling moment on day 7 the actual test substance ranged between 94 and 113% (mean: 106%).

The test period was 84 days. Within the microcosm study the fate of active substance was investigated.

The DT<sub>50</sub> of the test item under exposure condition was calculated based on analytical measurements during the study. The DT<sub>50</sub> of active substance was estimated to be 18.8 days.

## 5: Statistical evaluation of the observed effects

Effects on phytoplankton (species composition and abundance, chlorophyll-a), periphyton (chlorophyll-a), zooplankton (species composition and abundance), general water quality parameters (P-PO<sub>4</sub>, N-NH<sub>4</sub>, N-(NO<sub>3</sub>+NO<sub>2</sub>), and community metabolism parameters (temperature, pH, dissolved oxygen (DO) and electrical conductivity (EC<sub>25</sub>) were recorded.

One way analysis of variance (ANOVA) was used to determine if observed differences between endpoints were significant. The datasets of day 14 and 28 were used to perform multivariate analysis (Redundancy Analysis) to determine the presence of a direct effect of the application of the test substance. The significance of the observed correlations was tested using Monte Carlo simulation.

The RDA-biplot analysis was made on day 14 and 28 for the following tested groups: *Lemna gibba* growth rate, Flagellates—all groups (n/mL), *Oscillatoria* sp. (n/mL), *Microcystis* sp n/ml, chlorophyll-a (ug/L), *Elodea* weight per group, Patogenon weight per group, *Ceriodaphnia*(n/L), *Bosomia* (n/l), *Chydoridis* (n/L), Copepod adults (n/L), Copepod napuli (n/l), Rotifer (n/L) and Periphyton (ug chlorophyll-a/cm<sup>2</sup>).

*Daphnia magna* and *Daphnia longispina* were excluded from analysis due to absence of that days and periphyton on day 14. The data of day 14, 28, 42 and 84 are also analyzed together using all parameters. The observed correlation was tested with Monte Carlo simulation.

The summary of the biological parameters obtained in the study is presented below.

### Phytoplankton:

The following groups of alga and species were investigated in this study: Flagellates > 10 µm, Flagellates 3-10 µm, Flagellate <3µm, *Oscillatoria* sp., *Microcystis* sp. Figure 9.2.8-2. The other species were only observed in relatively low numbers. The green algae *Scenedesmus* sp. was the most dominant of less abundant taxa but these groups did not show any relation with the treatment.

There were indicated also groups of algae as *Peridinium* sp., *Anabaena* sp., *Malosina* sp., that only occurred into treated concentration but not in the controls.

The composition of phytoplankton community showed large inter-microcosm variance. Initially the community was dominated by flagellates >3 µm. From Day 0 onwards the flagellate density started to decline in most of the microcosm regardless of treatment. Especially smaller flagellates <3 µm remained in substantial numbers through to the test period. Throughout the test period the phytoplankton community was dominated by flagellates and the bluegreen algae *Microcystis* sp. with short period of dominance between days 14 and 42. On 14 day *Oscillatoria* sp. start to developed in 9 of 14 microcosms. With respect dominance of bluegreen algae *Microcystis* sp., no relation with treatment was observed.

Development of Flagellate community, blue green algae (*Microcystis* and *Oscillatoria*), green algae (*Scenedesmus*

subcapitatus) in the microcosmos and the average densities in the replicated test system in presented in the Figure 9.2.8-2 below:

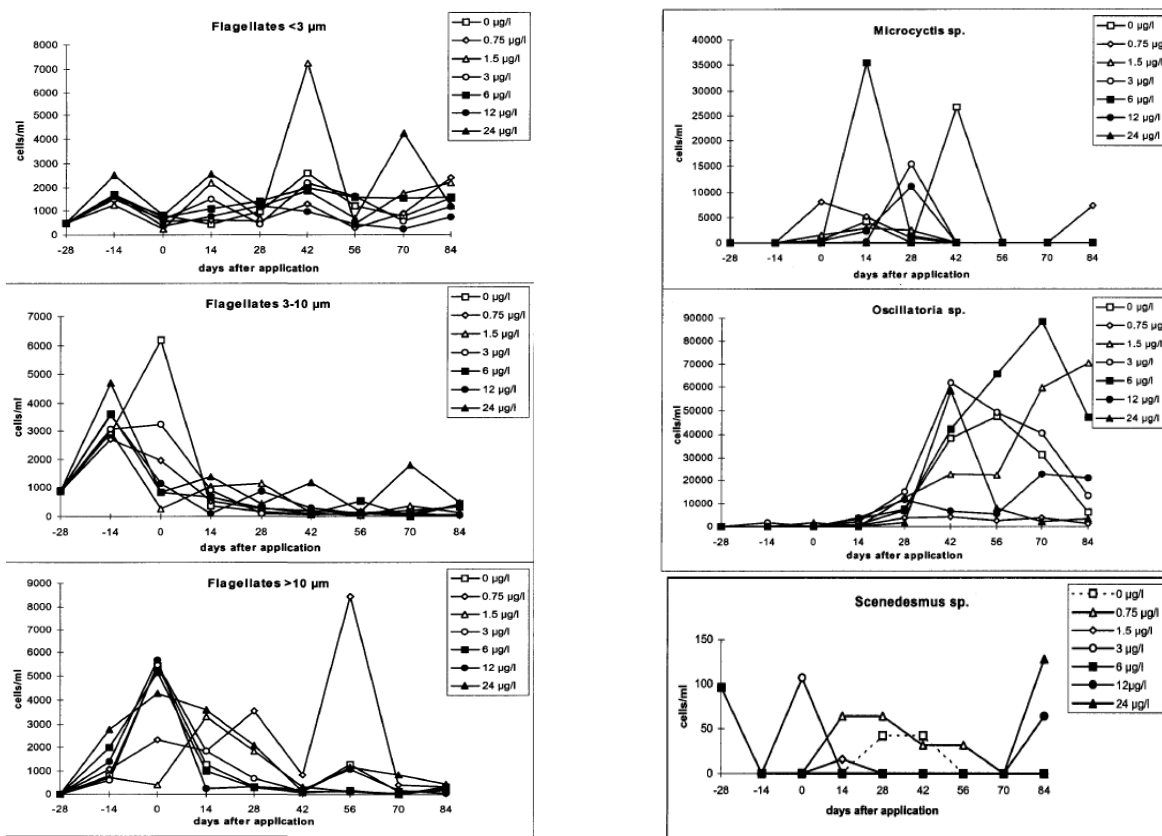


Figure 9.2.8-2. Development of Flagellate community, blue green algae (*Microcystis* and *Oscillatoria*), green algae (*Scenedesmus subcapitatus*) in the microcosmos ( average densities in the replicated test system).

**Table B. 9.2.8-3. Average composition of the phytoplankton community of the microcosms, expressed as the average number of cells/ ml during the whole test period (day 0-day 84).**

Microcosm code M	M2	M6	M11	M4	M8	M3	M7	M1	M9	M10	M14	M5	M13	M12
Nominal concentration	0 µg/L	0 µg /L	0 µg /L	0.75 µg/L	0.75 µg/L	1.5 µg L	1.5 µg /L	3 µg /L	3 µg /L	6 µg /L	6 µg L	12 µg /L	12 µg/L	24 µg/L
Flagellate > 10 µm	923	1410	1421	1518	3717	494	1301	1355	1093	379	1612	1045	1173	1786
Flagellate 3-10 µm	878	1196	1123	745	297	489	409	722	592	270	564	394	375	897
Flagellate < 3 µm	678	1777	1158	818	1088	1605	2606	853	1456	1230	1592	774	574	1792
<i>Oscillatoria</i> sp	13289	480	42989	1143	3456	0	53531	7794	44064	71918	521	5888	14606	10707
<i>Microcystis</i> sp.	11579	1874	0	4326	1760	421	1570	4466	0	2560	7954	3779	101	0
<i>Cryptomonas</i> sp	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Scenedesmus</i> sp	18	0	18	18	27	5	0	35	0	0	0	18	0	18
<i>Merismopedia</i> sp.	0	0	0	0	0	0	0	0	0	0	0	128	0	0
<i>Navicula</i> sp	9	0	27	5	9	0	14	0	0	9	5	5	9	0
<i>Peridinium</i> sp.	0	0	0	5	0	0	9	0	126	5	15	0	17	10
<i>Crucigenia</i> tetrapedia	0	0	274	0	0	0	0	0	0	0	0	0	0	0
<i>Dactylococcopsis</i> fadcularis	0	0	0	0	0	0	0	0	0	0	0	0	0	55
<i>Anabaena</i> sp.	0	0	0	0	0	0	0	0	0	27	0	59	0	18
<i>Melosira</i> sp	9	0	0	14	0	5	0	5	9	5	0	0	5	0
<i>Tetrastrum</i> sp.	0	37	0	0	0	0	0	0	0	0	0	0	0	0
<i>Frugiiaria</i> sp	0	0	0	0	5	5	0	0	0	0	0	0	0	9
Amoebe	0	0	0	0	0	0	0	0	0	0	0	6	0	0
<i>Amphora</i> ovalis	0	0	0	0	0	0	0	0	0	5	0	0	0	0
<i>Brachionus</i> sp.	5	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Chlorococcum</i> sp	0	0	0	0	0	0	0	5	0	0	0	0	0	0
<i>Diatoma</i> elongatum	0	0	5	0	0	0	0	0	0	0	0	0	0	0
<i>Nitschia</i> sp.	0	0	0	0	0	0	0	5	0	0	0	5	0	0
<i>Synedra</i> acus.	0	0	0	0	0	0	0	0	0	0	0	0	0	0
unknown green	0	0	0	0	0	0	0	0	0	0	0	4210	0	0

The algal biomass, expressed as the chlorophyll-a concentration in the water, reached its peak during the pre-test period and started to decline just about the time that the test substance was applied. This decline was observed in all microcosms, independent of treatment Figure 9.2.8-3. During the test period the algal biomass fluctuated between 5 and 15  $\mu\text{g/L}$  chlorophyll-a. There is no significant dose-dependency. However, it should be noted that there was high variability between replicates in tested microcosms.

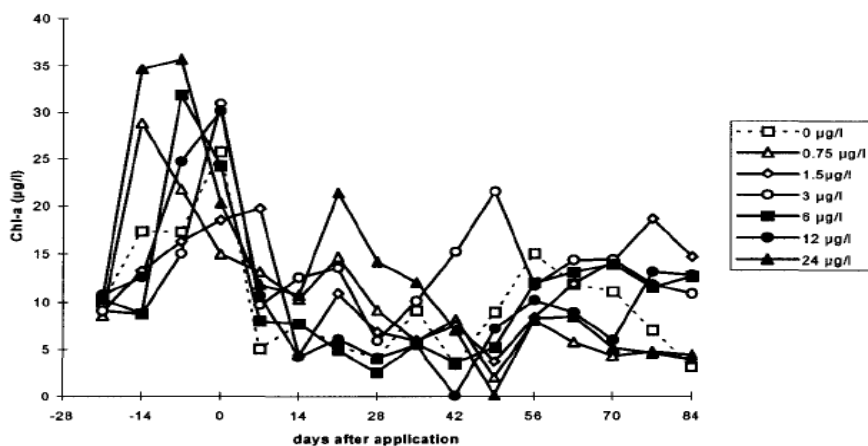


Figure 9.2.8-3. Development of the algal biomass (as chlorophyll-a) in the water column of microcosm. The average values of replicated test system are presented.

Table B. 9.2.8-4. Chlorophyll-a (µg/l) in microcosms water

		day -21	day-14	day -7	day 0	day 7	day 14	day 21	day 28	day 35	day 42	day 49	day 56	day 63	day 70	day 77	day 84
Microcosm	Nominal concentration a.s./L																
M2	0	8.78	16.55	16.34	32.81	7.69	5.66	12.37	6.44	0.59	3.40	19.50	11.86	11.85	7.38	4.80	4.80
M6	0	10.96	9.94	6.25	16.34	5.08	7.43	-0.51	10.13	2.87	-0.65	6.54	4.83	5.17	3.23	3.91	3.91
M11	0	10.16	25.51	29.21	28.01	2.31	10.00	4.33	10.74	6.68	24.01	19.05	18.68	16.24	10.36	0.71	0.71
M4	0.75 µg a.s./L	8.59	26.60	3.86	4.51	12.16	8.97	14.33	3.81	9.23	3.49	3.74	4.53	4.44	5.63	4.29	4.29
M8	0.75 µg a.s./L	8.56	31.06	39.72	25.41	14.06	11.71	15.03	7.95	7.05	0.53	12.34	6.93	4.22	3.86	4.53	4.53
M3	1.5 µg a.s./L	8.39	7.44	7.63	7.38	20.14	1.00	11.61	4.51	3.31	2.93	2.83	2.62	2.70	2.79	3.22	3.22
M7	1.5 µg a.s./L	9.69	19.04	24.93	29.55	19.27	7.73	10.19	7.41	11.76	4.52	13.90	21.20	25.64	34.46	26.13	26.13
M1	3 µg a.s./L	7.56	9.78	22.17	28.55	10.47	13.06	15.77	5.89	9.50	16.87	13.22	3.84	2.83	4.90	3.92	3.92
M9	3 µg a.s./L	10.61	7.72	7.88	33.30	8.90	12.02	11.34	14.35	20.90	26.19	10.10	24.85	25.93	18.69	17.78	17.78
M10	6 µg a.s./L	11.16	11.57	23.69	14.04	5.70	1.02	4.38	6.49	3.71	6.97	19.63	22.64	24.78	20.04	22.51	22.51
M14	6 µg a.s./L	9.52	5.97	40.03	34.42	10.26	14.42	5.52	4.63	3.51	3.47	4.33	3.53	3.02	2.98	2.80	2.80
M5	12 µg a.s./L	11.45	18.83	24.31	28.42	12.80	3.53	4.38	2.11	0.26	9.27	13.54	10.93	2.46	11.04	5.41	5.41
M13	12 µg a.s./L	9.98	6.37	25.11	31.80	8.44	4.89	7.78	8.79	-0.13	5.11	6.84	6.84	9.48	15.22	20.16	20.16
M12	24 µg a.s./L	10.31	34.66	35.67	20.26	11.80	10.73	21.39	12.03	6.99	0.13	8.26	8.44	5.13	4.59	3.94	3.94

### Periphyton

Periphyton biomass on glass side after 28 day exposure was relatively low on day 28 and ranged during the test period between 0 and 0.6  $\mu\text{g}$  chlorophyll-a/ $\text{cm}^2$ . On Day 42 there were some trend of dose related effect but average periphyton biomass was not significantly different from those in the control (one – side Annova,  $p > 0.05$ ). For the following days no statistically differences to control were observed, however on day 56, only one replicate of test concentration - 6  $\mu\text{g}$  /L was tested.

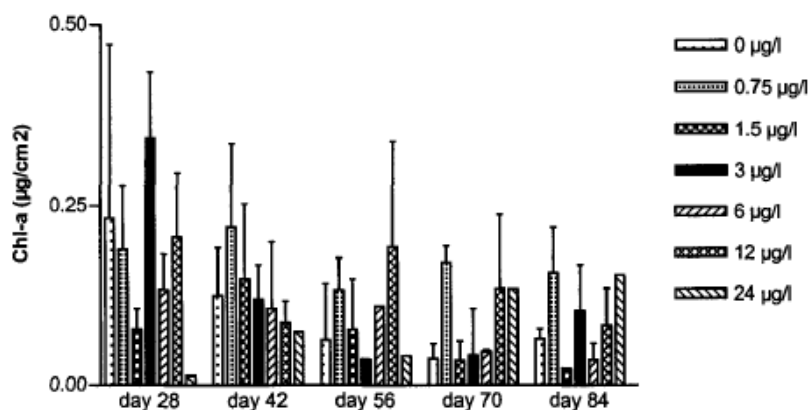


Figure 9.2.8-4. Periphyton biomass on glass slides (as  $\mu\text{g}$  chlorophyll-a/ $\text{cm}^2$ , after 4 week exposure in the microcosm). The average values of replicated test system. On day 56 the results of the 6  $\mu\text{g}$  /l treatment were not replicated.

### Lemna gibba

The relative increase of the number of fronts of *Lemna gibba* showed no clear relation with the test concentration.

Growth rates of *Lemna* in the duplicated treated concentrations were not statistically different from those found in the control (one-side Annova test,  $p > 0.05$ ). The lowest growth rate was observed at in the microcosm with the highest test concentration (one replicate, 24), except 56 day.

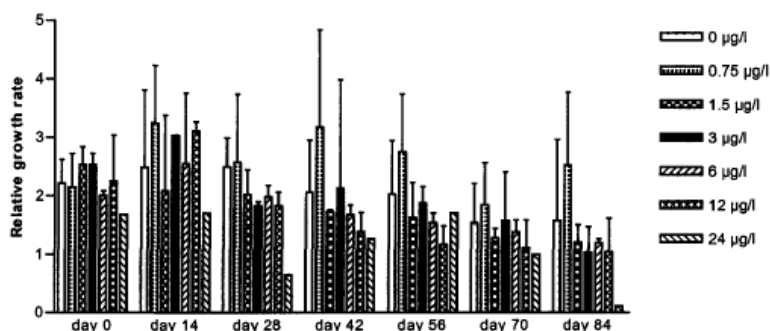


Figure 9.2.8-5. Development of duck weed in relation to the test concentration, expressed as the relative increase in the number of all fronts during two weeks exposure.

Taken into account the average dry weight of individual fronts no significant effects of the treatment was observed (one –side Anova,  $p < 0.05$ ) for replicated data. Data were not replicated in the following days: day 42 treatments (3 and 12  $\mu\text{g}$  a.s./L), day 56 (12  $\mu\text{g}$  a.s./L) and day 70 (3 and 12  $\mu\text{g}$  a.s./L).

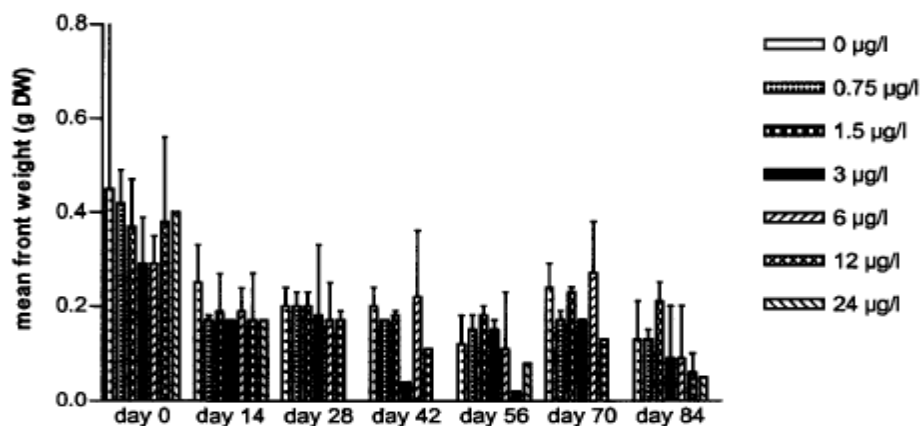


Figure 9.2.8-6. Average dry weight of individual duck weed fronts.

#### *Elodea densa*

The development of the *Elodea densa* was expressed as development of dry weight grouped plants or the length of individual.

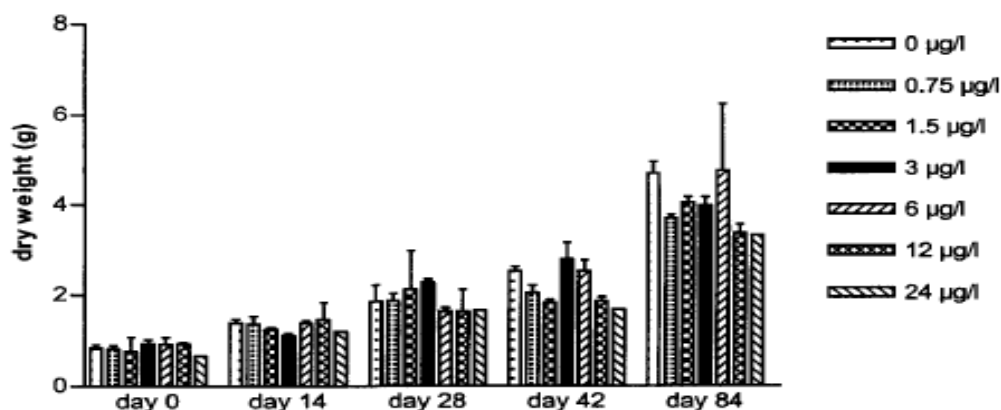


Figure 9.2.8-7. Development of the average dry weight with standard deviation of the grouped *Elodea*. Each group consisted of 4 plants.

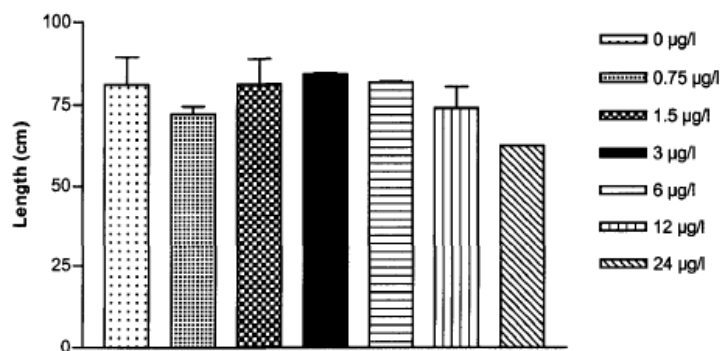
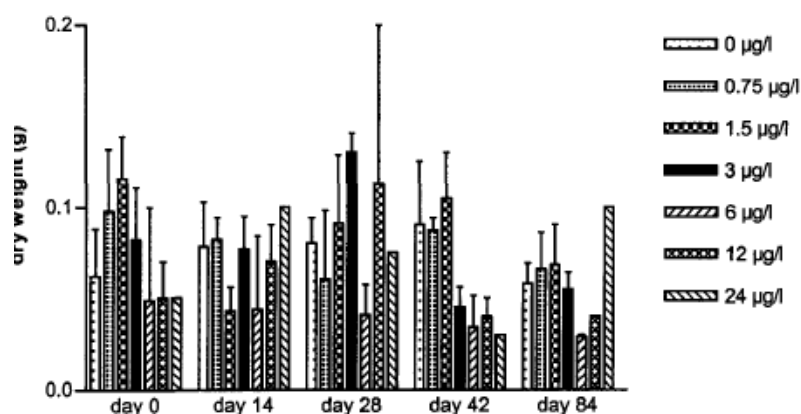


Figure 9.2.8-8. . Average length of *Elodea* sp. plants sampled from each microcosm on day 84.

Patomogeton pectitatus

Only four plants of Patomogeton were analyzed due to poor development of remaining plants in microcosm, in all test concentrations.



**Figure 9.2.8-9. Development of the average dry weight with standard deviation of the grouped *Patomogeton pectitatus*. Each group consisted of 4 plants.**

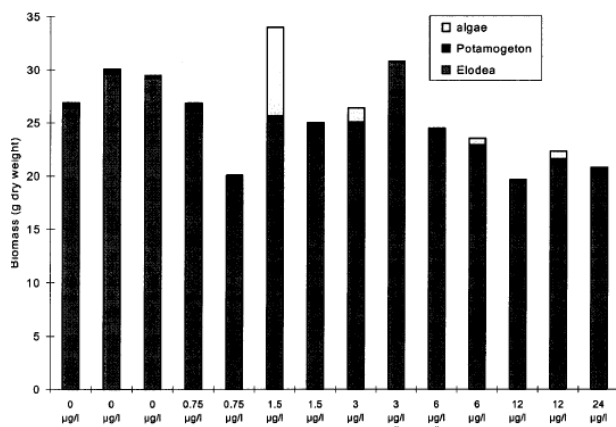
*Visual observation of macrophate*

During the entire test period the percentage of the microcosm surface that was covered with macrophytes was estimated and recorded. Although these data are rather arbitrary, they give an impression of the appearance of the microcosms. It has already been stated that especially *Elodea* developed well. The macrophyte coverage estimation can therefore be regarded as an indication of the *Elodea* development in the different microcosms. The development of the coverage of the microcosm area suggests a retarded development of *Elodea* in the microcosms with test concentrations of 6, 12 and 24 µg a.s./L. Differences between treatments had been noted 21 days after the application of the test substance. Especially in the highest dosed microcosm the development of *Elodea* seems to be delayed. The macrophyte coverage was low until day 63, although no differences between treatments could be observed at the end of the test. In addition, these observation were not confirmed by measurments such as *Elodea* biomass.

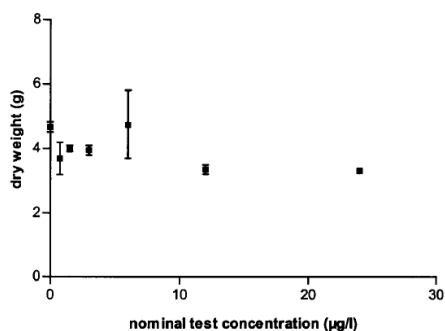
**Biomass of submerged macrophyte and filamentous algae at the end of test.**

The total biomass of *Elodea densa* at the end of the test in the different microcosm was evaluated as the average weight of of *Elodea* group, in which way the values were corrected for variation in introduced number of groups caused by mistake during inoculation (microcosm at tested rate of 6 ug a.s./L received 10 plants and microcosm at tested rate 3 ug a.s./L received 16 plants ). There was not differences between the weights of the different groups (Annova,  $p > 0.05$ ). At the end of test biomass of submerged macrophytes and filamentaus algae that was present in each microcosm was determined. *Elodea densa* contribuded more than 90 % of the total biomass of these plants in all test concentration except 1.5 µg a.s./L where filomenus algae were responsible for 24% of the

biomass..



**Figure 9.2.8-10. Total biomass of submerged macrophytes in the microcosm at the end of the test and the contribution of filamentous algae, Elodea densa and Potamogeton sp.**



**Figure 9.2.8-11. Relation between the nominal test concentration and the average weight of Elodea groups**

### Zooplankton

The most important group of Cladocerans was represented by *Ceriodaphnia* sp. and Chydorids, *Bosomia* and *Daphnia longissima*. At the moment of application the number of cladocerenas showed variation between microcosms. Before application the number of the numbers were higher in the lower dosed systems and the controls, than in the higher dosed systems. This difference between systems at the beginning of the test was caused by *Bosmina* sp. and *Daphnia longispina* (Figure 9.2.8-12). Both groups did not show further development in the microcosms, and had almost completely disappeared from all systems after day 14. The main cladoceran groups in the microcosms became *Ceriodaphnia* sp. and Chydorids, which were present in low numbers at the beginning of the test. Their development did not show a clear relation with the treatment. The average densities of *Ceriodaphnia* in the untreated systems showed a strong development during the first 28 days after the application of the test substance, which was not seen in the average densities in the treated microcosms. However, large variation appeared between the densities of this cladoceran species between the individual microcosms. The ANOVA ( $p > 0.05$ ) showed that the mean densities of the various treatments were not significantly different on days 14 or 28.

Initial densities of copepods (average densities per treatment) were low and rapidly increased during the pre-test period and in the first weeks after application (Figure 9.2.8-12). The copepod community consisted mainly of nauplius larvae(sub) adult copepodites were only present in low numbers. No trends with treatment levels were observed.

Rotifers were present in very high numbers in some microcosms, and densities showed large variation between microcosms. The rotifer density steadily decreased in all microcosms during the test period, seemingly without any relation with the test concentration.

Initial densities of copepods (average densities per treatment) were low and rapidly increased during the pre-test period and in the first weeks after application. The copepod community consisted mainly of nauplius larvae; (sub)adult copepodites were only present in low numbers.

The smallest zooplankton is formed by the unicellular ciliates. This group showed no sensitivity to the test substance and was found in the highest densities in the highest dosed microcosm. This could indicate that the ciliates took advantage from the highest test concentration. On the other hand it could also be just coincidence, since similar densities were observed in the 1.5 µg/L treatment on day 28.

No clear dose related direct or indirect effects of the test substance were observed on the densities of identified zooplankton groups.

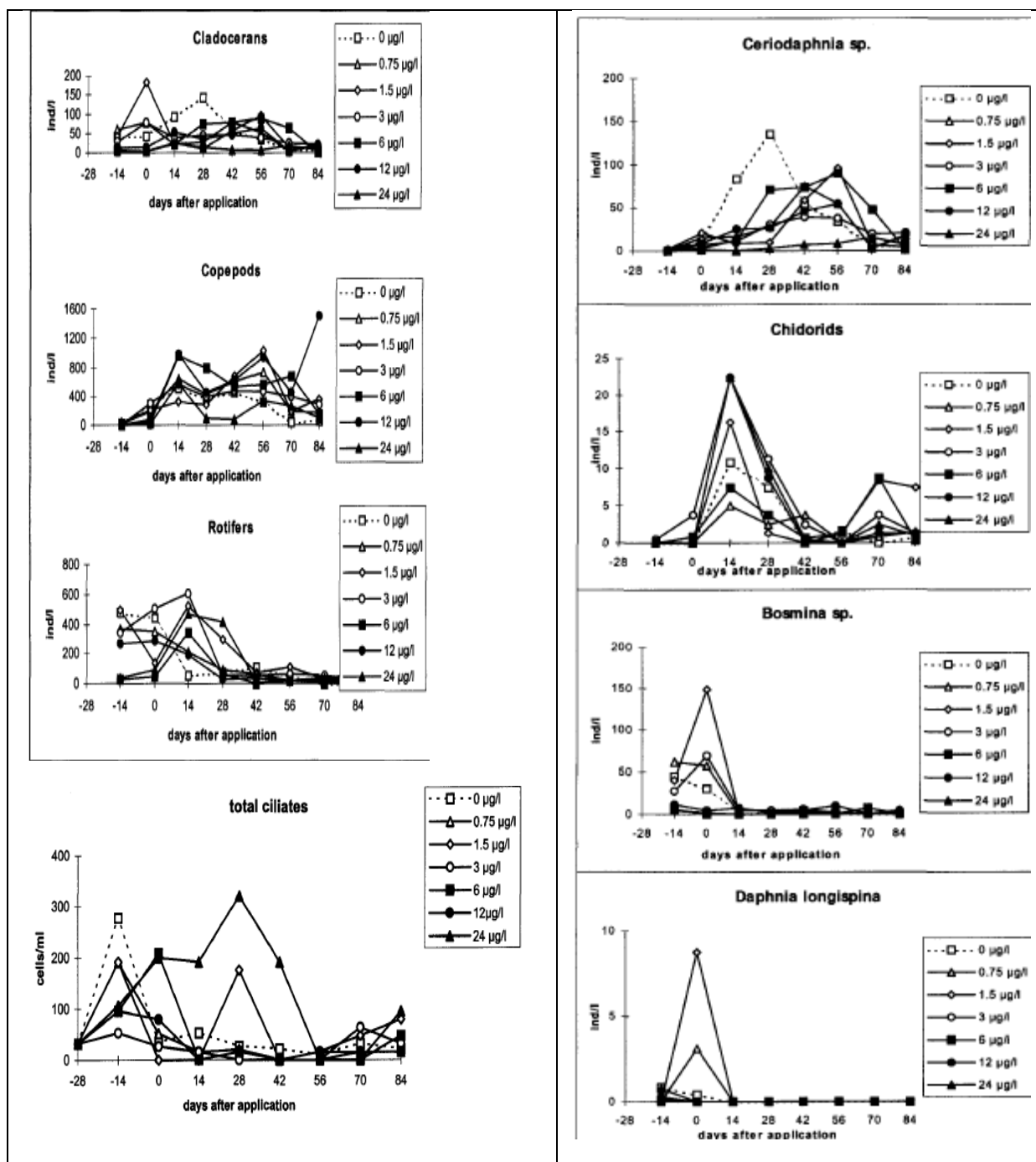


Figure 9.2.8-12. Dynamics of zooplankton groups averaged per treatment level of the test substance.

**Macrofauna:**

The macrofauna community at the end of the test was dominated in numbers by the snail *Valvata* sp. Other taxonomic groups that were found in relatively high numbers were the small sediment dwelling bivalve *Pisidium* sp., the crustacean *Gammarus* sp. and the small snail *Potamopyrgus jenkinsi*. In all microcosms small zebra mussels (*Dreissena polymorpha*) were found on the side of the wall. These animals must have entered the systems as larvae with the introduced water.

The large variation in the numbers of the snails *Lymnaea* sp., *Planorbis* sp. and *Bithynia tentaculata* between the different microcosms is remarkable. This can be explained by the introduction of egg masses with the sediment or plants into some of the microcosms. Between 7 and 11 macrofauna taxa were determined in the various microcosms, and the average numbers showed no relation with the microcosm treatment.

**Table B.9.2.8-5 Results of sampling of macrofauna on 84 day.**

	M2	M6	M11	M4	M8	M3	M7	M1	M9	M10	M14	M5	M13	M12
nominal conc. µg/l	0	0	0	0.75	0.75	1.5	1.5	3	3	6	6	12	12	24
<i>Gammarus</i> sp.	49	76	23	24	101	2	8	14	30	14	37	62	52	35
<i>Corixa</i> sp.	1	0	1	0	4	1	9	9	0	6	0	7	0	4
<i>Dreissena polymorpha</i>	19	22	19	15	27	10	7	16	25	11	18	21	17	15
<i>Pisidium</i> sp.	79	37	52	21	56	32	64	60	56	48	65	78	64	76
<i>Sphaerium</i> sp.	0	0	0	0	0	0	0	0	5	0	0	0	0	5
<i>Unio pictorum</i>	4	4	4	2	6	2	3	6	6	0	3	5	6	0
<i>Lymnaea</i> sp.	63	54	25	1	0	0	0	62	0	0	0	0	24	0
<i>Valvata</i> sp.	88	130	125	103	192	88	242	82	119	163	94	143	61	164
<i>Potamopyrgus jenkinsi</i>	13	7	41	11	36	7	24	36	15	35	47	35	10	28
<i>Bithynia tentaculata</i>	32	1	0	3	1	1	1	1	0	0	1	0	3	2
<i>Planorbis</i> sp.	2	1	0	0	1	0	19	0	2	0	33	51	0	1
<i>Theodoxus</i> sp.	0	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Physa</i> sp.	0	0	0	0	9	0	0	0	0	0	0	0	0	0
Tubificid worms	17	12	15	5	28	10	15	22	10	10	14	5	20	12
Chironomid larvae	0	0	0	0	0	0	0	0	0	0	0	1	0	0

**Chemical parameters:****Water temperature:**

The average temperature of the water in the microcosm was 16.4 °C. The highest temperature 18°C was recorded on day 28 .The other days with relative high temperature noticed on days 42 and 77.

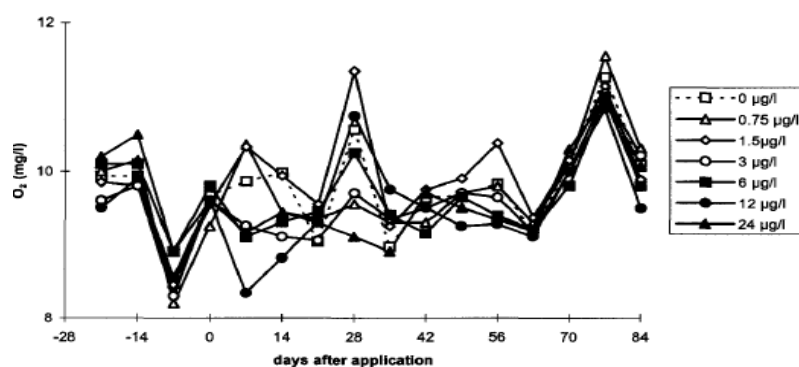
The high temperature was caused by extreme weather condition that hampered performance of air –conditioning device in experimental room. The lowest temperature was recorded on day 14 and 21 (15.3°C).

*Oxygen concentration*

The oxygen concentration in the water was relative high (8.2-11.8 mg/L) during test period and this was partly due to gentle aeration.

During to the study same differences in oxygen concentration were observed in treated microcosms.

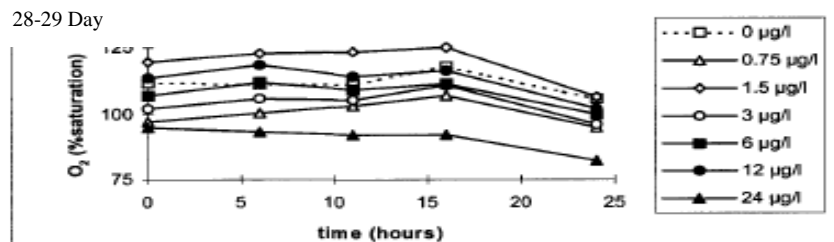
The differences was observed on day 7 after application. Taking into consideration results (one –side Annova,  $p > 0.05$ ) there was no significant different between treatments for this day.



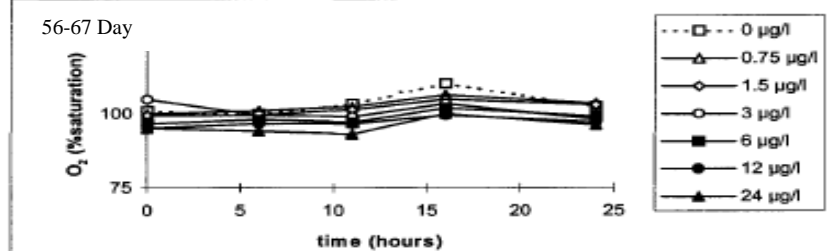
**Figure 9.2.8-13. Oxygen concentration on day 7, expressed as average values of replicated test system.**

On the days 28-29 during the first period of 24 hours with interrupted aeration the low oxygen concentration was recorded in highest dosed microcosm (24 µg/L). While oxygen saturation showed an increase in all other other microcosm during light period, it stabilized or even decreased in this particular microcosm. During the subsequent periods with interrupted aeration (Day 56-57 and 83-84) the oxygen concentration was comfortable in all microcosm (Figure 9.2.8-14 ).

28-29 Day



56-67 Day



83-84 Day

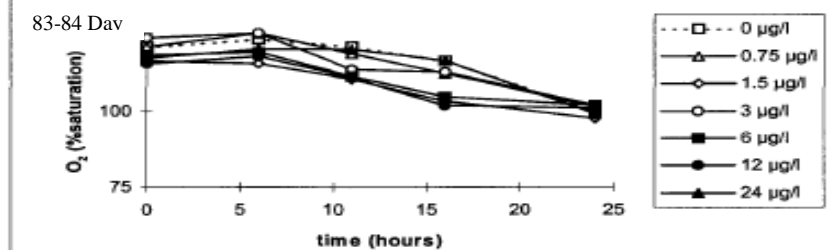


Figure 9.2.8-14. Development of the oxygen concentration in the microcosms during a 24 hour period without aeration; average values of replicate microcosms are presented. The systems were illuminated between 1 (09:00) and 15 (23:00) hours

## pH

The pH of the water in the microcosms was quite constant during the test period and ranged between 8.0 and 8.5 (Figure 9.2.8-15). In general, the development of the pH followed the same pattern in all microcosms. Between day 0 and day 28 some variation between the different microcosms was observed, but this did not lead to significant differences between treatments. On 21 and 28 days after the application of the test compound the pH of the highest dosed system was below that of the other microcosms.

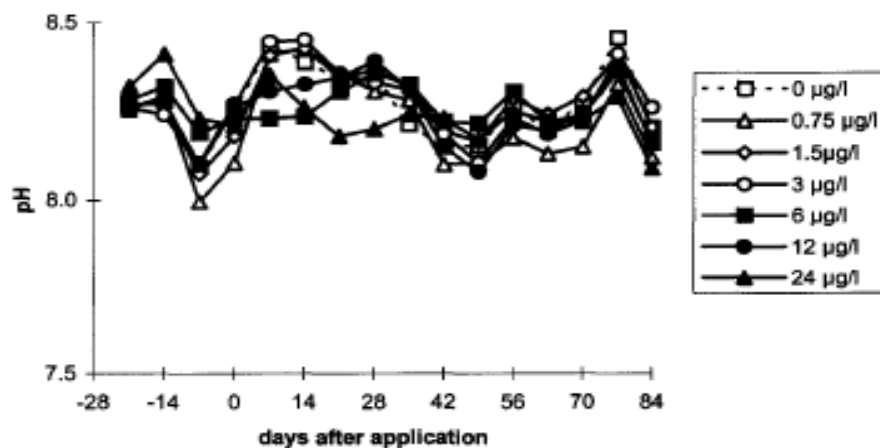


Figure 9.2.8-15. pH values of the water of microcosm, shown as average values of replicated test system.

## Conductivity

During test period conductivity ranged between 800 and 860 µS/cm.

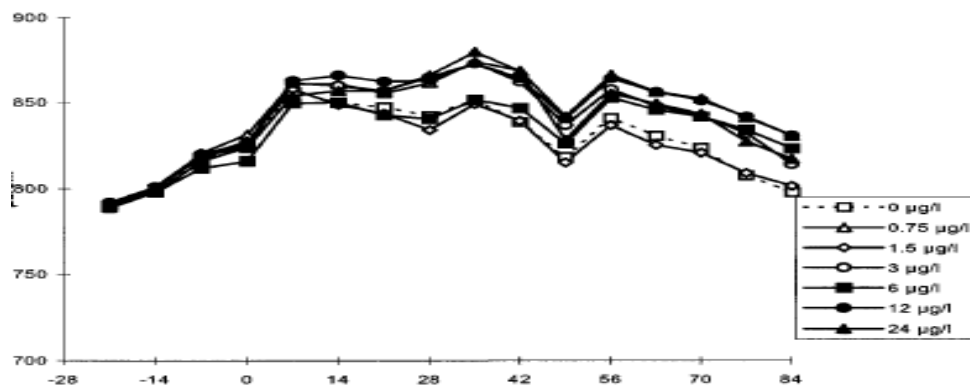


Figure 9.2.8-16. . Conductivity of the water microcosmos, shown as the average values of replicated test system.

### Nutrient concentration:

The nutrient concentrations in the tested microcosms were compared with the control and no statistical differences were observed for all tested parameters. A slight nitrite peak was observed on day -14, which indicates that the bacteria that were capable of transforming nitrite into nitrate had not yet adjusted to the new situation. At the end of the pre-test period the nitrite peak had disappeared, and the nitrogen concentrations varied (as nitrate) between 0 and 4 mg/L (Table 9.2.8-6.) in all microcosms during the rest of the test period.

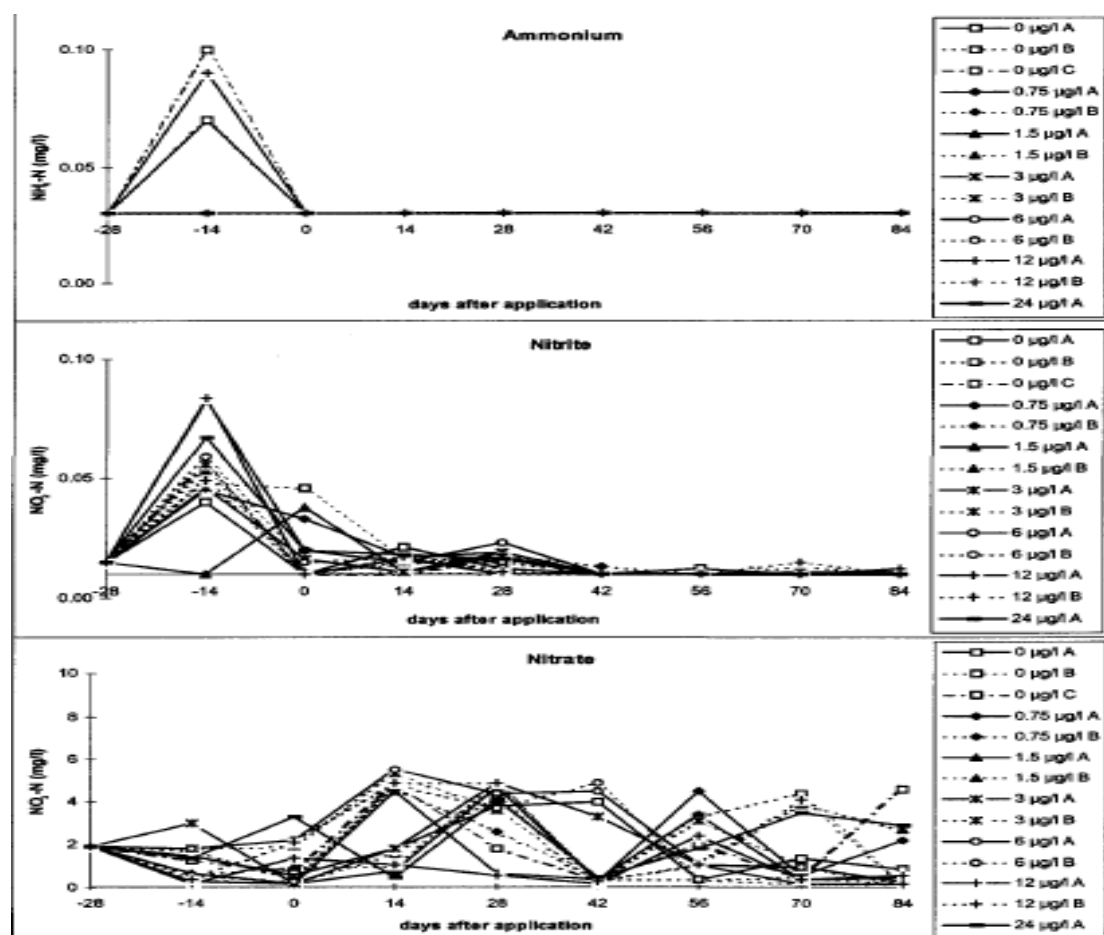


Figure 9.2.8-17. Development of inorganic nitrogen concentration (as ammonium, nitrite and nitrate), and orthophosphate and silicate in the water of microcosms. The crossing of x and y-axis indicates that detection limit of the analytical technique.

Table B.9.2.8-6. Nitrate (NO<sub>3</sub>-N) in mg N/L in microcosms water.

Test concentration (Nominal) µg a.s./L	Day -28	Day -14	Day 0	Day 14	Day 28	Day 42	Day 56	Day 70	Day 84
0	1.9	1.5	0.6	1.8	3.8	4.0	0.4	1.4	0.9
0	1.9	0.4	0.2	1.6	4.2	0.3	3.3	4.4	0.2
0	1.9	1.3	0.8	4.8	1.8	0.4	2.0	0.5	4.6
0.75	1.9	0.3	0.2	0.8	4.7	0.3	4.5	0.6	2.2
0.75	1.9	1.7	0.4	4.6	2.6	0.4	0.4	0.5	0.2
1.5	1.9	0.7	0.2	4.5	0.7	0.4	3.4	0.4	0.5
1.5	1.9	0.6	1.0	1.0	4.8	0.4	1.0	3.9	2.7
3	1.9	3.0	0.2	1.8	4.9	3.3	1.1	0.2	0.2
3	1.9	1.8	1.9	5.3	3.6	0.2	3.1	1.0	0.2
6	1.9	1.8	2.2	5.5	4.4	4.5	1.1	1.0	0.3
6	1.9	0.6	0.3	4.8	3.7	4.9	0.4	0.2	0.2
12	1.9	0.2	1.4	1.1	0.6	0.2	2.4	0.4	0.6
12	1.9	0.4	2.1	4.9	4.9	0.3	1.1	4.1	0.2
24	1.9	1.4	3.3	0.5	4.1	0.5	1.8	3.5	2.9

\*Limit dection 0.2 mg/l

### Phosphorus

The ortho-phosphate concentrations were rather low, and did not reach 0.1 mg P/L (Figure 9.2.8-18). Silicate is an essential element for diatoms, which use the silicate for the composition of a hard cell wall.

The highest silicate concentrations in the water of the microcosms were found around days 0 and 14. After this period the concentrations steadily declined to concentrations below 0.5 mg/l on day 42.

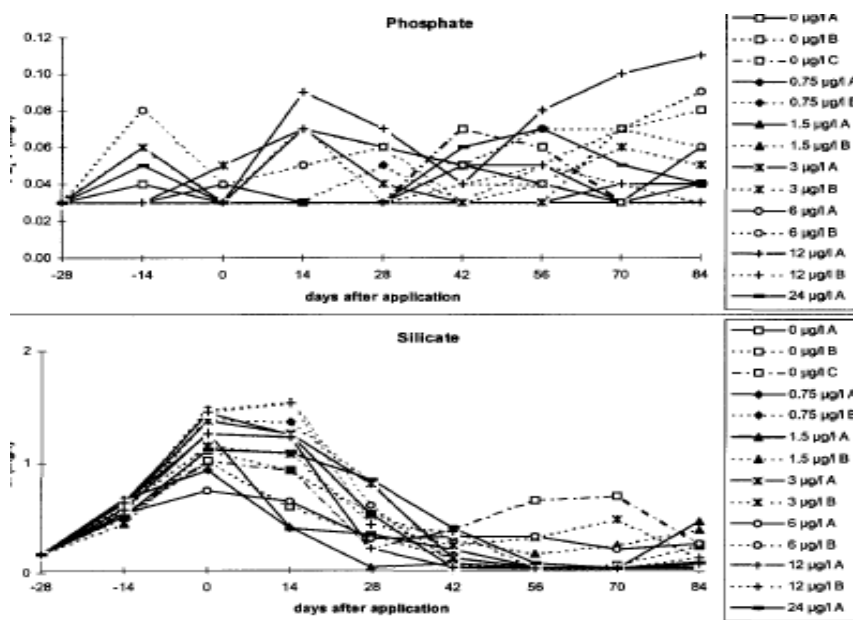


Figure 9.2.8-18. Development of orthophosphate and silicate in the water of microcosmos. The crossing of x and y-axis indicates that detection limit of the analytical technique.

**Table B.9.2.8-7. Phosphate (PO<sub>4</sub>) in mg P/L in microcosm water.**

Test concentration (nominal) µg a.s./L	Day -28	Day -14	Day 0	Day 14	Day 28	Day 42	Day 56	Day 70	Day 84
0	0.03	0.04	0.03	0.07	0.06	0.05	0.05	0.03	0.04
0	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.07	0.08
0	0.03	0.03	0.03	0.03	0.03	0.07	0.06	0.03	0.03
0.75	0.03	0.06	0.03	0.03	0.03	0.03	0.03	0.04	0.04
0.75	0.03	0.03	0.03	0.03	0.05	0.03	0.05	0.07	0.06
1.5	0.03	0.03	0.04	0.03	0.03	0.03	0.03	0.03	0.06
1.5	0.03	0.03	0.03	0.03	0.03	0.04	0.04	0.06	0.05
3	0.03	0.03	0.05	0.07	0.04	0.03	0.03	0.03	0.03
3	0.03	0.06	0.03	0.07	0.06	0.03	0.04	0.06	0.05
6	0.03	0.03	0.03	0.03	0.03	0.05	0.04	0.03	0.06
6	0.03	0.08	0.04	0.05	0.06	0.05	0.07	0.07	0.09
12	0.03	0.03	0.03	0.09	0.07	0.04	0.08	0.10	0.11
12	0.03	0.03	0.03	0.07	0.03	0.04	0.05	0.04	0.03
24	0.03	0.05	0.03	0.03	0.03	0.06	0.07	0.05	0.04

Det.limit 0.03 mg/l

**Table B.9.2.8-8. Silicate (Si) in g Si/L in microcosm water.**

Test concentration (nominal) µg a.s./L	Day -28	Day -14	Day 0	Day 14	Day 28	Day 42	Day 56	Day 70	Day 84
0	0.17	0.65	1.45	1.25	0.54	0.06	0.05	0.05	0.05
0	0.17	0.51	1.02	0.61	0.35	0.16	0.05	0.08	0.25
0	0.17	0.52	1.03	0.94	0.23	0.41	0.67	0.71	0.26
0.75	0.17	0.68	0.94	0.42	0.36	0.22	0.06	0.05	0.11
0.75	0.17	0.56	1.39	1.37	0.83	0.1	0.05	0.05	0.08
1.5	0.17	0.59	1.27	0.43	0.06	0.1	0.05	0.05	0.48
1.5	0.17	0.46	1.12	0.94	0.54	0.31	0.18	0.26	0.4
3	0.17	0.64	1.38	1.26	0.82	0.15	0.06	0.05	0.08
3	0.17	0.61	1.16	1.09	0.46	0.26	0.33	0.5	0.14
6	0.17	0.55	0.76	0.66	0.32	0.34	0.34	0.22	0.27
6	0.17	0.61	1.48	1.54	0.62	0.09	0.05	0.05	0.11
12	0.17	0.65	1.26	1.23	0.23	0.06	0.05	0.05	0.09
12	0.17	0.58	1.46	1.53	0.45	0.39	0.05	0.05	0.15
24	0.17	0.52	1.13	1.1	0.85	0.42	0.1	0.05	0.1

Det.limit 0.05 mg/l

**Results:**

Results of the study indicated that no significant treatment related effects could not be observed at any treatment level, although some slight differences in community metabolism (O<sub>2</sub> and pH) were noted in the highest treatment level 24 (µg a.s./L), together with a slightly reduced growth of some macrophytes and periphyton.

The observed effects for the highest test concentration were listed below:

- The oxygen production was quite low during the 24 hour period without aeration on day 28-29; but this reduction was not seen at the following period without aeration on days 56-57.
- The pH was relatively low on days 21 and 28. On day 35 and beyond, the pH followed the same pattern as in all other microcosms.
- Periphyton biomass was relatively low on day 28. But the periphyton biomass did not differ from the other concentrations at the subsequent sampling on day 42.
- The growth rate of *Lemna gibba* was relatively low on day 28 but no differences were found before day 28.
- The macrophyte coverage was low until day 63, although no differences between treatments could be observed at the end of the test on day 84. Since the macrophyte coverage was visually estimated it cannot be considered as

a quantitative measurement. In addition, these observations were not confirmed by measurements such as *Elodea* biomass.

### **Comments RMS**

For the above mentioned indoor microcosm study a NOEC<sub>(periphyton, macrophyte)</sub> of 12 µg a.s./L was agreed during the first inclusion of Flufenacet into the Annex I without setting the assessment factor.

The following 5 questions were answered in order to draw a conclusion whether the study can be used in the risk assessment.

#### **1.- Is the test system adequate and does the test system represent a relevant freshwater community?**

##### **Answer: unclear**

The study was performed as indoor aquatic microcosms under controlled temperature and light conditions.

Enclosures contained macrophytes and communities of phytoplankton, zooplankton and macrofauna.

Consequently, different trophic levels were present in the microcosms. The sediment and biota originated from natural sources. The communities in the enclosures are characteristic for shallow, static water bodies without fish.

However, in the opinion of the RMS, the microcosm was not sufficiently representative for the freshwater community due to the following reasons:

- The phytoplankton composition consisted of only three taxonomic groups: mainly *Flagellates*, blue-green algae (*Oscillatoria* sp., *Microcystis* sp.) and *Cryptomonas* species.

*Pseudokirchinella subcapitata*, which had been shown in the standard laboratory tests to be the most sensitive species was not observed in the microcosm. The second green algae –*Scenedesmus* sp. was observed in low numbers

The other alga species were recorded also in very low numbers and non homogenous distribution was observed within ponds.

- The zooplankton was represented by eight species at test start (with low numbers of species)

The macrophytes were represented by 3 plants mainly by *Lemna gibba*, *Elodea* sp., *Potamogeton pectinatus*.

However, *Potamogeton pectinatus* species did not develop sufficiently well (only four species in each group were recorded).

#### **2.- Is the description of the experimental set-up adequate and its description unambiguous?**

##### **Answer: Yes .The description of the experimental set-up is clear**

Five concentrations were tested, with the number of replicates for each tested concentrations (two replicates for treatment, 3 for control) fulfilling the minimum requirements in the current aquatic guidance (AGD, 2009). Only one replicate was provided for the highest application rate of 24 µg a.s./L. Therefore, the observed effects for that concentration bear significant level of uncertainty.

#### **3.- Is the exposure regime adequately described?**

##### **Answer: yes**

The Flufenacet 60 WG formulation, containing only one active substance -flufenacet (600g/l, nominal), was used in the study. The method of application is described in detail in the study report. However, it should be noted

that the nominal concentration of flufenacet four hours after application exceed significantly the level of 100% of theoretically applied probably due to incomplete mixing of the water. However, for the next sampling date, the test concentration ranged between 94 and 113% (mean: 106%).

The treatment validation and application checks were performed. Concentrations of the active substance in water were monitored to the end of experiment and DT<sub>50</sub> value was determined.

#### 4.- Are the investigated endpoints sensitive and in accordance with the working mechanism of the compound?

**Answer: unclear** ( the most sensitive species- *Pseudokirchneriella subcapitata* are not present in the study)

Laboratory single-species tests performed with aquatic organisms indicate that the aquatic primary producers: aquatic macrophytes and algae are the most sensitive species. In the tested microcosms all groups were investigated. However, the lack of the most sensitive species of green algae- *Pseudokirchneriella subcapitata* recorded in the laboratory studies, does not allow to draw reliable conclusion about sufficient representativeness of the phytoplankton community. Generally, a low number of species were observed in the microcosm tested, but the ones present did not show a treatment related effect up to 12 µg/L.

Three species of macrophyte were investigated in the microcosms.

In case of *Potamogeton pectinatus*, the development of the plants introduced into the test system was poor – some introduced plants did not survive, while in case of the others the growth, expressed in terms of the amount of produced biomass, was inhibited. All that resulted in practically no increase of biomass in any of the groups of four plants tested, what makes difficult to determine the extent of the would-be inhibitory/toxic effect of flufenacet on that particular macrophyte (*Potamogeton pectinatus*). At the same time it was stated that there was no indication that the test compound affected anyhow the development of the test plants.

RMS, having analysed the conclusions drawn by the Applicant and the graphical presentation of the results, arrived to conclusion that for *Potamogeton* the whole picture was unclear. For that reason no unambiguous conclusions for that species can be drawn.

For *Lemna* sp. according to the graphs, effects can be seen on growth rate and frond weights at the highest tested concentration at the last time point. Therefore, the NOEC for *Lemna* sp. could be set at 12 µg/L.

For *Elodea* sp., the dry weight of plants exposed to 12 and 24 µg/L seems to be clearly reduced in comparison to the control. About 30% effects were observed for these concentrations which is considered biologically relevant.

The length of plants exposed to 24 µg/L is clearly reduced at 24 µg/L.

Finally according to the Figure 9.2.8-10 the total biomass of submerged macrophytes (*Potamogeton pectinatus* and *Elodea*) seems to be clearly affected at 12 and 24 µg/L.

Therefore, the NOEC of 6 µg/L should be set based on the effects of dry weight for *Elodea* sp.

**Therefore, it should be indicated that the results from this study may suggest that rooted macrophytes could be slightly more sensitive to flufenacet than *Lemna* sp.**

The indirect effects of exposure to herbicide may potentially also affect the non-sensitive taxa, such as zooplankton (sampled every two weeks, 7 times after treatment) and macrofauna (sampled once at the end of the study). No significant treatment related effects were observed.

In this microcosmos study, due to the high variability between individual microcosms and relatively low

abundance of zooplankton, RMS found it difficult to determine the influence of treatment with Flufenacet 60 WG on zooplankton community. Therefore, the significant effects on tested zooplankton could not always be satisfactorily demonstrated.

The level of nutrients such as phosphorus and nitrogen, fluctuated during the study both in the controls and in the treated samples, without any clear time-trend. Only silicate decreased from day 14 onwards.

**- Is it possible to evaluate the observed effects statistically?**

**Answer: No statistical effects were observed during the study.**

In the current microcosm the results were analysed using one way analysis of variance (ANOVA) to determine if observed differences between endpoints were significant. The datasets of day 14 and 28 were used to perform multivariate analysis (Redundancy Analysis) in order to determine the presence of a direct effect of the application of the test substance. The significance of the observed correlations was tested using Monte Carlo simulation.

For all tested parameter (periphyton biomass (chlorophyll-a), algal abundances (chlorophyll-a), metabolism community: O<sub>2</sub> content, pH, conductivity) and of macrophyte's development, no significant effects in replicated samples were observed up to 12 µg a.s./L.

**Overall conclusion:**

**The study report does not facilitate the interpretation of the study.**

**A principal component analysis of the results would have been appreciated for phyto/zooplankton and macroinvertebrates. According to the study report, it seems that no statistically significant effects have been found, even at the highest tested concentration, which would not demonstrated the ability of the study to detect effects on organism.**

**The figures are rather small. The respective contribution of *Elodea sp.* and *Potamogeton sp.* can not be distinguished in Figures.**

**The results have not been reanalyzed on the basis of the MDD and effects class approach.**

**With regard to algae, it is difficult to make clear conclusion because of the low representation of green algae in the cosm, identified in lab study. In addition, it should be emphasized, that the experiment was carried out in two replicates only, yielding a high variability of the results obtained.**

**Therefore, the results from this study can not be used in the risk assessment for algae.**

**However, the results obtained for *Lemna sp.* and *Elodea sp.* may be used to derive final NOEC for macrophytes.**

**RMS would propose a NOEC<sub>macrophytes</sub> of 6 µg/L based on the effects on the dry weight of *Elodea sp.* at the end of the study: ~30% effects were observed at 12 µg/L and 24 µg/L which is considered biologically relevant.**

**Given that no MDD analysis has been provided RMS proposed AF of 5.**

**Agreed endpoint:**

**NOEC<sub>macrophytes</sub> = 6 µg a.s./L, AF=5**

**B.9.2.8.1. Additional data from open literature**

Based on literature search, several publication relevant to flufenacet evaluation were performed, which need further consideration. Their summarised and evaluations are included below:

---

<b>Reference:</b>	Comparison of developmental toxicity of seven perfluoroalkyl acids to zebrafish embryos
<b>Author(s), year:</b>	Ulhaq, M., Carlsson, G., Örn, S., Norrgren, L., 2013
<b>Report/Doc. number:</b>	Refernce BCS No: M-462660-01-1
<b>Guideline(s):</b>	Not stated
<b>GLP:</b>	Not stated

---

**EXECUTIVE SUMMARY**

The toxicity of individual perfluoroalkyl acids (PFAAs) has been suggested to be determined by the carbon chain length as well as the functional group attached. In this study, seven different PFAAs including both sulfonic and carboxylic PFAAs were tested with different chain length to evaluate the developmental toxicity in zebrafish embryos. Generally, the acute toxicity of PFAAs including TFA is relatively low to zebrafish embryos. The EC<sub>50</sub> values ranged from 1.5 to 2200 mg/L. A relationship between higher toxicity with longer carbon chain was observed. In addition, also a higher toxicity for sulfonic PFAAs than for carboxylic PFAAs was observed.

**MATERIAL AND METHODS**

Since the purpose of the literature review is to select literature relevant for the environmental risk assessment under Regulation (EC) No 1107/2009 for the metabolite trifluoroacetic acid (TFA), the study summary contains primarily the results for the compound of concern.

**A. Material**1. Test material

Test item:	Perfluoroalkyl acids (PFAAs) including trifluoroacetic acid (TFA)
Active substance(s):	See above
Chemical state and description:	liquid
Source of test item:	TFAA: Sigma-Aldrich, Germany
Batch number:	Not stated
Purity:	Not stated
Storage conditions:	Not stated
Water solubility:	Not stated

## 2. Test solutions

Vehicle/solvent:	Not stated
Source of vehicle/solvent:	Not stated
Concentration of vehicle/solvent:	Not stated
Method of preparation:	Not stated
Evidence of unsolved material:	Not stated

## 3. Test organism(s)

Species:	Zebrafish ( <i>Danio rerio</i> )
Common name:	See above
Source of test species:	Not stated

## 4. Test conditions of test organism(s)

Culture medium:	Reconstituted water (ISO, 1996)
Temperature:	Not stated
Photoperiod:	Not stated
Light intensity:	Not stated
pH:	Not stated
Oxygen saturation:	Not stated
Food and feeding regime:	Not stated
Acclimatisation prior to testing:	Not stated
Observations during acclimatisation:	Not stated

## B. Study design and methods

### 1. Test procedure

Test system:	Laboratory test, fish embryo acute toxicity
Test concentration(s):	TFAA: 10 - 3000 mg/L
Control(s):	Reconstituted water without test item
Number of replicates:	4 replicates with 6 embryos per replicate for each treatment group and control (= 168 embryos per PFAA)
Test conditions:	Zebrafish eggs within 15 min after collection were exposed to a series of concentrations of the test substance dissolved in reconstituted water. Fertilized eggs were randomly distributed individually into flat bottom, 48-well polystyrene plates along with 750 µL of the exposure medium. The PFAAs were tested at six consecutive concentrations differing by a factor of 3.3 based on logarithmic scale fitting. For each PFAA test four 48-well plates were used, with a total of 24 embryos per PFAA

concentration as well as 24 in the water control group. The plates were covered with parafilm and the embryos were exposed to the chemical until 144 h post fertilization (hpf). Observations of mortality and sublethal endpoints (see below) were made after 24, 48, 120 and 144 hpf using a stereomicroscope according to endpoints presented in Carlsson et al. (2013). Test was done under the following environmental conditions: water temperature:  $26 \pm 1^\circ\text{C}$ ; pH: 7.2-7.6; 14 h light cycle.

Feeding:	Not stated
Medium renewal:	No renewal
Frequency of test item application:	One application
Test duration:	144 h
Endpoints:	Mortality and sublethal endpoints (presence of edema, malformations, not-hatched eggs, lack of circulation, reduced pigmentation)
Statistics:	The 50% effective concentration ( $\text{EC}_{50}$ ) values with 95% confidence intervals were calculated for categorical data using probit analysis and defined as the concentration when 50% of the embryos displayed sublethal or lethal effects. The continuous data were analyzed using one-way ANOVA with two-sided Dunnett's post hoc test. LOEC and NOEC parameters were determined on the basis of Dunnett's test.

## 2. Measurements during the test

Water/medium parameters: Not stated

## 3. Sampling

Sampling frequency: No samples

Transport/storage of samples: See above

## 4. Chemical analysis

Guideline/protocol: No chemical analysis was done. Explanation given in the study: PFAA concentrations have been reported to be stable in similar exposure studies or considered so where actual concentrations were not measured.

Method: See above

Pre-treatment of samples: See above

Conduction: See above

Reference item: See above

Recovery: See above

Limit of detection: See above

Limit of quantification: See above

## RESULTS

### 1. Validity criteria:

An official OECD guideline for a fish embryo toxicity test (OECD 236) will be available soon. However, no information were given whether the study from Ulhaq *et al.* (2013) meets the validity criteria set forth in the new guideline.

### 2. Analytical findings:

No chemical analysis was done. It was stated that PFAA concentrations have been reported to be stable in similar exposure studies or considered so where actual concentrations were not measured.

### 3. Other measurements:

Please refer to point 3 'Biological findings'. Measurement of other parameters was not reported.

### 4. Biological findings:

TFA and the other tested PFAAs are not highly toxic to early life stage zebrafish. Results are in agreement with those reported in the literature. Evaluation of the PFAAs in the present study followed established endpoints.

The statistical evaluations are based on the sum of total effects since the statistical power was too low for making correlations between individual endpoints and chemical concentrations. EC<sub>50</sub> and NOEC/LOECs of TFAA and other PFAAs are presented in the table below.

**Table 1 (taken from Uhlaq *et al.*, 2013):** Chemical information and measurements of toxicity of PFAAs including TFA in zebrafish embryos

PFC	Chemical name	Formula	Cas#	Test range (mg/L)	EC <sub>50</sub> 144 h	LC <sub>50</sub> 144 h	NOEC/LOEC (mg/L)	
					(mg/L, 95% C.I.)		Heart rate	Hatching time
TFAA	Trifluoroacetic acid	CF <sub>3</sub> COOH	76-05-1	10-3000	700 (460-1000)	>3000	ne	300/1000
PFBA	Perfluorobutyric acid	C <sub>4</sub> F <sub>7</sub> COOH	375-22-4	10-3000	2200 (1200-22000)	>3000	ne	ne
PFCA	Perfluorooctanoic acid	C <sub>8</sub> F <sub>15</sub> COOH	335-67-1	3-1000	350 (290-430)	430 (290-710)	ne	ne
PFNA	Perfluorononanoic acid	C <sub>9</sub> F <sub>17</sub> COOH	375-95-1	0.03-10	16 (7.7-450)	>10	ne	ne
PFDA	Perfluorodecanoic acid	C <sub>10</sub> F <sub>19</sub> COOH	335-76-2	0.1-30	5.0 (3.8-66)	8.4 (5.3-15)	ne	ne
PFBS	Perfluorobutane sulfonic acid	C <sub>4</sub> F <sub>9</sub> SO <sub>3</sub> H	375-73-5	10-3000	450 (350-600)	1500 (1100-1900)	300/1000	ne
PFOS	Perfluorooctane sulfonic acid	C <sub>8</sub> F <sub>17</sub> SO <sub>3</sub> H	1763-23-1	0.03-10	1.5 (1.1-19)	>10	ne	ne
ne = no effect.								

group seem to be related to the developmental toxicity of PFAAs in zebrafish embryos. Generally, PFAAs with longer carbon chain lengths had higher toxic potential than PFAAs with shorter chain length (e.g. TFAA). Further, PFAAs with a sulfonic group were more toxic than PFAAs with a carboxylic group of the same carbon chain length.

### Summary of the results provided by RMS:

The following results were obtained during the study:

144 h EC<sub>50</sub> and LC<sub>50</sub> of zebrafish embryos was 700 mg TFA/L and 3000 mg TFA/L and the NOEC for heart rate and hatching time was established at 3000 mg TFA/L and 300 mg TFA/L.

The comparison of the toxic effects levels calculated in the study with measured levels in natural water the metabolite of flufenacet does not indicate the acute risk for zebra fish embryos.

**RMS comments:**

The study was taken from the open literature. No guideline was reported.

However, the study seems to follow new OECD 236 test guideline but due to that the lack of information it is not possible to verify the validity criteria of this study according to the current OECD 236 test guideline (no full report is available).

Moreover, the chemical analysis was not carried out and is not possible to verify the validity criteria of this study.

In opinion of RMS, the results obtained from the study can be considered as supplemental information.

**B.9.2.8.1.2.**

<b>Reference:</b>	Haloacetic acids in the aquatic environment - Part I: macrophyte toxicity
<b>Author(s), year:</b>	Hanson, Mark L., Solomon, Keith R., 2004
<b>Report/Doc. number:</b>	Referebce BCS No: M-455787-01-1 (doi:10.1016/j.envpol.2003.12.016)
<b>Guideline(s):</b>	Lemna: Greenberg et al. (1992), ASTM (2000); Myriophyllum spp.: ASTM (1999)
<b>GLP:</b>	Not stated

**EXECUTIVE SUMMARY**

Laboratory tests were conducted with 3 macrophytes (*Lemna gibba*, *Myriophyllum sibiricum*, and *Myriophyllum spicatum*) to assess the toxicity of 5 HAAs. The HAAs in the present experiments were monochloroacetic acid (MCA), dichloroacetic acid (DCA), trichloroacetic acid (TCA), trifluoroacetic acid (TFA), and chlorodifluoroacetic acid (CDFA). MCA was the most toxic to *Myriophyllum* spp. with EC<sub>50</sub> values ranging from 8 to 12.4 mg/L depending on the endpoint, followed by DCA (EC<sub>50</sub> range 62-722.5 mg/L), TCA (EC<sub>50</sub> range 49.5-1702.6 mg/L), CDFA (EC<sub>50</sub> range 105.3 to greater than 10,000 mg/L), and with TFA (EC<sub>50</sub> range 222.1 to 10,000 mg/L) the least toxic. Generally, *L. gibba* was less sensitive to HAA toxicity than *Myriophyllum* spp., with the difference in toxicity between them approximately 3-fold. The range of toxicity within *Myriophyllum* spp. was normally less than 2-fold. Statistically, plant length and node no. were the most sensitive endpoints as they had the lowest observed coefficients of variation, but they were not the most sensitive to HAA toxicity. Toxicological sensitivity of endpoints varied depending on the measure of effect chosen and the HAA, with morphological endpoints usually an order of magnitude more sensitive than pigments for all plant species. Overall, mass and root measures tended to be the most sensitive indicators of HAA toxicity.

## MATERIAL AND METHODS

Since the purpose of the literature review is to select literature relevant for the environmental risk assessment under Regulation (EC) No 1107/2009 for the metabolite trifluoroacetic acid (TFA), the study summary contains only the results for the compound of concern.

### A. Material

#### 1. Test material

Test item:	Haloacetic acids (HAAs) including TFA, tested as neutralized sodium salts
Active substance(s):	See above
Chemical state and description:	Not stated
Source of test item:	TFA: Aldrich Chemicals (Milwaukee, WS, USA)
Batch number:	Not stated
Purity:	99 + % (spectrophotometric grade)
Storage conditions:	Not stated
Water solubility:	Not stated

#### 2. Test solutions

Vehicle/solvent:	Not stated
Source of vehicle/solvent:	Not stated
Concentration of vehicle/solvent:	Not stated
Method of preparation:	Not stated
Evidence of unsolved material:	Not stated

#### 3. Test organism(s)

Species:	<i>Myriophyllum spicatum</i> L., <i>M. sibiricum</i> , <i>Lemna gibba</i>
Common name:	Not stated
Source of test species:	Not stated

#### 4. Culture conditions of test organism(s)

Culture medium:	<i>Myriophyllum</i> spp. cultured according to standard methods (ASTM, 1999); <i>L. gibba</i> cultured axenically according to Greenberg et al. (1992) with Hunter's media containing 10 g/l sucrose.
Temperature:	25:20°C during light and dark phases
Photoperiod:	16 h light:8 h
Light intensity:	Not stated
pH:	pH 5.8
Oxygen saturation:	Not stated

Acclimatisation prior to testing:	The test conditions appear to be similar to the culture conditions, thus acclimatization was not necessary. However, approximately 10 days prior to a <i>L. gibba</i> toxicity test, plants were transferred from growth media containing sucrose to media without sucrose. This was done so that the plants would switch from heterotrophic to autotrophic energy production.
Observations during acclimatisation:	Not stated

## B. Study design and methods:

### 1. Test procedure

Test system:

Test concentration(s):

*Myriophyllum* spp.: 10, 30, 100, 300, 1000, 3000, 10,000 mg/L.

*Lemna gibba*: 10, 30, 100, 300, 1000, 3000 mg/L

Control(s):

Yes: Test media without test item

Number of replicates:

*Myriophyllum* spp.: Controls: n = 10; exposed plants: n = 5 per treatment. *Lemna gibba*: Controls: n = 5; treated plants: n = 3

Test conditions:

*Myriophyllum* spp.: Conducted axenically in the environmental growth chamber for 14 days and under the environmental conditions described above. All plants were trimmed to a 3 cm apical length so that all plants would have the same initial status, with no roots or side shoots evident. Range-finding studies were conducted and used to determine the final range of concentrations chosen for the definitive tests (see above). At the end of the 14-day test period, plants were evaluated for several parameters (see below).

*Lemna*: Each test solution (see above) was transferred to a 10-ml plastic Petri dish and two plants, each with four fronds, for a total of eight fronds, were introduced and monitored. Tests were conducted in the growth chamber for 7 days and under environmental conditions described above.

Medium renewal:

*Myriophyllum* spp: No renewal reported

*Lemna gibba*: Test solutions were changed on day 3 and 5 to maintain consistent levels of the compound under study.

Frequency of test item application:

See above

Test duration:

*Myriophyllum* spp.: 14 days

*Lemna gibba*: 7 days

Endpoints:

*Myriophyllum* spp.: Plant length, node number, root number, total

	root length, longest root length, wet mass, drymass, and chlorophyll a, chlorophyll b, and carotenoid concentrations
	<i>Lemna gibba</i> : frond number, colony number, wet mass, frond mass, frond growth rate and chlorophyll a, chlorophyll b, and total chlorophyll concentrations.
Statistics:	<p><u>Regression analysis</u>: Data evaluated from toxicity testing with all three plant species were evaluated using non-linear regression techniques described in Stephenson et al. (2000). Only new growth (e.g., shoot length, wet/dry mass, nodes) was used in the models so that a more sensitive and conservative estimate of toxicity was obtained.</p> <p><u>NOEC / LOEC calculations</u>: NOEC and LOEC were calculated with a one-way ANOVA in a completely randomized design in SAS Version 8.2 (SAS Institute, Cary NC, USA) using General Linear Models with no adjustments for new growth as was done for the nonlinear regression analysis.</p>

## 2. Measurements during the test

Water/medium parameters: Not stated

## 3. Sampling

Sampling frequency: *Myriophyllum* spp.: Endpoints were evaluated at the end of the test (after 14 days).

*Lemna gibba*: Not stated / most probably endpoints were only evaluated at the end of the test (after 7 days)

Transport/storage of samples: Not stated

## 4. Chemical analysis

Guideline/protocol:	Concentrations of HAAs could not be verified analytically due to interference by the growth media with the ion chromatographic methods used to quantify the HAAs in other studies
Method:	See above
Pre-treatment of samples:	See above
Conduction:	See above
Reference item:	See above
Recovery:	See above
Limit of detection:	See above
Limit of quantification:	See above

## RESULTS

1. Validity criteria:

Not stated

2. Analytical findings:

Concentrations of HAAs could not be verified analytically due to interference by the growth media with the ion chromatographic methods used to quantify the HAAs in other studies.

3. Other measurements:

Please refer to point 3 'Biological findings'. Measurement of other parameters was not reported.

4. Biological findings:

TFA was the least toxic compound to *Myriophyllum* spp. with EC<sub>50</sub> values ranging from 222.1 to > 10000 mg/L depending on the endpoint. *L. gibba* was less sensitive to TFA toxicity than *Myriophyllum* spp., with EC<sub>50</sub> values ranging from 618.3 to > 3000 mg/L. Overall, mass and root measures tended to be the most sensitive indicators of HAA toxicity.

Laboratory-derived EC<sub>x</sub> values with 95 % confidence intervals for 14 day *Myriophyllum sibiricum* toxicity tests with TFA

Endpoint	EC <sub>10</sub>	EC <sub>25</sub>	EC <sub>50</sub>	Model	Variables	r <sup>2</sup>
Plant length	31.8 (0, 64.1)	155.9 (53.0, 258.7)	765.0 (444.7, 1085.3)	Logistic	$t=4.943$ $x=765.001$ $b=0.691$	0.88
Node number	97.1 (0, 203.2)	392.2 (121.1, 633.3)	1583.6 (897.5, 2269.7)	Logistic	$t=17.876$ $x=1583.553$ $b=0.787$	0.83
Root number	90.5 (24.0, 157.0)	251.7 (130.5, 372.9)	700.0 (477.9, 922.1)	Logistic	$t=8.446$ $x=700.020$ $b=1.074$	0.91
Root length	81.7 (18.7, 144.7)	166.9 (83.6, 250.1)	340.7 (224.4, 456.9)	Logistic	$t=34.163$ $x=340.657$ $b=1.539$	0.88
Longest root length	91.0 (26.2, 155.9)	237.2 (126.1, 348.3)	618.1 (425.6, 810.7)	Logistic	$t=6.806$ $x=618.135$ $b=1.147$	0.91
Wet mass	36.3 (3.5, 69.1)	113.8 (45.8, 181.8)	357.0 (216.3, 497.6)	Logistic	$t=436.060$ $x=356.991$ $b=0.961$	0.88
Dry mass	21.9 (0, 52.7)	134.1 (12.5, 255.6)	822.6 (354.0, 1291.2)	Logistic	$t=73.885$ $x=822.621$ $b=0.606$	0.80
Chlorophyll <i>a</i>	4460.3 (1849.8, 7070.7)	7890.4 (6082.0, 9698.8)	13,958.4 (9702.7, 18214.2)	Logistic	$t=0.749$ $x=13958.416$ $b=1.926$	0.66
Chlorophyll <i>b</i>	> 10,000	> 10,000	> 10,000	nc <sup>a</sup>	nc	nc
Carotenoids	> 10,000	> 10,000	> 10,000	nc	nc	nc

<sup>a</sup> The effect measure could not be calculated for these endpoints.

Laboratory-derived EC<sub>x</sub> values with 95 % confidence intervals for 14 day *Myriophyllum spicatum* toxicity tests with TFA.

Endpoint	EC <sub>10</sub>	EC <sub>25</sub>	EC <sub>50</sub>	Model	Variables	r <sup>2</sup>
Plant length	43.4 (15.7, 71.1)	196.2 (115.3, 227.1)	886.6 (654.9, 1118.3)	Logistic	$t=6.698$ $x=886.599$ $b=0.728$	0.95
Node number	53.8 (1.6, 106.0)	225.8 (84.1, 367.6)	947.9 (570.5, 1325.3)	Logistic	$t=18.201$ $x=947.871$ $b=0.766$	0.87
Root number	88.5 (7.9, 169.1)	243.2 (97.9, 388.4)	668.0 (404.5, 931.6)	Logistic	$t=7.142$ $x=668.032$ $b=1.087$	0.87
Root length	37.9 (15.8, 59.9)	91.7 (56.8, 126.7)	222.1 (166.1, 278.2)	Logistic	$t=31.467$ $x=222.137$ $b=1.242$	0.95
Longest root length	52.4 (23.8, 81.0)	129.3 (83.0, 175.5)	318.8 (242.4, 395.1)	Logistic	$t=7.731$ $x=318.790$ $b=1.217$	0.95
Wet mass	41.8 (8.8, 74.8)	114.4 (55.0, 173.8)	312.9 (205.0, 420.8)	Logistic	$t=377.373$ $x=312.908$ $b=1.092$	0.90
Dry mass	46.3 (0, 95.4)	144.5 (51.6, 237.3)	450.3 (265.1, 635.5)	Logistic	$t=72.078$ $x=450.311$ $b=0.966$	0.77
Chlorophyll <i>a</i>	672.4 (0, 1478.7)	5052.5 (2343.9, 7761.2)	37,965.4 (2877.0, 73053.7)	Logistic	$t=0.0963$ $x=37965.380$ $b=0.545$	0.68
Chlorophyll <i>b</i>	> 10,000	> 10,000	> 10,000	nc <sup>a</sup>	nc	nc
Carotenoids	> 10,000	> 10,000	> 10,000	nc	nc	nc

<sup>a</sup> The effect measure could not be calculated for these endpoints.

Laboratory-derived EC<sub>x</sub> values with 95 % confidence intervals for 7 day *Lemna gibba* toxicity tests with TFA

Endpoint	EC <sub>10</sub>	EC <sub>25</sub>	EC <sub>50</sub>	Model	Variables	r <sup>2</sup>
Frond number	388.8 (306.9, 470.8)	512.3 (407.9, 616.8)	884.0 (654.3, 1113.6)	Hormetic	$t = 59.415$ $h = 0.011$ $x = 883.961$ $b = 0.829$	0.94
Colony number	541.1 (407.2, 675.0)	693.2 (516.3, 870.1)	1140.4 (757.5, 1524.3)	Hormetic	$t = 17.876$ $h = 0.009$ $x = 1140.410$ $b = 0.897$	0.87
Wet mass	192.8 (104.1, 281.5)	298.5 (191.0, 406.0)	618.3 (421.1, 815.5)	Hormetic	$t = 265.412$ $h = 0.009$ $x = 618.269$ $b = 0.662$	0.91
Frond mass	11.2 (0, 44.2)	506.6 (0, 1189.8)	22965.3 (0, 70230.3)	Logistic	$t = 3.940$ $x = 22965.257$ $b = 0.288$	0.71
Growth rate	445.2 (342.8, 547.6)	790.4 (638.5, 942.3)	2505.2 (1761.1, 3249.3)	Hormetic	$t = 0.445$ $h = 0.017$ $x = 2505.208$ $b = 0.361$	0.95
Chlorophyll <i>a</i>	> 3000	> 3000	> 3000	nc <sup>a</sup>	nc	
Chlorophyll <i>b</i>	> 3000	> 3000	> 3000	nc	nc	
Total chlorophyll	> 3000	> 3000	> 3000	nc	nc	

<sup>a</sup> The effect measure could not be calculated for these endpoints.

NOEC for *Myriophyllum sibiricum* exposed to HAAs including TFA. Values in brackets are the percent change from control as either stimulation (+) or inhibition (-) for untransformed data.

Endpoint	MCA	DCA	TCA	TFA	CDFA
Plant length	10 (-44) <sup>a</sup>	10 (-7) <sup>b</sup>	10 (+1)	100 (-6)	30 (-7)
Node number	10 (-38) <sup>a</sup>	10 (-2)	10 (-4)	100 (+1)	30 (-5)
Root number	5 (-22) <sup>a</sup>	100 (-41) <sup>a</sup>	100 (-51) <sup>a</sup>	100 (-7)	300 (-58) <sup>a</sup>
Root length	5 (-32) <sup>a</sup>	100 (-51) <sup>a</sup>	100 (-57) <sup>a</sup>	100 (-12) <sup>a</sup>	300 (-76) <sup>a</sup>
Longest root length	5 (-14) <sup>a</sup>	100 (-34) <sup>c</sup>	30 (-19)	100 (-3)	300 (-45) <sup>a</sup>
Wet mass	2.5 (-4)	10 (-9)	3 (+7)	100 (-10) <sup>b</sup>	10 (+4)
Dry mass	5 (-17)	10 (-11)	10 (-9)	100 (-7)	10 (+2)
Chlorophyll <i>a</i>	10 (-54) <sup>a</sup>	100 (+4)	1000 (-49) <sup>a</sup>	3000 (-5)	1000 (0)
Chlorophyll <i>b</i>	10 (-58) <sup>a</sup>	100 (+6)	1000 (-34) <sup>a</sup>	3000 (+113)	3000 (-5)
Carotenoids	10 (-53) <sup>a</sup>	100 (+4)	1000 (-31) <sup>a</sup>	3000 (0)	3000 (-1)

Values in parentheses are the percentage change from control as either stimulation (+) or inhibition (-) for untransformed data.

<sup>a</sup> This analysis was run as a non-parametric Kruskal–Wallis on Ranks.

<sup>b</sup> The data were ln transformed.

<sup>c</sup> The data were square transformed.

NOEC for *Myriophyllum spicatum* exposed to HAAs including TFA. Values in brackets are the percent change from control as either stimulation (+) or inhibition (-) for untransformed data.

Endpoint	MCA	DCA	TCA	TFA	CDFA
Plant length	5 (-6)	10 (-4)	30 (-20) <sup>a</sup>	30 (-5)	10 (+1)
Node number	5 (-6)	10 (-7)	3 (0)	100 (-2)	30 (-5)
Root number	2.5 (-12)	10 (-23) <sup>a</sup>	10 (-4)	100 (-18)	300 (-63) <sup>a</sup>
Root length	5 (-33) <sup>a</sup>	3 (-3)	10 (-17) <sup>a</sup>	30 (-7) <sup>a</sup>	30 (-24) <sup>b</sup>
Longest root length	10 (-49) <sup>a</sup>	10 (-13)	300 (-43)	30 (-1)	300 (-49) <sup>a</sup>
Wet mass	5 (-17) <sup>b</sup>	3 (-1)	10 (-12)	30 (-3)	10 (0)
Dry mass	10 (-45) <sup>a</sup>	3 (+4)	10 (-4)	100 (-16)	30 (-5)
Chlorophyll <i>a</i>	10 (-31) <sup>a</sup>	300 (-18)	30 (-15)	300 (-5)	1000 (-13)
Chlorophyll <i>b</i>	10 (-30) <sup>a</sup>	300 (-4)	300 (-20)	> 10000 (0)	3000 (-10)
Carotenoids	10 (-32) <sup>a</sup>	300 (-8)	300 (-20)	> 10000 (+2)	3000 (-5)

<sup>a</sup> This analysis was run as a non-parametric Kruskal–Wallis on Ranks.

<sup>b</sup> The data were square root transformed.

NOEC for *Lemna gibba* exposed to HAAs including TFA. Values in brackets are the percent change from control as either stimulation (+) or inhibition (-) for untransformed data.

Endpoint	MCA	DCA	TCA	TFA	CDFA
Frond number	10 (-6)	50 (+9)	30 (+8)	300 (+5)	30 (+1)
Colony number	10 (-3)	100 (-21)	100 (-19)	< 1000	100 (+2) <sup>c</sup>
Wet mass	3 (-9)	50 (-16)	100 (-17) <sup>b</sup>	100 (+6)	30 (0) <sup>c</sup>
Frond mass	3 (-12)	25 (0)	> 800 (+19) <sup>b</sup>	30 (-11)	100 (-11)
Growth rate	10 (-3)	50 (+5)	30 (+9) <sup>c</sup>	300 (+3)	30 (0)
Chlorophyll <i>a</i>	20 (-16) <sup>a</sup>	400 (0) <sup>a</sup>	nc <sup>d</sup>	3000 (+9) <sup>b</sup>	1000 (+5)
Chlorophyll <i>b</i>	20 (-7) <sup>a</sup>	400 (0) <sup>a</sup>	nc <sup>d</sup>	3000 (+7)	1000 (+4)
Total chlorophyll	20 (-14) <sup>a</sup>	400 (0) <sup>a</sup>	nc <sup>d</sup>	3000 (+9) <sup>b</sup>	1000 (+5)

<sup>a</sup> This analysis was run as a non-parametric Kruskal–Wallis on Ranks.

<sup>b</sup> The data were reciprocal transformed.

<sup>c</sup> The data were square transformed.

<sup>d</sup> Only the 100 mg/l TCA showed a significant difference from control, with concentrations on both sides not being significantly different from controls.

<sup>e</sup> The data were ln transformed.

## RESULTS SUMMARY

Under the conditions of this study, the overall lowest 14 day EC<sub>50</sub> of *Myriophyllum* spp. was 222.1 mg TFA/L (based on root length) and the NOEC was established at 30 mg TFA/L. For *Lemna gibba*, the overall lowest 7 day EC<sub>50</sub> was 618.3 mg TFA/L (based on wet mass) and the NOEC was established at 30 mg TFA/L (based on front mass). In conclusion, tested HAAs including TFA do not exhibit a high degree of toxicity to *Myriophyllum* spp. or *L. gibba* under laboratory conditions. In general, *L. gibba* was less sensitive to TFA toxicity than *Myriophyllum* species.

### RMS comments

The study is taken from the open literature. No full report is available and many informations are missing. Moreover, chemical analysis aimed on the verification of the concentrations of the test item was not performed. That was explained by the fact of the strong matrix effect of the growth medium. Since, no raw data was available; the validity criteria could be not checked. In opinion of RMS the study can be considered as additional information only.

## B.9.3.EFFECTS ON ARTHROPODS

### B.9.3.1. Effects on bees

#### B.9.3.1.1. Acute toxicity to adult honeybees

For the first EU approval of the active substance several acute oral and contact studies with the active substance were submitted and evaluated in the DAR.

The new studies for active substance flufenacet were submitted for the process of renewal. These studies were performed according to current OECD test guidelines - OECD 213 and 214 (1998). The toxicity endpoints obtained from these studies LD<sub>50</sub> > 109.2 µg a.s./bee and LD<sub>50</sub> > 100 µg a.s./bee was considered valid and appropriate for the risk assessment.

Moreover, an acute contact toxicity study in bumble bees has been conducted in order to benchmark potential sensitivity differences to honey bees. One new study with the representative formulation DFF+FFA SC 600 was submitted for the renewal.

In addition, a chronic 10 day adult feeding limit test was conducted with technical flufenacet as well as bee brood feeding test in order to investigate potential side effects of flufenacet on immature honey bee life stages. The respective study summaries are presented below:

Table B.9.3.1.1-1: Summary of the results

Test substance	The toxicity endpoints		Reference
Acute oral and contact toxicity (laboratory) in honey bees			
Flufenacet, tech.	LD <sub>50</sub> -contact 24 h	> 25 µg a.s./bee	Mayer (1994) M-004922-01-1 <sup>1</sup>
Flufenacet, tech.	LD <sub>50</sub> -oral 48 h LD <sub>50</sub> -contact 48 h	> 340.4 µg a.s./bee > 400 µg a.s./bee	Tornier (1995) M-004920-01-1
Flufenacet, tech.	LD <sub>50</sub> -oral 48 h LD <sub>50</sub> -contact 48 h	> 175.56 µg a.s./bee > 200 µg a.s./bee	Nengel (1995) <sup>2</sup> M-004919-01-1
Flufenacet, tech.	LD <sub>50</sub> -contact 48 h	> 25 µg a.s./bee	Mayer (1996) <sup>3</sup> M- 004918-01-1
Flufenacet, tech.	LD <sub>50</sub> -oral, 48 h LD <sub>50</sub> -contact, 48 h	> 109.2 µg a.s./bee > 100 µg a.s./bee	Schmitzer (2011) <sup>4</sup> M-421687-01-1
Acute contact toxicity (laboratory) in bumble bees			
Flufenacet, tech.	LD <sub>50</sub> -contact, 48 h	LD <sub>50</sub> > 100 µg a.s./bee	Vergé (2014) M-478564-01-1
Chronic toxicity in adult honey bees (laboratory)			
Flufenacet, tech.	10 d chronic adult feeding study	LD <sub>50</sub> > 4.42 µg a.s./bee/d NOEC =4.42 µg a.s./bee/d	Kling (2014) M-477339-01-1
Bee brood feeding test			
Flufenacet SC 508.8	Honey bee brood feeding (Oomen <i>et al.</i> , 1992)	No adverse effects on mortality, bee brood development (eggs, young larvae, old larvae, pupae) and colony development by feeding honey bee colonies sugar syrup with a flufenacet -concentration typical for/exceeding the concentration of flufenacet in the spray tank (1500 ppm).	Hecht-Rost (2012) M-456504-01-1

1 Study was not considered valid.

2 The endpoint is the same as determined during the previous evaluation.

3 The study listed as one of the studies submitted for the current assessment, but RMS was not able to find its full report. RMS stated that the dossier contains the alternative studies, performed fully in line with the current Guidelines. Therefore RMS is of the opinion that there is no need to take this study into account, nor to require its equivalent, because the available data base is sufficient to finalise the whole assessment, even without it.

4 New endpoint for the active substance.

**B.9.3.1.1.1. Acute oral toxicity of flufenacet tech. on bees.**

<b>Reference:</b>	FOE 5043/Honey bees acute toxicity.
<b>Author(s), year:</b>	Mayer D. F., 1994
<b>Report/Doc. number:</b>	Study Number, Report No.: 106765, Reference No. M-004922-01-1
<b>Guideline(s):</b>	EPA Guideline 141-1, 1982
<b>GLP:</b>	Yes

Material and methods:

Test substance:	FOE 5043 technical, batch No: not reported, purity: 95.5%
Reference:	None
Solvent:	Acetone
Test species:	<i>Apis mellifera L.</i> , adult worker honeybees
Type of test:	Acute contact toxicity test
Test cages:	The screen cages made with the tops and bottoms of 150 x 15 mm plastic petri plates. Wire screen was cut into a strip 46 x 5 cm and the ends stapled to form a cylinder. Petri plates serve as top and bottom of the cage.
Number of organism:	three replicates with 10 bees for the control and test item treatment groups
Food:	Bees were fed during testing by providing cotton squares (5x5 cm) soaked with 50 percent sugar syrup and placed in the cages
Test condition:	Temperature: 70-80 F (equivalent to 21-27°C) Relative humidity: not reported Photoperiod: not reported
Test parameter:	Mortality counts were made after exposure for 24 h.
Applied concentration:	Control: acetone Test item: 5, 10, 15, 20 and 25 µg a.s./bee
Exposure:	A 2 µL droplet of the appropriate solution of the test item dissolved in acetone was administered to the thoracic surface of CO <sub>2</sub> -anaesthetised bees with a handheld applicator. Solvent control bees were similarly dosed with pure acetone. Solvent control bees were similarly dosed with pure acetone.
Statistic:	Even at the highest tested concentration neither in the oral nor in the contact toxicity test a 50 % mortality was reached. By this, the exact LD <sub>50</sub> could not be calculated.

Findings:

Contact test:	After 24 hours the 10% mortality was observed in the highest test concentration and in the control.
---------------	---

Effects on the survival of the honeybees see Table B.9.3.1.1.1-1.

**Table B. 9.3.1.1.1-1: Effects of flufenacet tech. on *Apis mellifera* following 48-h contact exposure in an acute toxicity test (averages from 3 replicates per dosage/control).**

Nominal dose [µg a.s./bee]	Mortality [%]
	24h
Control (acetone)	10
Treatment	
5	0
10	0
15	0
20	10
25	0

**Conclusion:**

24 h LD<sub>50</sub> > 25 µg a.s./bee (contact toxicity)

**Comment RMS:**

The study was conducted according to the US EPA 170 test guideline (1985). In the US EPA guideline (1985) no validity criteria are stated.

The validity criteria given in the current valid guideline according to OECD 214 (1998) was used to validate the acute contact toxicity study. The mean mortality of the control after 24 hour was maximal 10 %. However, no mortality after 48 hour was determined as recommended in the OECD 214 test guideline.

In addition no toxic reference was used to address the sensitivity of the test.

In general, the study is very poor reported. No information is given regarding the humidity, starving period, photoperiod and sublethal effects after application of test item.

Under consideration of the deficiencies of the study results the study is considered **not valid**.

**B.9.3.1.1.2. Assessment of Side Effects of FOE 5043 (tech.) to the Honey bee, *Apis mellifera* L. in the laboratory.**

<b>Reference:</b>	Assessment of Side Effects of FOE 5043 (tech.) to the Honey bee, <i>Apis mellifera</i> L. in the Laboratory.
<b>Author(s), year:</b>	Nengel, S., 1995
<b>Report/Doc. number:</b>	Report No: 94137/01-BLEU, Reference No. M-004919-01-1
<b>Guideline(s):</b>	EPPO No. 170, 1992
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet :FOE 5043,tech., CAS No: 142459-58-3, Batch No: 898313105, purity: 96.90%
Reference:	Rogor ( a.s.: Dimethoate), Batch No: 1019401, 404.g s.a./l Tested concentration: 0.25 µ.g a.s./bee (oral test), 0.16 µ.g a.s./bee (contact test)
Solvent:	Acetone
Test species:	<i>Apis mellifera</i> L., adult worker honeybees
Type of test:	Acute oral and contact toxicity test
Test cages:	In both test procedures the bees were kept in cages (width: 10 cm; depth: 5.5 cm; height: 8.5 cm). The front side of the cages were equipped with a glass pane so that the bees could be observed. The bottom of the cages consisted of a perforated board, which guaranteed sufficient air supply for the test animals. The test cages were lined with filter paper.
Number of organism:	Three replicates with 10 bees for control and test item treatment groups
Food:	50% sucrose solution. Before the feeding started the bees starved of half an hour.
<u>Oral toxicity test:</u>	
Applied concentration:	Control: 50% (w/w) aqueous sucrose solution with solvent. Test item: 12.5, 25, 50 and 100, and 200 µg a.s./bee
Exposure route:	The test item was dissolved in acetone and a 50% sucrose solution and 250 µL was offered to each bees. Once the treatment had been consumed (after approximately 2 hours), the feeder was removed and untreated 50% sucrose solution fed ad libitum for the duration of the study.
Test condition:	Temperature: 24-28°C, Relative humidity: 30 - 70 %, Darkness (except during observation).

Test parameter:	Mortality counts and checks for behavioural abnormalities were made after exposure for 1, 4, 24 and 48 h.
<u>Contact toxicity test:</u>	
Applied concentration:	Control: 50% (w/w) aqueous sucrose solution with solvent Test item: 12.5, 25, 50 and 100, 200 µg a.s./bee
Exposure:	A 4 µL droplet of the appropriate solution of the test item dissolved in acetone was administered to the thoracic surface of CO <sub>2</sub> -anaesthetised bees with a handheld applicator. Solvent control bees were similarly dosed with pure acetone. After application the bees were returned to the test cages and feed with 50% v solution of sucrose ad libitum.
Test condition:	Temperature: 24°C-28°C, Relative humidity: 30 - 70 %, Darkness (except during observation).
Test parameter:	Mortality counts and checks for behavioral abnormalities (e.g. apathy, intensive cleaning, vomiting) were made after exposure for 1, 4, 24 and 48 h.
Statistic:	Even at the highest tested concentration neither in the oral nor in the contact toxicity test a 50 % mortality was reached. By this, the exact LD <sub>50</sub> could not be calculated
<u>Findings:</u>	Oral toxicity test: Up to the highest concentration tested 200 µg a.s./bee (nominal), the mortality after 48 hours did not exceed 10.0 %. In the control a mortality of 6.7 % had been observed.  No test item induced behavioural effects were observed at any time in the oral toxicity test

Effects on the survival of the honeybees see Table B.9.3.1.1.2-1.

**Table B. 9.3.1.1.2-1: Effects of flufenacet tech. on *Apis mellifera* following 48-h oral exposure in an acute toxicity test (averages from 3 replicates per dosage/control).**

Nominal dose (consumed) [µg a.s./bee]	Mortality [%]				
	1 h	2h	4h	24h	48h
Control (sugar solution+ solvent)	0		0	0	6.7
Toxic reference „Rogor” (s.a.: dimethoate) 0.25	0	0	90	98.3	100
Treatment					
12.5 (14.21)	0	0	0	0	6.7
25 (28.46)	0	0	0	0	10
50 (51.81)	0	0	0	0	6.7
100 (100.52)	0	0	0	0	0
200 (175.56)	0	0	0	0	6.7
Test substance 24/48h LD <sub>50</sub> >200 µg prod./bee ( nominal) Test reference item 24/48h LD <sub>50</sub> >0.25 µg a.s./bee ( nominal),					

Contact toxicity test: Up to the highest concentration tested 200 µg a.s./bee (nominal), the mortality after 48 hours did not exceed 10.0 %. In the control group a mortality of 10 % had been observed.

No test item induced behavioural effects were observed at any time in the contact toxicity test.

Effects on the survival of the honeybees see Table B.9.3.1.1.2-2.

**Table B. 9.3.1.1.2-2: Effects of flufenacet tech. on *Apis mellifera* following 48-h contact exposure in an acute toxicity test (averages from 3 replicates per dosage/control).**

Nominal dose [µg a.s./bee]	Mortality [%] (no. dead bees/no. treated bees)				
	1 h	2 h	4h	24h	48h
Control (sugar solution + solvent)	0		0	0	10
Toxic reference, „Rogor “ ( a.s.: Dimetoathe) 0.16		3.33	96.6	96.6	100
Treatment					
12.5	0	0	0	0	3.33
25	0	0	0	0	6.7
50	0	0	0	0	6.7
100	0	0	0	0	10
200	0	0	0	0	6.7
Test substance 24/48h LD <sub>50</sub> >200 µg prod./bee ( nominal), Test reference item 24/48h LD <sub>50</sub> >0.16 µg a.s./bee ( nominal)					

**Conclusion:**

48 h LD<sub>50</sub> > 175.56 µg a.s./bee (oral toxicity), correspond to 48 h LD<sub>50</sub> > 170 µg a.s./bee (oral toxicity)

48 h LD<sub>50</sub> > 200 µg a.s./bee (contact toxicity), correspond to 48 h LD<sub>50</sub> > 194 µg a.s./bee (oral toxicity)

**Comment RMS:**

The study was conducted according to the EPP0 170 test guideline (1992). The validity criteria given in the current valid guidelines according OECD 213 and 214 (1998) were used to validate the acute oral and contact toxicity study. The mean mortality of the control in the oral and contact toxicity test was maximal 10 % which is in the line with the recommended maximum mortality of 10 % according to the OECD guidelines.

It was noted that concentration of the toxic standard used in the acute oral and contact study was such, that it caused almost 100% mortality within 24 h/48h period for rate 0.25 µg a.s./bee (oral test ) and 0.16 µg a.s./bee (contact test). As a result it may be stated that the value of LD<sub>50</sub> of the reference item (dimethoate) were within the recommended range of 0.10 - 0.35 µg a.s./bee ( oral test) and of 0.10-0.30 µg a.s./bee ( contact test).

Some deviations to the OECD guidelines were identified. However, these deviations are not considered of relevance for the results of the acute oral and contact toxicity test.

The relative humidity in the oral and contact toxicity test is between 30 and 70% which is below the

recommendations given in the OECD guidelines (relative humidity between 50 and 70%).

Based on the results of the oral and contact toxicity test the deviation of relative humidity is considered to have no impact on the honeybees. In the oral toxicity test the bees were starved for 30 minutes only and not for 2 hours as recommended in the OECD guideline. However, this may not have an effect on the results of the study considering the amount of consumed sugar solution. In addition, 250 µg /l of 50% sucrose solution containing test substance was offered to bees instead of 100-200 µg /l as is recommended in test guideline.

In the contact toxicity test a 4 µL droplet was used in deviation to the OECD guideline recommendation of a 1 µL droplet.

This deviation is considered acceptable since a higher volume ensured a more reliable dispersion of the test item. The study is considered acceptable.

**Agreed endpoints:**

**48 h LD<sub>50</sub> > 170 µg a.s./bee (oral toxicity)**

**48 h LD<sub>50</sub> > 194 µg a.s./bee (contact toxicity)**

**B. 9.3.1.1.3. Results of the screening test on the honey bee *Apis mellifera* L.**

<b>Reference:</b>	Results of the screening test on the honey bee <i>Apis mellifera</i> L.
<b>Author(s), year:</b>	Tornier L., 1995
<b>Report/Doc. number:</b>	Study Number: B-958264, Reference No. M-004920-01-1
<b>Guideline(s):</b>	EPA Guideline Number 141-1.
<b>GLP:</b>	No

Material and methods:

Test substance:	FOE 5054 tech., Batch No: 898313195, purity: not reported
Reference:	Rogor ( a.s.: Dimethoate), Batch No: 1019401, 404.g s.a./l
	Tested concentration: 0.20 µ.g a.s./bee
Solvent:	Acetone
Test species:	<i>Apis mellifera</i> L., adult worker honeybees Acute oral and contact toxicity test.
Type of test:	Acute oral and contact toxicity test
Test cages:	In both test procedures the bees were kept in cages (width: 10 cm; depth: 5.5 cm; height: 8.5 cm). The front side of the cages were equipped with a glass pane so that the bees could be observed. The bottom of the cages consisted of a perforated board, which guaranteed sufficient air supply for the test animals. The test cages were lined with filter paper.
Number of organism:	Three replicates with 10 bees for control and test item treatment groups
Food:	50% sucrose solution. Before the feeding started the bees starved for about 2 hours.

Oral toxicity test:

Applied concentration:	Control: 50% (w/w) aqueous sucrose solution with solvent. Test item: 12.5, 25, 50 and 100 and 200 µg a.s./bee
Exposure route:	The test item was dissolved in acetone and a 50% sucrose solution and 250 µL was offered to each bees. Once the treatment had been consumed (after approximately 2 hours), the feeder was removed and untreated 50% sucrose solution fed <i>ad libitum</i> for the duration of the study.
Test condition:	Temperature: 25°-28°C, Relative humidity: 40 - 60 %, Darkness (except during observation).
Test parameter:	Mortality counts and checks for behavioural abnormalities were made after exposure for 1, 4, 24 and 48 h.

Contact toxicity test:

Applied concentration:	Control: 50% (w/w) aqueous sucrose solution with solvent Test item: 12.5, 25, 50 and 100 and 200 µg a.s./bee
Exposure:	A 1 µL droplet of the appropriate solution of the test item dissolved in acetone was administered to the thoracic surface of CO <sub>2</sub> -anaesthetised bees with a handheld applicator. Solvent control bees were similarly dosed with pure acetone. After application the bees were returned to the test cages and feed with 50%v solution of sucrose <i>ad libitum</i> .
Test condition:	Temperature: 25-28°C, Relative humidity: 40 - 60 %, Darkness (except during observation).
Test parameter:	Mortality counts and checks for behavioral abnormalities were made after exposure for 1, 4, 24 and 48 h.
Statistic:	Even at the highest tested concentration neither in the oral nor in the contact toxicity test a 50 % mortality was reached. By this, the exact LD <sub>50</sub> could not be calculated.

Findings:

<u>Oral toxicity test:</u>	Up to the highest concentration tested 400 µg a.s./bee (nominal), the mortality after 48 hours did not exceed 3.3 %. In the control no mortality had been observed.  The mortality in the toxic standard amounted to 100 % after 24 and 48 hours. No test item induced behavioural effects were observed at any time in the oral toxicity test.
----------------------------	---

Effects on the survival of the honeybees see Table B.9.3.1.1.3-1.

**Table B. 9.3.1.1.3-1: Effects of flufenacet tech. on *Apis mellifera* following 48-h oral exposure in an acute toxicity test (averages from 3 replicates per dosage/control)**

Nominal dose (consumed) [µg a.s./bee]	Mortality [%]	
	24h	48h
Control (sugar solution + solvent)	0	0
Toxic reference Rogor (a.s.:dimethoate) 0.43 (0.20)	100	100
Treatment		
25.5 (16.2)	0	3.3
50 (35.2)	0	0
100 (81.9)	0	0
200 (168.2)	0	0
400 (340.4)	0	3.3

Contact test:

Up to the highest concentration tested 400 µg a.s./bee (nominal), the mortality after 48 hours did not exceed 3.3 %. Corresponding with the results of the oral toxicity test no mortality was noticed in the control group.

The mortality in the toxic standard variant amounted to 100 % after 24 and 48 hours. No test item induced behavioural effects were observed at any time in the acute contact toxicity test.

Effects on the survival of the honeybees see Table B.9.3.1.1.3-2.

**Table B. 9.3.1.1.3-2: Effects of flufenacet tech. on *Apis mellifera* following 48-h contact exposure in an acute toxicity test (averages from 3 replicates per dosage/control).**

Nominal dose [µg a.s./bee]	Mortality [%]	
	24h	48h
Control (sugar solution + solvent)	0	10
Toxic reference „Rogor”(a.s.:Dimethoate) 0.20	100	100
Treatment		
25.5	0	3.3
50	0	0
100	0	3.3
200	3.3	3.3
400	3.3	3.3

## Conclusion:

48 h LD<sub>50</sub> > 340.4 µg a.s./bee (oral toxicity)

48 h LD<sub>50</sub> > 400 µg a.s./bee (contact toxicity)

**Comment RMS:**

The study was conducted according to the EPP0 170 test guideline (1992). The validity criteria given in the current valid guidelines according to OECD 213 and 214 (1998; were used to validate the acute oral and contact toxicity study. The mean mortality of the control in the oral and contact toxicity test was maximal 10 % which is in the line with the recommended maximum mortality of 10 % according to the OECD guidelines.

It was noted that concentration of the toxic standard used in the acute oral and contact study was such, that it caused 100% mortality within 24 h/48h period for rate 0.20 µg a.s./bee (oral and contact test ) As a result it may be stated that the value of LD<sub>50</sub> of the reference item (dimethoate) were within the recommended range of 0.10 - 0.35 µg a.s./bee (oral test) and of 0.10-0.30 µg a.s./bee (contact test).

Some deviations to the OECD guidelines were identified. The relative humidity in the oral and contact toxicity test is between 40 and 60% which is below the recommendations given in the OECD guidelines (relative humidity between 50 and 70%). Based on the results of the oral and contact toxicity test the deviation of relative humidity is considered to have no impact on the honeybees. In addition 250 µg/L of 50% sucrose solution containing test substance was offered to bees instead of 100-200 µg/L as is recommended in test guideline.

This deviation is considered acceptable since a higher volume ensured a more reliable dispersion of the test item.

In the study protocol only data of mortality after 24 and 48 hour was presented. No information of mortality after 1 and 4 hours were reported in the study. In addition no purity of active substance flufenacet was reported.

The deviations are but taking into consideration that the validity criteria was met, they are not considered of relevance for the results of the acute oral and contact toxicity test.

The study is considered acceptable.

**Agreed endpoints:**

48 h LD<sub>50</sub> > 340.4 µg a.s./bee (oral toxicity)

48 h LD<sub>50</sub> > 400 µg a.s./bee (contact toxicity)

#### B. 9.3.1.1.4. Effects of flufenacet tech. (Acute Contact and Oral) on Honey Bees (*Apis mellifera* L.) in the Laboratory.

<b>Reference:</b>	Effects of flufenacet tech. (Acute Contact and Oral) on Honey Bees ( <i>Apis mellifera</i> L.) in the Laboratory.
<b>Author(s), year:</b>	Schmitzer, S.; 2011
<b>Report/Doc. number:</b>	IBACON Project 6768103, Reference No. M-421687-01-1
<b>Guideline(s):</b>	OECD Guideline 213 and 214 (1998)
<b>GLP:</b>	Yes

#### Material and methods:

Test substance:	Flufenacet tech.: Batch No. AE F133402-01-02, purity: 97.5% w/w (analysed)
Reference:	Perfekthion (BAS 152 11 I), Batch No: 90924-06: s.a.:414.8 g dimethoate/L (analysed)
Solvent:	Acetone
Test species:	<i>Apis mellifera</i> L., adult worker honeybees
Type of test:	Acute oral and contact toxicity test
Test cages:	In both test procedures the bees were kept in cages (width: 10 cm; depth: 5.5 cm; height: 8.5 cm). The front side of the cages were equipped with a glass pane so that the bees could be observed. The bottom of the cages consisted of a perforated board, which guaranteed sufficient air supply for the test animals. The test cages were lined with filter paper.

Number of organism: 3 replicates with 10 honey bees each per test item dose level, controls and per reference item dose level, respectively.

Food: Commercial ready-to-use syrup (Apiinvert; 30 % sucrose, 31 % glucose, 39 % fructose) ad libitum.

Starvation Time: 20 minutes for all treatment groups in the oral test, prior to application.

#### Oral toxicity test:

<u>Applied concentration:</u>	Control: 50% (w/w) aqueous sucrose solution (50% tap water, 50% ready-to-use syrup) Solvent control: 50% (w/w) sugar solution with solvent (45% tap water, 5% acetone, 50% ready-to-use syrup). Test item: 100.0, 50.0, 25.0, 12.5 and 6.3 µg a.s./bee (nominal) Test item: 109.2, 54.3, 26.9, 13.8 and 6.8 µg a.i./bee (measured)  Reference item: 0.30, 0.20, 0.15, 0.05 µg dimethoate/bee (nominal)
-------------------------------	---

---

	Reference item: 0.27, 0.16, 0.08, 0.06 µg dimethoate/bee (measured)
Exposure route:	The test item was dissolved in acetone before the ready-to-use syrup was added and offered to the bees. The test bees were starved for 20 minutes before they were fed with the solutions. After 2 hours and 5 minutes, the feeding troughs were exchanged with clean feeders containing ready-to-use syrup and the retrieved containers re-weighed to determine the quantity of feed consumed.
Test condition:	Temperature: 25°C, Relative humidity: 48 - 83 %, Darkness (except during observation).
Test parameter:	Mortality counts and checks for behavioural abnormalities (e.g. apathy, intensive cleaning, vomiting) were made after exposure for 1, 4, 24 and 48 h.
<u>Contact toxicity test:</u>	
Applied concentration:	Control: tap water with 0.5% Adhäsit (wetting agent to improve spreading of the test droplet on the water-repellent hairs on the thorax of bees). Solvent control: Acetone (applied after anesthetization with CO <sub>2</sub> ) Test item: 100.0, 50.0, 25.0, 12.5 and 6.3 µg a.s./bee (nominal) Reference item: 0.30, 0.20, 0.15, 0.10 µg dimethoate/bee (nominal)
Exposure:	A 5.0 µL droplet of the appropriate solution of the test item dissolved in acetone was administered to the thoracic surface of CO <sub>2</sub> -anaesthetised bees with a handheld applicator. Solvent control bees were similarly dosed with a) tap water containing the wetting agent Adhäsit (0.5%) and b) pure acetone. The reference item was also applied in a 5 µL droplet (dimethoate made up in acetone). After application the bees were returned to the test cages and feed with ready-to use syrup <i>ad libitum</i> .
Test condition:	Temperature: 25°C, Relative humidity: 48 - 83 %, Darkness (except during observation).
Test parameter:	Mortality counts and checks for behavioral abnormalities were made after exposure for 1, 4, 24 and 48 h.
Statistic:	The contact and oral LD <sub>50</sub> values of the reference item were estimated using the binomial distribution (according to Stephan, 1977). If necessary, the LD <sub>50</sub> calculation was carried out taking into account the mortality data corrected by control mortality using Abbott's formula (1925). The NOED was estimated using Fisher Exact Test (pairwise comparison, one-sided greater, $\alpha = 0.05$ ). The software used to perform the statistical analysis was ToxRat Professional, Version 2.10.05, ® ToxRat Solutions GmbH.
<u>Findings:</u>	
<u>Oral test:</u>	Actual oral doses of 109.2, 54.3, 26.9, 13.8 and 6.8 µg a.s./bee resulted in no mortality in any of the dose levels until the end of the test (48 hours after

application). Also no mortality occurred in the solvent control group and in the water control group, respectively.

Contact test:

In the contact toxicity test, mortality occurred in the 50.0 and 25.0 µg a.s./bee dose levels, when one out of the 30 treated bees were found dead, respectively. In the other dose levels (100, 12.5 and 6.3 µg a.s./bee) no mortality occurred. 3.3 % mortality occurred in the water control group (water + 0.5 % Adhäsit) and there was no mortality in the solvent control group (acetone).

Effects on the survival of the honeybees see Table B.9.3.1.1.4-1 and Table B.9.3.1.1.4-2.

**Table B. 9.3.1.1.4-1: Effects of flufenacet tech. on *Apis mellifera* following 48-h oral exposure in an acute toxicity test (average from 3 replicates per dosage/control).**

Nominal dose (consumed) [µg a.s./bee]	%Effects					
	4 hours		after 24 hours		after 48 hours	
	mortality	behavioural abnormalities	mortality	behavioural abnormalities	mortality	behavioural abnormalities
Treatment						
100 (109.2)	0.0	0.0	0.0	0.0	0.0	0.0
50 (54.3)	0.0	0.0	0.0	0.0	0.0	0.0
25 (26.9)	0.0	0.0	0.0	0.0	0.0	0.0
12 (13.8)	0.0	0.0	0.0	0.0	0.0	0.0
6.3(6.8)	0.0	0.0	0.0	0.0	0.0	0.0
Control (sugar solution)	0.0	0.0	0.0	0.0	0.0	0.0
Solvent control (acetone)	0.0	0.0	0.0	0.0	0.0	0.0
Reference item						
0.27	0.0	100.0	100.0	0.0	100.0	0.0
0.16	0.0	23.3	100.0	0.0	100.0	0.0
0.08	0.0	0.0	0.0	0.0	6.0	30.0
0.06	0.0	0.0	0.0	0.0	0.0	0.0
Test substance: 24h / 48h LD <sub>50</sub> > 109.2 µg a.s./bee Test substance: 24h / 48h LD <sub>20</sub> > 109.2 µg a.s./bee Test substance: 24h / 48h LD <sub>10</sub> > 109.2 µg a.s./bee Reference: 24 h / 48 h LD <sub>50</sub> = 0.11 µg a.s./bee (95 % C.I: 0.08 – 0.16 µg a.s./bee)						

**Table B. 9.3.1.1.4-2. Mortality and behavioural abnormalities of the bees in the contact toxicity test.**

Nominal dose [µg a.s./bee]	% Effects					
	after 4 hours		after 24 hours		after 48 hours	
	Mortality	Behavioural abnormalities	Mortality	Behavioural abnormalities	Mortality	Behavioural abnormalities
Treatment						
100.0	0.0	0.0	0.0	0.0	0.0	0.0
50.0	0.0	3.3	3.3	0.0	3.3	0.0
25.0	0.0	0.0	3.3	0.0	3.3	0.0
12.5	0.0	0.0	0.0	0.0	0.0	0.0
6.3	0.0	0.0	0.0	0.0	0.0	0.0
Control (tap water+wetting agent)	0.0	0.0	3.3	0.0	3.3	0.0
Solvent control (acetone)	0.0	0.0	0.0	0.0	0.0	0.0
Reference item						
0.30	3.3	53.3	100.0	0.0	100.0	0.0
0.20	6.7	20.0	43.3	13.3	73.3	6.7
0.15	3.3	13.3	26.7	0.0	40.0	6.7
0.10	0.0	3.3	3.3	0.0	3.3	0.0
Test substance: 24h / 48h LD <sub>50</sub> > 100 µg a.s./bee Test substance: 24h / 48h LD <sub>20</sub> > 100 µg a.s.t./bee Test substance: 24h / 48h LD <sub>10</sub> > 100 µg a.s./bee Reference: 24 h LD <sub>50</sub> = 0.21 µg a.s./bee (95 % C.I: 0.15 – 0.30 µg a.s./bee) Reference: 48 h LD <sub>50</sub> = 0.16 µg a.s./bee (95 % C.I: 0.10 – 0.20 µg a.s./bee)						

Conclusion: 48 h LD<sub>50</sub> > 109.2 µg a.s./bee (oral toxicity)  
 48 h LD<sub>50</sub> > 100 µg a.s./bee (contact toxicity)

#### Comment RMS:

All validity criteria according to the OECD guidelines 213 and 214 are met.

The mean mortality of the controls (water and solvent) in the oral and contact toxicity test was maximal 3.3 % which is in the line with the recommended maximum mortality of 10 % according to the OECD guidelines.

The 24 h LD<sub>50</sub> values of the reference item (dimethoate) in the oral (24 h LD<sub>50</sub> = 0.11 µg a.s./bee) and contact (24 h LD<sub>50</sub> = 0.21 µg a.s./bee) toxicity tests were within the recommended range of 0.10 – 0.35 µg a.s./bee (oral) and 0.10 – 0.30 µg a.s./bee (contact), respectively.

Some deviations to the OECD guidelines were identified. The relative humidity in the oral and contact toxicity test is between 48 and 83% which is below the recommendations given in the OECD guidelines (relative humidity between 50 and 70%). Based on the results of the oral and contact toxicity test the deviation of relative humidity is considered to have no impact on the honeybees.

In the oral toxicity test the bees were starved for 20 minutes only and not for 2 hours as recommended in the OECD guideline.

However, this may not have an effect on the results of the study considering the amount of consumed sugar solution. In the contact toxicity test a 5 µL droplet was used in deviation to the OECD guideline recommendation of a 1 µL droplet. This deviation is considered acceptable since a higher volume ensured a more reliable

dispersion of the test item. The study is considered acceptable and the RMS is of the opinion that the results of the study should be used in the risk assessment.

**Agreed endpoints:**

**48 h LD<sub>50</sub> > 109.2 µg a.s./bee (oral toxicity)**

**48 h LD<sub>50</sub> > 100 µg a.s./bee (contact toxicity)**

**B. 9.3.1.1.5. Flufenacet (tech.): Acute contact toxicity to the bumble bee, *Bombus terrestris* L. under laboratory conditions.**

<b>Reference:</b>	Flufenacet (tech.): Acute contact toxicity to the bumble bee, <i>Bombus terrestris</i> L. under laboratory conditions.
<b>Author(s), year:</b>	Vergé E., 2014
<b>Report/Doc. number:</b>	Study No:S13-0176, Reference BCS No. M-478564-01-1
<b>Guideline(s):</b>	No specific guidelines are available, OEPP/EPPO 170 (4) (2010), OECD Guideline 214 (1998), and on the review article of VAN DER STEEN (2001).
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet tech., Batch No: NK61CX0650, Purity: 98.18 % w/w (analysed)
Reference:	Perfekthion, (BAS 152 11 I), Batch No: 0001017331 411.7 g dimethoate/L (analysed)
Solvent:	Acetone
Test species:	<i>Bombus terrestris</i> L. (Hymenoptera, Apidae)
Type of test:	Acute contact toxicity test
Test cages:	The bumble bees were kept in plastic boxes (base: 13 x 17 cm; height: 6 cm). The upper side of the boxes were equipped with a transparent perforated cover so that the bumble bees could be observed. The base of the test cages were lined with filter paper.
Number of organism:	Three replicates with 10 bees each for controls, the test item and toxic reference treatment group.
Food:	50% (w/v) aqueous sucrose solution.

Contact toxicity test:

Applied concentration:	Control: tap water with 0.5% aqueous sucrose solution.
------------------------	--

	Solvent control: Acetone
Test item:	100 µg a.s./bee (nominal) added to acetone
Reference item:	11 µg dimethoate/bumble bee
Exposure:	After the bumble bees had been anaesthetized with carbon dioxide for approximately 17 seconds, they were treated individually by topical application with a micro-applicator. An aliquot of 2 µL of the control, test and reference item solutions were applied dorsally to the thorax of each bumble bee. After the application, the bumble bees were returned to the test cages and fed with a 50 % aqueous sucrose solution ad libitum. Between each application, the outside of the micro applicator needle was cleaned with a mixture of water and a water-wetting agent. This reduced the surface tension of the applied solution and ensured that the drop of the test item solution spread out immediately after the application.
Test condition:	Temperature: 24.2-26.0°C, Relative humidity: 55.3 – 61.4 %, Darkness (except during observation).
Test parameter:	Mortality counts and checks for behavioral abnormalities were made after exposure for 24 and 48 h.

**Findings:** In both control groups treated either with tap water or acetone, no mortality was observed during the 48 h test period. In the test item treatment group, no mortality and no sub-lethal effects were observed until the final assessment 48 hours after start of the experimental phase.

#### B. 9.3.1.1.5-1: Mortality of the bumble bees

	Mortality	
Nominal dose	24 h	48 h
Nominal dose [µg a.s./bee]	0.0	0.0
Treatment 100.0	0.0	0.0
Control (tap water)	0.0	0.0
solvent control	36.7	60
reference item (dimethoate) 11		
LD <sub>50</sub> > 100 µg a.s. /bumble bee		

**Comment RMS:**

There is no guideline available for bumble bees at the moment of evaluation of study by RMS.

The recommendation given to EFSA the current OECD 214 guideline was used to evaluate the study provided by Applicant.

In this study the mean mortality of the controls (water and solvent) in the contact toxicity test was 0% which is in the line with the recommended maximum mortality of 10 % according to the 214 OECD guidelines.

The following deviations from the OECD 214 guidelines were identified:

- 2 µL droplet was used in deviation to the OECD 214 guideline recommendation of a 1 µL droplet.

This deviation is considered acceptable since a higher volume ensured a more reliable dispersion of the test item.

- the reference item group, mortality was  $\geq 50$  % at rate 11 µg dimethoate/bumble bees at the end of test (after 48 h). According to the guideline the 24 h LD<sub>50</sub> value for toxic reference should be at the range between 0.10 to 0.30 µg a.s./bee in the contact test.

In this study for application dose at 11 µg/bees no mortality >50 % was identified. After 24 h after application (being 36.7%) however after 48h the mortality was at level of 60%.

Due to the fact that the range of 24 h LD<sub>50</sub> of the reference item (dimethoate) was only a recommended value, it is the opinion of the RMS that the sensitivity of the test is confirmed after 48 hours which is considered to be sufficient to considered the study as acceptable.

**B. 9.3.1.2. Chronic toxicity to adult honeybees**

According to the data requirements for active substances (Commission Regulation (EU) 283/2013) and/or plant protection products (Commission Regulation (EU) 284/2013) the chronic risk to adult honeybees has to be evaluated. However, no valid test guidelines are available to address this point. In the draft EFSA guidance document on risk assessment on honeybees (EFSA Journal 2013;11(7):3295) a study protocol (Appendix O) is given as support on how to perform a chronic oral toxicity test. The protocol is based on information from Decourtye et al. (2005), Suchail et al. (2001), Thompson H. (Food and Environment Research Agency, 2012) and CEB (2012).

**B. 9.3.1.2.1. Flufenacet (tech.) - Assessment of chronic effects to the honeybee, *Apis mellifera* L., in a 10 days continuous laboratory feeding limit test.**

<b>Reference:</b>	Flufenacet (tech.) - Assessment of chronic effects to the honeybee, <i>Apis mellifera</i> L., in a 10 days continuous laboratory feeding limit test.
<b>Author(s), year:</b>	Kling A., 2014
<b>Report/Doc. Number:</b>	Study No: S13-00145, Doc. No: M-477339-01-1
<b>Guideline(s):</b>	(EFSA Journal 2013;11(7):3295) a study protocol (Appendix O) The protocol is based on information from Decourtye et al. (2005), Suchail et al. (2001), Thompson H. (Food and Environment Research Agency, 2012) and CEB (2012).
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet (tech.), Batch No: NK61CK0650, purity: 98.18 % w/w (analysed)
Reference:	None (no validated reference substance available for this type of study)
Solvent:	Acetone
Test species:	<i>Apis mellifera</i> L., adult worker honeybees (1 to 4 days old). Four days prior to test start, brood combs containing capped cells with an expected hatch on the same day were taken out of a honey bee colony and transferred into the climatic chamber. Additionally a pollen and honey comb was placed beside the brood combs as food for the hatched bees. One day prior to test start the 1 - 3 days old bees were picked off the combs, transferred to the test cages and kept under test conditions until test start. Moribund bees were rejected and replaced by healthy bees before starting the test.
Type of test:	Chronic 10 days continuous feeding test (limit test)

---

Test cages:	Cages made of stainless steel (base: 8 cm x 4 cm; height: 6 cm). The front side of the cages were equipped with a transparent pane so that the bees could be observed. The bottom of the cages consisted of a perforated board, which guaranteed sufficient air supply for the test organisms. The test units were lined with filter paper.
Number of organism:	Ten replicates (cages) with 10 bees each for controls and the test item treatment groups.
Food:	50% (w/v) aqueous sucrose solution.
Applied concentration:	Control: 50% (w/v) aqueous sucrose solution containing 3% acetone. Test item: 120 mg a.s./kg
Exposure:	Over a period of 10 days, honeybees were exposed to 50 % (w/v) aqueous sucrose application (feeding) solution, containing nominally 120 mg a.s./kg of the test item flufenacet by continuous and ad libitum feeding. Because the test item was first dissolved in acetone and then diluted with aqueous sucrose solution, the final test item application (feeding) solution contained 3 % acetone. The application (feeding) solutions were offered ad libitum to each cage of 10 bees in plastic syringes. Every morning the syringes of all test cages (i.e. test item and control) were replaced by new syringes, filled with freshly prepared application solution over a period of 10 days. The weight of the syringes was determined before and after feeding to determine the mean food consumption of the bees per replicate.
Test condition:	Temperature: 31.9-33.2°C, Relative humidity: 55.4-69.9 %. Darkness (except during observation).
Test parameter:	Mortality counts and checks for behavioural abnormalities (e.g. apathy, intensive cleaning, vomiting) were made every 24 hour during the 10 days test period.
Statistic:	Fisher's Exact Test (Bonferroni-Holms corrected, one-sided, $p \leq 0.05$ ) was used to evaluate whether there are significant differences between the mortality data of the test item treatment group and the control group and to determine the NOEC based on mortality.  For the statistical comparison of the food consumption, non-rounded mean values per replicate over the entire test period were taken.  Data of food consumption were statistically analysed by using the Mann-Whitney, t-Test Satterthwaite or t-Test pooled (left-sided, $p \leq 0.05$ ) depending on the results of the pre-tests of Shapiro Wilks and F-Test ( $p \leq 0.05$ ).  Statistical calculations were made by using the statistical program SAS release Version 9.2.

## Findings:

Analytical data:

The actual concentration of flufenacet in the feeding solutions, determined for each preparation day, was in the range from 86 to 95% of the nominal concentration.

## Test parameter:

After 10 days of continuous exposure, mortality at the test item treatment level of 120 mg a.s./kg of flufenacet (tech.) was not statistically significantly different when compared to the control group.

The cumulative control mortality was 0.0%, as determined at the final assessment after 10 days. The cumulative mortality at the treatment level of 120 mg a.s./kg flufenacet (tech.) was 3.0% at the final assessment.

At 120 mg a.s./kg flufenacet (tech.), no remarkable sub-lethal effects or behavioural abnormalities were observed throughout the entire observation period of 10 days.

After 10 days of continuous exposure, by considering the actual food consumption of the honey bees, the accumulated nominal intake of the test item flufenacet (tech.) at the treatment level of 120 mg a.s./kg was 44.2 µg a.s./bee, the corresponding average daily dose was therefore 4.4 µg a.s./bee.

The overall mean daily consumption of the application (feeding) solution (i.e. the average value over 10 days) in the test item treatment group was not statistically significantly different (lower) when compared to the untreated control group (36.8 mg/bee at 120 mg a.s./kg, compared to 38.4 mg/bee in the control group).

The mean daily consumption of the aqueous sucrose application (feeding) solution was not statistically significantly different (lower) between the control group and the test item treatment group throughout the entire testing period (day-by-day comparison), except for the first day and the 8<sup>th</sup> day of exposure.

Effects on the survival of the bumble bees see Table B.9.3.1.2-1.

**Table B. 9.3.1.2-1: Effects of flufenacet tech. on *Apis mellifera* following 10 days oral exposure in a chronic toxicity test.**

Nominal dose mg a.s./kg	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
<b>Cumulative mortality [%]</b>										
Control <sup>1</sup> (sugar+acetone)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Treatment <sup>2</sup> 120 mg a.s./kg	0.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0	3.0
<b>Mean consumption of feeding solution per day [mg/bee]</b>										
Control <sup>1</sup> (sugar+acetone)	41	34	35	41	37	38	38	40	38	42
Treatment 120 mg a.s./kg <sup>2</sup>	34*	36	35	37	35	37	38	36**	41	39
<b>Mean accumulated nominal intake of active substance [µg a.s./bee]</b>										
Control (sugar+acetone)	-	-	-	-	-	-	-	-	-	-
Treatment 120 mg a.s./kg	4.08	8.40	12.6	17.0	21.2	25.7	30.2	34.6	39.5	44.2

<sup>1</sup> Application (feeding) solution: 50 % (w/v) aqueous sucrose solution with 3 % acetone

<sup>2</sup> Application (feeding) solution: 50 % (w/v) aqueous sucrose solution with 3 % acetone and flufenacet (tech.)

\* Statistically significantly lower compared to the control group; t-Test (pooled, left sided;  $p \leq 0.05$ )

\*\* Statistically significantly lower compared to the control group; Mann-Whitney (pairwise, N-Approx, left sided;  $p \leq 0.05$ ), D1-D10-day of study

#### Conclusions:

It can be concluded that the continuous *ad libitum* feeding of honey bees in the laboratory over a period of 10 consecutive days with the test item flufenacet (tech.) at the treatment level of 120 mg a.s./kg caused no adverse effect regarding mortality, sub-lethal effects and behaviour.

The overall mean daily consumption of application (feeding) solution (i.e. the average value over 10 days) in the test item treatment group was not statistically significantly lower compared to the untreated control group. Further, on every single day during the 10 day continuous exposure period the mean food consumption per bee was not statistically significantly different (lower) in the test item treatment group compared to the control group, except for the first day and the 8<sup>th</sup> day of exposure.

As the overall mean daily food uptake in the test item treatment group was not statistically significantly lower compared to the control group, it can be concluded that there was no repellent effect of the test item at the treatment level of 120 mg a.s./kg (44.2 µg a.s./bee corresponding to 4.42 µg a.s./bee/day)

The NOEC for mortality was determined at the end of the test period to be 120 mg a.s./kg (nominal).

The LC<sub>50</sub> after 10 days of continuous exposure was determined to be > 120 mg a.s./kg (nominal).

**Comment RMS:**

No test guideline is available to address the chronic risk to adult honeybees.

The protocol given in the draft EFSA Guidance Document on honeybees (EFSA Journal 2013;11(7):3295), Appendix O was used by RMS to evaluate the chronic oral toxicity test provided by Applicant.

In this study control mortality was 3% which is in line with study protocol (should be ≤15%).

The following deviations from the study protocol are noted:

- during the study no pollen was available ad libitum (should be available sucrose solution, water and pollen).
- groups of 10 honeybees were used instead of at least 20 grouped bees per treatment and control.
- no effect on the on the hypopharyngeal glands (HPG) was determined.
- the endpoints are expressed in mg/kg instead of µg/bee/day.
- 1-4 days old honey bees were used instead of newly emerged honeybees to ensure that the age of the test bees is homogenous.

Even though, some deviations were identified the chronic oral toxicity study is considered to be valid, because of the mean mortality in the control was ≤15% at the end of the test.

This criterion is taken from the acute toxicity study outlined in EPPO 170.

**Agreed endpoints:**

The LC<sub>50</sub> > 120 mg a.s./kg (nominal) corresponding to **LD<sub>50</sub> > 4.42 µg a.s./bee/day**

The NOEC = 120 mg a.s./kg (nominal) corresponding to **NOEC=4.42 µg a.s./bee/day**

**B.9.3.1.3. Effects on honeybee development and other honeybee life stages**

A honeybee brood study with the flufenacet as formulated product (Flufenacet SC 508.8) ) according to the test guideline by Oomen *et al.* was conducted to address the risk on the honey bee brood.

**B.9.3.1.3-1: Flufenacet SC 508.8: A honeybee brood feeding study to evaluate the effects on brood development of the honeybee, *Apis mellifera* L. (Hymenoptera: Apidae).**

<b>Reference:</b>	Flufenacet SC 508.8: A honeybee brood feeding study to evaluate the effects on brood development of the honeybee, <i>Apis mellifera</i> L. (Hymenoptera: Apidae).
<b>Author(s), year:</b>	Hecht-Rost, S.; 2012
<b>Report/Doc.</b>	IES Study 20110057, Number: M-456504-01-1
<b>Guideline(s):</b>	EPPO Bulletin 22 (Oomen <i>et al.</i> , 1992)
<b>GLP:</b>	Yes

In course of its evaluation of this study RMS stated that there were discrepancies between the study protocol, numerical results and their graphical presentation. Additionally it was stated that there were no statistically significant differences between response in the negative control and the positive control (reference) in case of eggs and young larvae, although that was observed for the old larvae. RMS indicated that problem to the Applicant receiving the information that the study will be updated and in that form it should be ready for commenting period.

**B.9.3.2. Effects on non-target arthropods other than bees.**

The studies with the representative formulation Flufenacet+Diflufenican 600 SC were submitted by see Vol. 3 CP B9 for formulated product.

#### B.9.4. EFFECTS ON NON-TARGET SOIL MESO-AND MACROFAUNA

##### B.9.4.1. Earthworm - sub-lethal effects.

In the new requirements (EU Commission regulation No. 283/2013) the acute toxicity to earthworm needs not be studied any longer.

One study (Heimbach, 1997) on the reproductive toxicity of the active substance flufenacet (tested as Flufenacet WG 60) to earthworms was submitted for the first EU approval. The NOEC was estimated to be 3 kg a.s./ha corresponding to 4 mg a.s./kg soil dw.

The new statistical analysis done by Kratz A. (1997) based on the original data from study Heimbach (1997) indicated the endpoint to earthworm - NOEC=0.605 kg flufenacet/ha (based on growth).

The endpoint was considered not valid by RMS.

The new standard laboratory study on the reproductive toxicity of representative formulation DFF+FFA SC 600 to earthworms was performed.

In this study (Leicher T., 2010) the effects on growth and reproduction were determined in two runs of five different concentrations of formulation DFF+FFA 600 SC (the first run: 48, 85, 15.2, 27 and 48 mg product/ha, the second run with 0.8, 1.5, 2.6, 4.7 and 8.4 mg item/kg soil dw) incorporated into artificial soil, resulting in an  $NOEC_{reproduction}=2.6$  mg product/kg soil dry weight. The study summaries for the studies with representative formulation DFF+FFA SC 600 are given in Volume 3 (CP), B 9.

Two field studies examined the influence on the population of earthworms.

One of them was the one-year earthworm field study with Flufenacet SC 500 (Leicher, 2008) applied on an arable field up to an application rate of 1.2 L/ha (600 g flufenacet/ha).

It was performed to examine the effect of the increasing application rate on the toxicity of flufenacet to natural earthworm population. Based on the results of that study it can be concluded that there was no long-term adverse effects, determined 5 and 11 months after application, in population of juvenile and adult earthworms resulting from application of 1.2 L Flufenacet 500 SC/ha.

Therefore, the determined NOAER = 1.2 L Flufenacet 500 SC/ha, corresponding to NOAER =0.438 mg flufenacet/kg soil dw (measured value). However it should be indicated that the study was not used because the most sensitive species to flufenacet - *Octolasion lacteum*, identified as such in another field study, was not tested. Therefore, the NOAER value of 0.438 mg flufenacet/kg dw was considered not appropriate to use in the risk assessment.

A one-year earthworm field study with the representative formulation DFF+ FFA SC 200+400 G was conducted in Southern Germany under field conditions, after one autumn application of Diflufenican SC 500A on bare soil, at a rate of 243.75 g diflufenican/ha (application 1), followed by once application of DFF+ FFA SC 200+400 G (diflufenican+flufenacet, application 2-DDA2): at different rates (0.6 L product/ha, 1.2 L product/ha and 1.8 L product/ha. Not statistically significant reduction in numbers and in biomass of total earthworms, total juveniles, total adults and single species occurred at any post treatment sampling (35, 183, 364 days) after application of the test item at rates of 0.6, 1.2 and 1.8 L/ha, following the plateau application of diflufenican at a rate of 243.77 g a.s/ha. However, it should be noted that biological significant effects (19-33%) could still be observed on the population *Octolasion lacteum* after 364 d at rates of 1.2. and 1.8 L/ha.

At rate of 0.6 L DFF+ FFA SC 200+400 G /ha biological significant but transient effects for this species were observed. Therefore, NOAER of 0.6 L DFF+ FFA SC 200+400 G /ha (leading to 0.203 mg flufenacet/kg soil dw, measured value) was estimated from the study and this value was used in the risk assessment.

Studies with the soil metabolites such as: FOE oxalate, FOE sulfonic acid-Na-salt, FOE methylsulfone, TFA, FOE 5043-trifluoroethane sulfonic acid and FOE-Thiadone were conducted addressing the risk soil organisms including earthworms.

The study summaries for studies with the active substance flufenacet and the soil metabolites are given in the Table B.9.4.1-1.

**Table B.9.4.1-1: Sub-lethal ecotoxicological endpoint for flufenacet and its metabolites for earthworms.**

Test substance	Test species	Endpoint		Reference
FFA SC 500 (Flufenacet)	Earthworm field study	NOAER	1.2 L prod/ha 0.6 kg a.s./ha 0.438 mg a.s./kg dws <sup>1</sup>	Leicher (2008) M-307211-01-1
FOE oxalate	Earthworm, reproduction (10% peat in test soil)	NOEC	≥100 mg /kg	Leicher (2010) M-398163-01-1
FOE sulfonic acid-Na-salt	Earthworm, reproduction (5% peat in test soil)	NOEC	500 mg/kg	Leicher (2009) M-358264-01-1
FOE methylsulfone	Earthworm, reproduction (5% peat in test soil)	NOEC	125 mg /kg	Leicher (2010) M-362081-01-1
TFA	Earthworm, reproduction (10% peat in test soil)	NOEC	320 mg/kg <sup>2</sup>	Luehrs (2005) M-251328-01-1
FOE 5043-trifluoroethane sulfonic acid	Earthworm, reproduction (5% peat in test soil)	NOEC	100 mg/kg	Kratz (2012) M-436340-01-1
FOE-Thiadone	Earthworm, reproduction (5% peat in test soil).	NOEC	3.2 mg/kg	Kratz (2012) M-442579-01-1

pm Pure metabolite

<sup>1</sup> The study not included because the most sensitive species to flufenacet - *Octolasion lacteum*, identified as such in another field study for representative formulation, was not tested. Therefore, the NOAER value of 0.438 mg flufenacet/kg dws was (measured value) not used in the risk assesmet.

<sup>2</sup> NOEC reduced to 320 mg/kg based on the effects on body weight in the concentration 1000 mg/kg

**Active substance****B.9.4.1.1. Influence of FOE5043 WG 60 on the reproduction of earthworm (*Eisenia fetida*).**

<b>Reference:</b>	Influence of FOE5043 WG 60 on the reproduction of earthworm ( <i>Eisenia fetida</i> ).
<b>Author(s), year:</b>	Heimbach F., 1997
<b>Report/Doc. number:</b>	Study No.: <b>HBF/Rg 251</b> , Reference No M-004878-01-1
<b>Guideline(s):</b>	ISO 11268-2: (1995):Part 2: ISO/DIS 11268-2 (1995), BBA –PART VI,2-2, January 1994
<b>GLP:</b>	Yes

The original study (Heimbach F., 1997, references above) showed that there was no (statistically) significant changes in body at any concentration, except of the highest application rate (5 kg/ha), for which a slight, although statistically significant reduction in weight increase, compared to control, was observed. This reduction is not considered as biologically significant since the results of the U-test show only a very small difference to control findings ( $p = 0.043$  instead of  $p = 0.050$ ).

New statistical calculations with the data obtained in Heimbach (1997), M-004878-01-1), with new NOEC value, were presented in the following document:

<b>Reference:</b>	Influence of FOE5043 WG 60 on the reproduction of earthworm ( <i>Eisenia fetida</i> ).
<b>Author(s), year:</b>	M.A. Kratz, 1997
<b>Report/Doc. number:</b>	Amended to M-004878-02-1 Amended to Report No: HBF/Rg 251, Reference BCS NO: M-004878-01-1
<b>Guideline(s):</b>	ISO 11268-2: (1995):Part 2: ISO/DIS 11268-2 (1995), BBA –PART VI, 2-2, January 1994
<b>GLP:</b>	Yes

The summary of the study (Heimbach, 1997) with the new statistical calculations submitted in the report: Kratz M.A. (1997) was presented below:

<b>Test substance:</b>	Formulation: FOE5043 60 WG 04317/0131, Product No: 015816, content of a.s -60.5 %.
<b>Test species:</b>	Earthworm <i>Eisenia fetida</i> ( <i>Eisenia fetida Andrei</i> )
<b>Number of organism:</b>	4 replicates per treatment group and control group, each with 10 individuals
<b>Weight, age:</b>	370 mg/worm (mean), adults
<b>Type of test, duration:</b>	Laboratory sub-lethal test, 8 weeks (4 weeks adult mortality, 4 weeks juvenile development)

Applied concentrations:

Nominal:	0 (control), 1x1, 1x2 and 1x5 kg product/ha, sprayed onto the soil surface.
Solvent:	None
Toxic standard:	Derosal (36% of Carbendazin), tested at concentrations of 0.10, 0.25 and 0.5 kg product/ha
Test substrate:	Artificial soil: 10 % sphagnum peat, 20 % kaolin clay, 69 % industrial quartz sand, 1 % calcium carbonate
Substrate/vessel:	The plastic boxes (length x width x height <i>ca.</i> 16 cm x 12cm x 6 cm, area approximately 200 cm <sup>2</sup> (198 cm <sup>2</sup> ) were used as test vessels. Each test vessel contained an amount of approximately 500 g dry weight artificial soil.
Temperature:	20 ±2 °C
Light regime:	16 hours light/8 hours dark. Light intensity (400-800 Lux)
Relative humidity:	70-90%
Water content:	Test start : 33.6-34 % (equivalent to 53.3 -53.7 % of WHC) Test end: 32.9-3
pH:	Test start :6.2 Test end: 6.1-6.2
Feeding:	Air-dried and finely ground cattle manure; feeding interval was weekly during the first 4 weeks, weekly amount of ground cattle manure (5 g) depended on the feeding activity.
Test parameters:	Temperature was recorded continuously during the whole test period. The moisture content of the artificial soil were determined at start of test. The pH measurements were determinate at the start and the end of test for all treatment levels. Mortality of adults (assessed after 28 days), mean body weight of adults (measured at day 0 and after 28 days), morphological and behavioural changes of adults (observed at day 28) number of juvenile earthworms (counted after further 28 days) . Cumulative amount of food added to each test container during the test period.
Statistic:	The endpoints were mortality, change of biomass (difference in fresh weight of surviving worms between test start and four weeks after treatment) and reproduction (the number of juveniles present). The arithmetic mean and the standard deviation per treatment and per control for reproduction and biomass were calculated. The reproduction of the surviving test organisms per test vessel at the end of the study was compared to the control values. Changes in body weight values of the surviving test organisms of the treatment groups during the test period were compared to the values of the control group. The normal distribution of the data was tested by Kolmogorov-Smirnov test. The normality hypothesis was accepted. The

homogeneity of variances of the data was checked by Cochran's test. The homogeneity hypothesis was rejected. Therefore the data were transformed ( $y' = \ln(y)$ ). The homogeneity of variances of these transformed data was given. The data were statistically evaluated by means of a Williams multiple sequential t-test, two-sided,  $\alpha = 0.05$ . The reproduction of the surviving test organisms per test vessel at the end of the study was compared to the control values. The normal distribution of the data was tested by Kolmogorov-Smirnov test. The normality hypothesis was accepted. The homogeneity of variances of the data was checked by Cochran's test. The homogeneity hypothesis was accepted. The homogeneity hypothesis was accepted. The data were statistically evaluated by means of a Williams multiple sequential t-test, one – sided smaller,  $\alpha = 0.05$ . The statistical software package ToxRatPro Version 2.09 was used for the calculation. The data on food addition was not statistically analyzed since 100% consumption in all the occasions.

**Findings:**

After 28 days of exposure no worms died in the control group and no mortality was observed at any test item concentration.

In respect to growth, there was statistically significant different to the control for at test concentrations of 2 and 5 kg test item/ha.

No statistically significant different values for the number of juveniles relative to the control were observed at all test concentrations.

There was no significant difference in food consumption at all tested level when compared to the control.

All the treatment groups recorded complete consumption of food during the four weeks of exposure.

Effects on mortality and changes in body weight of the adults after an exposure period of 28 days and the number of offspring per test vessel after 56 days are shown in the Table 9.4.1.1-1

**Table 9.4.1.1-1: Effects on mortality and reproduction of *Eisenia fetida* in a sub-chronic test.**

	<i>Eisenia fetida</i>			
	(kg Flufenacet 60 WG /ha)			
Exposure	Control	1x1	1x2	1x5
Mortality of adult earthworms [%] after 28 days		0	0	0
Mean change of body weight of the adults from day 0 to day 28 [%] (Standard deviation)	48.692 (± 9.711)	49.054 (±1.200 )	35.431* (± 1.621 )	30.401* (± 5.481)
Mean number of offspring per test vessel after 56 days (Standard deviation)	49.3 (± 10.3)	49.0 (± 9.8)	48.8 (± 1.7)	48.5 (± 1.3)
% reproduction compared to control	100	99.40	99	98.37

\* Statistically significant different to the control (Williams multiple sequential t-test, two-sided,  $\alpha = 0.05$ )

In the positive control (Carbendazim) the application of 0.079 kg a.s./ha and 0.158 kg a.s./ha caused reduced the numbers of juvenile (by 39 and 63% respectively). The NOEL was estimated to be 0.032 kg a.s./ha and LOEL 0.079 kg a.s./ha.

**Conclusion:**

NOEC<sub>growth</sub> = 1 kg product/ha corresponding to 0.605 kg a.s./ha and 1.2 mg a.s./kg dws

NOEC<sub>reproduction</sub> = 5 kg product/ha corresponding to 3 kg a.s./ha

LOEC = 2 kg product/ha corresponding to 1.21 kg a.s./ha

**Comment RMS:**

The earthworm reproduction study was conducted according to BBA-PART VI, 2-2 (1994) guideline.

Taking into account validity criteria given in the test guideline OECD 222 (2004) the study is considered acceptable.

The mortality of adults in the control was below 10% (being: 0%). The number of juveniles per control replicate was greater than 30 (being 35-59 juveniles per replicate).

Mean change in growth of the adult earthworms in the control during the exposure period of four weeks  $\geq 20\%$  (being 48.69 %).

The coefficient of variation of reproduction in the control was  $\leq 30\%$  (being 5.07%).

The following deviations from the recommendations given in the OECD 222 test guideline were recorded:

-The WHC<sub>max</sub> at the end of the test was not measured.

- The information of age of earthworms was missing.

- Only four control replicates were used in the reported study instead of eight as recommended by OECD 222 guideline.

-- the formulated product was sprayed onto the soil instead of being incorporated in the soil

However, according to the Regulation (EU)283/2013 the test substance should be incorporated into the soil to obtain a homogenous soil concentration.

**For this reason the study is considered not valid.**

**Metabolites:****B.9.4.1.2.FOE 5043 – oxalate: Effects on survival, growth and reproduction on the earthworm*****Eisenia fetida* tested in artificial soil with 10% peat.**

<b>Reference:</b>	FOE 5043 – oxalate: Effects on survival, growth and reproduction on the earthworm <i>Eisenia fetida</i> tested in artificial soil with 10% peat.
<b>Author(s), year:</b>	Leicher T., 2010
<b>Report/Doc. number:</b>	Report No:LRT-RG-R-68/09, Reference BCS No: M-398163-01-1
<b>Guideline(s):</b>	ISO 11268-2 (1998), OECD Guideline 222 (2004), ISO 11268-2: 1998 (E) and OECD 222 (April 13, 2004)
<b>GLP:</b>	Yes

**Material and methods:**

Test substance:	Test item: FOE 5043-oxalate, Batch code: BCS-AB16305-01-01, Purity: 92.2 %w/w (analysed).
Test species:	Earthworm, <i>Eisenia fetida</i> ( <i>Eisenia fetida Andrei</i> ).
Number of organism:	8 replicates per treatment group and control group, each with 10 individual
Age:	Mean: 250-440 mg/worm, adults with clitellum, approximately 9 months
Type of test, duration:	Laboratory sub-lethal test, 8 weeks (4 weeks adult mortality, 4 weeks juvenile development).
Applied concentrations:	Nominal: 0 (control, artificial soil and dilution water), 100 mg test item/kg soil dw. The test item was mixed into the soil.
Solvent:	None
Toxic standard:	Derosal flüssig (BAS 346F, 360 g Carbendazim/L), tested at concentrations of 1.25, 2.5, and 10 mg a.s./kg soil dw.
Test substrate:	Artificial soil, 10 % sphagnum peat, 20 % kaolinite clay, 68.65% industrial quartz sand, 0.34% calcium carbonate, 1% dried ground cow manure (food).
Substrate/vessel:	The plastic boxes (length x width x height <i>ca.</i> 16.5cm x 12cm x 6 cm, area approximately 200 cm <sup>2</sup> ) were used as test vessels. Each test vessel contained an amount of approximately 500 g dry weight artificial soil to obtain a depth of approximately 5 cm soil in the test vessels.
Temperature:	18-22 °C
Light regime:	16 hours light/8 hours darkness. Light intensity: mean measured: 539 lux at day 0, 472 Lux at day 28 and 479 Lux

	at day 56
Water content:	Test start: 29% (corresponding to 56.82% of WHC) Test end: 28.8 % (corresponding to 56.43% of WHC)
pH:	Prior the test: 6.14 Test end: 5.62-5.97
Feeding:	Air-dried and finely ground cow manure; feeding interval was weekly during the first 4 weeks, weekly amount of manure (5 g) depended on the feeding activity. The off spring were fed only once at the start of the second week.
Test parameters:	Temperature was recorded continuously during the whole test period. The moisture content of the artificial soil were determined prior to test start, at day application Day 0 and day 56 of the test. The ph measurements were made prior the test and the end on Day 56 of the study. Mortality of adults (assessed after 28 days), mean body weight of adults (measured at day 0 and after 28 days), morphological and behavioural changes of adults (observed at day 28) number of juvenile earthworms (counted after 8 weeks).
Statistic:	The endpoints were mortality, change of biomass (difference in fresh weight of surviving worms between test start and four weeks after treatment) and reproduction the number of juveniles present). Kolmogorov-Smirnov test and Cochran's test were used respectively, to test the data for normality and homogeneity of variance. The data were statistically evaluated by means of a STUDENT-t test for homogeneous variances. The statistical software package ToxRatPro Version 2.09 was used for the calculation. Since there was no mortality observed in any of the treatments, statistical analysis was not necessary for mortality. Similarly the data on food addition was not statistically analyzed since 100% consumption in all the occasions.
Findings:	No mortality was observed after 28 days of exposure at the control group and at the tested concentration of 100 mg test item/kg dry weight artificial soil. No statistically significant different values for the growth relative to the control were observed at the tested concentration of 100 mg test item /kg dws. No statistically significant different values for the number of juveniles per test vessel relative to the control were observed at the tested concentration of 100 mg FOE 5043-oxalate/kg dry weight artificial soil.

There was no difference in food consumption at tested concentration 100 mg FOE test item/kg dry weight artificial soil. The treatment group recorded complete consumption of food during the four weeks of exposure.

Effects on mortality and changes in body weight of the adults after an exposure period of 28 days and the number of offspring per test vessel after 56 days are shown in the Table B. 9.4.1.2-1 below:

**Table B.9.4.1.2-1: Effects on mortality and reproduction of *Eisenia fetida* in a sub-chronic test.**

	<i>Eisenia fetida</i>	
	Control	FOE 5043-oxalate mg test item /kg soil dw
Exposure	-	100
Mortality of adult earthworms [%] after 28 days	0	0
Mean change of body weight of the adults from day 0 to day 28 [%] (Standard deviation)	21.2 (±4.8)	24.5 (±4.8) <sup>1</sup>
Mean number of offspring per test vessel after 56 days (Standard deviation)	76.4 (±11.9)	68.1 (±18.3) <sup>2</sup>
% reproduction compared to control		89.2 <sup>2</sup>

1 Not statistically significantly different from the control (Student t-t rest, two-sided,  $\alpha=0.05$ ,  $p \geq 0.05$ )

2 Not statistically significantly different from the control (Student t-t rest, one-sided,  $\alpha=0.05$ ,  $p \geq 0.05$ )

#### Toxic standard

The results of positive control (carbendazim) indicated that the number of juveniles was reduced by 58.1 and approximately 100% at concentration of 2.5 and 5.0 mg carbendazim./kg soil dw (mean number of juveniles was 95.3 and 0.8) after 56 days of test duration, when compared to the control (mean number of juveniles =227.4).

#### Conclusion:

The NOEC<sub>reproduction</sub>  $\geq$  100 mg test item/kg soil dw

The NOEC<sub>growth</sub>  $\geq$  100 mg test item/kg soil dw

The LOEC<sub>reproduction</sub> > 100 mg test item/kg soil dw

The LOEC<sub>growth</sub> >100 mg test item/ kg soil dw

#### Comment RMS:

The earthworm reproduction study was conducted according to the OECD test guideline 222 (2004).

Taking into account validity criteria given in the test guideline OECD 222 (2004) the study is considered acceptable.

The mortality of adults in the control was below 10% (being: 0%). The number of juveniles per control replicate was greater than 30 (being 59-94 juveniles per replicate). The coefficient of variation of reproduction in the control was  $\leq$  30% (being 15.6%).

**Agreed endpoints:**

The NOEC<sub>reproduction</sub>  $\geq$  100 mg test item/ kg soil dw

The NOEC<sub>growth</sub>  $\geq$  100 mg test item/kg soil dw

The LOEC<sub>reproduction</sub>  $>$  100 mg test item/kg soil dw

The LOEC<sub>growth</sub>  $>$  100 mg test item/kg soil dw

**B.9.4.1.3. Flufenacet (FOE 5043) – Sulfonic acid Na-salt: Effects on survival, growth and reproduction on the earthworm *Eisenia fetida* tested in artificial soil with 5% peat.**

<b>Reference:</b>	Flufenacet (FOE 5043) – Sulfonic acid Na-salt: Effects on survival, growth and reproduction on the earthworm <i>Eisenia fetida</i> tested in artificial soil with 5% peat.
<b>Author(s), year:</b>	Leicher, T., 2009.
<b>Report/Doc. number:</b>	Report No.: LRT-RG-R-68/09, Reference BCS no. M-358264-01-1
<b>Guideline(s):</b>	ISO 11268-2: 1998 (E) and OECD 222: April 13, 2004
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet-Sulfonic acid Na-salt, Batch code: AE 0841914-01-03, Origin Batch No.: SES 10294-6-2., purity: 92.4% (analysed).
Test species:	Earthworm <i>Eisenia fetida</i> ( <i>Eisenia fetida Andrei</i> )
Number of organism:	4 replicates per treatment group and 8 replicates per control group, each with 10 individuals
Weight, age:	Mean: 300-500 mg/worm, adults with clitellum, approximately 8 months
Type of test, duration:	Laboratory sub-lethal test, 8 weeks (4 weeks adult mortality, 4 weeks juvenile development)

Applied concentrations:

Nominal:	0 (control), 62.5, 125, 250, 500, and 1000 mg test item/kg dry weight artificial soil. The test item was mixed into the soil.
Solvent:	None
Toxic standard:	Derosal flüssig (BAS 346F, 360 g Carbendazim/L), tested at concentrations of 1.25, 2.5, and 5 mg a.s./kg dws
Test substrate:	Artificial soil, 5 % sphagnum peat, 20 % kaolinite clay, 73.82 % industrial quartz sand, 0.18 % calcium carbonate, 1% dried ground cow manure (food)
Substrate/vessel:	Non-re-usable plastic boxes (length x width x height <i>ca.</i> 16.5 cm x 12 cm x 6 cm, area approximately 200 cm <sup>2</sup> ) were used as test vessels. Each test vessel contained an amount of approximately 500 g dry weight artificial soil to obtain a depth of approx. 5 cm soil in the test vessels.

	<p>The artificial soil was prepared by mixing the dry components intensely in a laboratory mixer. Then, the soil was moistened with deionised water to reach a water content of 57 % of the maximum water holding capacity.</p>
Temperature:	18-22 °C
Light regime:	16 hours light/8 hours darkness
	Light intensity: 552 Lux at day 0, 560 Lux at day 28 and 646 Lux at day 56 of the study.
Water content:	<p>Test start: 24.75 % (corresponding to 60.8% of WHC)</p> <p>Test end: 21.3 % (corresponding to 52.33% of WHC)</p>
pH:	<p>Prior the test: 6.32</p> <p>Test end: 6.77- 6.98</p>
Feeding:	Air-dried and finely ground cow manure; feeding interval was weekly during the first 4 weeks, weekly amount of manure (5 g) depended on the feeding activity. The off spring were fed only once at the start of the second week.
Test parameters:	<p>Temperature was recorded continuously during the whole test period. The moisture content of the artificial soil were determined prior to test start, at day application Day 0 and day 56 of the test. The pH measurements were made prior the test and the end on Day 56 of the study.</p> <p>Mortality of adults (assessed after 28 days), mean body weight of adults (measured at day 0 and after 28 days), morphological and behavioural changes of adults (observed at day 28) number of juvenile earthworms (counted after 8 weeks) .</p>
Statistic:	<p>The endpoints were mortality, change of biomass (difference in fresh weight of surviving worms between test start and four weeks after treatment) and reproduction (the number of juveniles present).</p> <p>The reproduction of the surviving test organisms per test vessel at the end of the study was compared to the control values. The normal distribution of the data was tested by Kolmogorov-Smirnov test. The homogeneity of variances of the data was checked by Cochran's test. The data were statistically evaluated by means of a Williams multiple sequential t-test, one – sided smaller, <math>\alpha = 0.05</math>. The statistical software package ToxRatPro Version 2.09 @ was used for the calculation. The data on food addition was not statistically analyzed since 100% consumption in all the occasions.</p>
Findings:	<p>Mortality of adult earthworms was observed after 28 days of exposure only at the highest test concentration of 1000 mg test item/kg dry weight artificial soil. The 5% mortality is below the allowed maximum mortality for the control and is therefore not considered as an adverse effect.</p> <p>Statistically significant different values for the growth relative to the control were observed at all tested concentrations.</p>

Since the growth in all tested concentrations of the test item were higher than in the control this was not considered as an adverse effect.

No statistically significant different values for the number of juveniles per test vessel relative to the control were observed at the test concentrations up to and including 500 mg test item/kg dry weight artificial soil.

A statistically significant different value for the number of juveniles per test vessel relative to the control was observed at the test concentrations of 1000 mg test item/kg dry weight artificial soil.

There was no significant difference in food consumption in treatments up to and including 1000 mg Flufenacet-Sulfonic acid Na-salt/kg soil dry weight, when compared to the control. All the treatment groups recorded complete consumption of food during the four weeks of exposure.

Effects on mortality and changes in body weight of the adults after an exposure period of 28 days and the number of off spring per test vessel after 56 days are shown in the Table 9.4.1.3-1 below:

**Table B.9.4.1.3-1: Effects on mortality and reproduction of *Eisenia fetida* in a sub-chronic test.**

	<b><i>Eisenia fetida</i></b>					
	<b>Flufenacet-Sulfonic acid Na-salt (mg test item /kg soil dw)</b>					
Exposure	Control	62.5	125	250	500	1000
Mortality of adult earthworms [%] after 28 days	0	0	0	0	0	5
Mean change of body weight of the adults from day 0 to day 28 [%]	+ 20.0 (± 2.1)	+ 40.9* (± 9.6)	+ 44.3* (± 1.8)	+ 44.4* (± 9.0)	+ 34.2* (± 5.9)	+ 36.7* (± 3.2)
Mean number of offspring per test vessel after 56 days	161.8 (± 16.8)	153.5 (± 25.0)	163.8 (± 15.5)	155.0 (± 10.7)	167.5 (± 24.3)	120.8** (± 5.1)
% reproduction compared to control	100	94.9	101.2	95.8	103.52	74.7**

\* Mean value statistically significant different compared to the control (Williams Multiple Sequential t-test, two-sided,  $\alpha = 0.05$ ,  $p < 0.05$ )

\*\* Mmean value statistically significant different compared to the control (Williams Multiple Sequential t-test, one-sided smaller,  $\alpha = 0.05$ ,  $p < 0.05$ )

**Toxic standard:**

The results of positive control (carbendazim) indicated that the number of juveniles was reduced by 58.1 and approximately 100% at concentration of 2.5 and 5.0 mg carbendazim/kg soil dw (mean number of juveniles=95.3 and 0.8) after 56 days of test duration, when compared to the control (mean number of juveniles =227.4).

Conclusions:

The  $\text{NOEC}_{\text{reproduction}} = 500 \text{ mg test item/kg soil dw}$

$\text{LOEC}_{\text{reproduction}} = 1000 \text{ mg test item/kg soil dw}$

$\text{NOEC}_{\text{growth}} \geq 1000 \text{ mg test item/kg soil dw}$

$\text{LOEC}_{\text{growth}} > 1000 \text{ mg test item/kg soil dw}$

In accordance with the new data requirement (Commission Regulation EU No 283/2013), the  $\text{EC}_{10}$ ,  $\text{EC}_{20}$ , values should be calculated.

A valid  $\text{EC}_x$  calculation was not possible due to the lack of a significant concentration/response function. As at the  $\text{NOEC}$  of 500 mg/kg the number of juveniles reach 103.5% of the control, it can be reasonably assumed that the  $\text{EC}_{10}$  would be less critical than the  $\text{NOEC}$ . Therefore the  $\text{NOEC}$  can be considered as the relevant endpoint in the risk assessment as a worst case approach.

**Comment RMS:**

The earthworm reproduction study was conducted according to the OECD 222 (2004) test guideline.

Taking into account validity criteria given in the test guideline OECD 222 (2004) the study is considered acceptable.

The mortality of adults in the control was below 10% (being: 0%). The number of juveniles per control replicate was greater than 30 (being 135-189 juveniles per replicate). The coefficient of variation of reproduction in the control was  $\leq 30\%$  (being 10.4%).

**Agreed endpoints:**

The  $\text{NOEC}_{\text{reproduction}} = 500 \text{ mg test item/kg soil dw}$

$\text{LOEC}_{\text{reproduction}} = 1000 \text{ mg test item/kg soil dw}$

$\text{NOEC}_{\text{growth}} \geq 1000 \text{ mg test item/kg soil dw}$

$\text{LOEC}_{\text{growth}} > 1000 \text{ mg test item/kg soil dw}$

**B.9.4.1.4. Flufenacet (FOE 5043)–Methylsulfone: Effects on survival, growth and reproduction on the earthworm *Eisenia fetida* tested in artificial soil with 5% peat.**

<b>Reference:</b>	Flufenacet (FOE 5043)–Methylsulfone: Effects on survival, growth and reproduction on the earthworm <i>Eisenia fetida</i> tested in artificial soil with 5% peat.
<b>Author(s), year:</b>	Leicher, T., 2010
<b>Report/Doc. number:</b>	Report No.: LRT-RG-R-68/09, Reference BCS No. M-362081-01-1
<b>Guideline(s):</b>	ISO 11268-2: 1998 (E) and OECD 222: April 13, 2004
<b>GLP:</b>	Yes

Material and methods:

Test substance: Flufenacet-methylsulfone, Batch code: BCSCO62475-01-01, purity: 97.6 % w/w (analysed).

Test species:	Earthworm <i>Eisenia fetida</i> ( <i>Eisenia fetida</i> Andrei)
Number of organism:	8 replicates per treatment group and control group, each with 10 individuals
Weight, age:	mean: 250-450 mg/worm, adults with clitellum, approximately 8 months old
Type of test, duration:	Laboratory sub-lethal test, 8 weeks (4 weeks adult mortality, 4 weeks juvenile development)
<u>Applied concentrations:</u>	
Nominal:	0 (control, quartz sand), 62.5, 125, 250, 500, and 1000 mg test item/kg dry weight artificial soil. The test item was mixed into the soil.
Solvent:	None
Toxic standard:	Derosal flüssig (BAS 346F, 360 g Carbendazim/L, tested at concentrations of 1.25, 2.5 and 5 mg a.s./kg dws
Test substrate:	Artificial soil, 5 % sphagnum peat, 20 % kaolinite clay, 73.82 % industrial quartz sand, 0.18 % calcium carbonate, 1% dried ground cow manure (food). The artificial soil was prepared by mixing the dry components intensely in a laboratory mixer. Then, the soil was moistened with deionised water to reach a water content of 58 % of the maximum water holding capacity.
Substrate/vessel:	Non-re-usable plastic boxes (length x width x height <i>ca.</i> 16.5 cm x 12 cm x 6 cm, area approximately 200 cm <sup>2</sup> ) were used as test vessels. Each test vessel contained an amount of approximately 500 g dry weight artificial soil.
Temperature:	18-22 °C
Light regime:	16 hours light/8 hours dark. Light intensity: 538 Lux at day 0, 580 Lux at day 28 and 556 Lux at day 56 of the study
Water content:	Test start: 21.5 % (corresponding to 54.93 % of WHC) Test end: 21.2 % (corresponding to 54.16% of WHC)
pH:	Test start: 6.38 Test end: 6.08-6.68
Feeding:	Air-dried and finely ground cow manure; feeding interval was weekly during the first 4 weeks, weekly amount of manure (5 g) depended on the feeding activity. The off spring were fed only once at the start of the second week.
Test parameters:	Temperature was recorded continuously during the whole test period. The moisture content of the artificial soil were determined prior to test start, at day application Day 0 and day 56 of the test. The pH measurements were made prior the test and the end on Day 56 of the study. Mortality of adults (assessed after 28 days), mean body weight of adults (measured at day 0 and after 28 days), morphological and behavioural changes of adults (observed at day 28) number of juvenile earthworms (counted after 8 weeks)

---

Statistic:	<p>The endpoints were mortality, change of biomass (difference in fresh weight of surviving worms between test start and four weeks after treatment) and reproduction (the number of juveniles present).</p> <p>The reproduction of the surviving test organisms per test vessel at the end of the study was compared to the control values.</p> <p>The normal distribution of the data was tested by Kolmogorov-Smirnov test. The homogeneity of variances of the data was checked by Cochran's test.</p> <p>The data were statistically evaluated by means of a Williams multiple sequential t-test. The statistical software package ToxRatPro Version 2.09 was used for the calculation. The data on food addition was not statistically analyzed since 100% consumption in all the occasions.</p>
<u>Findings:</u>	<p>No mortality of adult earthworms was observed after 28 days of exposure at the control group and at the test concentrations 62.5, 125, 250 and 500 mg test item/kg dry weight artificial soil. In the highest test concentration 1000 mg test item/kg dry weight artificial soil 75% (30 worms) died.</p> <p>A statistically significant different value for the growth relative to the control were observed at the tested concentrations 250, 500 and 1000 mg test item/kg dry weight artificial soil. A statistically significant different value for the number of juveniles per test vessel relative to the control was observed at the test concentrations of 250, 500 and 1000 mg test item/kg dry weight artificial soil. There was no significant difference in food consumption in treatments up to and including Flufenacet-Methylosulfone/kg soil dry weight, when compared to the control. All the treatment groups recorded complete consumption of food during the four weeks of exposure.</p>

Effects on mortality and changes in body weight of the adults after an exposure period of 28 days and the number of offspring per test vessel after 56 days are shown in the Table B.9.4.1.4-1 below:

**Table B. 9.4.1.4-1: Effects on mortality and reproduction of *Eisenia fetida* in a sub-chronic test.**

	<b><i>Eisenia fetida</i></b>					
	<b>Flufenacet-Methylosulfone ( mg test item/kg soil dw)</b>					
Exposure	Control	62.5	125	250	500	1000
Mortality of adult earthworms [%] after 28 days	0	0	0	0	0	75
Mean change of body weight of the adults from day 0 to day 28 [%] (Standard deviation)	+ 62.7 (± 5.2)	+ 61.2 (± 15.3)	+ 63.0 (± 9.7)	+ 45.6* (± 5.4 )	+ 10.1* (± 5.7)	-64.6* (± 9.6 )
Mean number of offspring per test vessel after 56 days (Standard deviation)	103.3 (± 228.3)	115.5 (± 25.0)	101.5 (± 15.5)	51** (± 10.7)	3.5** (± 24.3)	0
% reproduction compared to control	100	112	98.3	49.4**	3.4**	0**

\* Mean value statistically significant different compared to the control (Bonferoni-Holm Multiple Sequential u-test)

\*\* Mean value statistically significant different compared to the control (Williams Multiple Sequential t-test, one-sided smaller,  $\alpha = 0.05$ ,  $p < 0.05$ ).

Toxic standard:

The results of positive control (carbendazim) indicated that the number of juveniles was reduced by 58.1 and approximately 100% at concentration of 2.5 and 5.0 mg carbendazim/kg soil dw (mean number of juveniles = 95.3 and 0.8) after 56 days of test duration, when compared to the control (mean number of juveniles was 227.4).

#### Conclusion:

NOEC<sub>growth</sub> = 125 mg test item/kg soil dw

LOEC<sub>growth</sub> = 250 mg test item/kg soil dw

NOEC<sub>reproduction</sub> = 125 mg test item/kg soil dw

LOEC<sub>reproduction</sub> = 250 mg test item/kg soil dw

In accordance with the new data requirement ( Commission Regulation EU No 283/2013), the EC<sub>10</sub>, EC<sub>20</sub>, EC<sub>50</sub> values should be calculated.

Please provided relevant calculations of EC<sub>10</sub>, EC<sub>20</sub>, EC<sub>50</sub> values.

#### **Comment RMS:**

The earthworm reproduction study was conducted according to the OECD test guideline 222 (2004).

Taking into account validity criteria given in the test guideline OECD 222 (2004) the study is considered acceptable.

The mortality of adults in the control was below 10% (being: 0%). The number of juveniles per control replicate was greater than 30 (being 53-144 juveniles per replicate).

Mean change in growth of the adult earthworms in the control during the exposure period of four weeks  $\geq 20\%$  (being 62.7%). The coefficient of variation of reproduction in the control was  $\leq 30\%$  (being 27.4%).

**Agreed endpoints:**

NOEC<sub>growth</sub> = 125 mg test item/kg soil dw

NOEC<sub>reproduction</sub> = 125 mg test item/kg soil dw

LOEC<sub>growth</sub> = 250 mg test item /kg soil dw

LOEC<sub>reproduction</sub> = 250 mg test item/kg soil dw

**B.9.4.1.5. Effects of AE C502988 00 1B99 0001 on reproduction and growth of earthworms *Eisenia fetida* in artificial soil.**

<b>Reference:</b>	Effects of AE C502988 00 1B99 0001 on reproduction and growth of earthworms <i>Eisenia fetida</i> in artificial soil.
<b>Author(s), year:</b>	Luehrs, U., 2005
<b>Report/Doc. number:</b>	Report No, C048065, Reference BCS No: M-251328-01-1
<b>Guideline(s):</b>	ISO 11268-2: 1998 (E) and BBA 1994: "Effects of pesticides on the reproduction and growth of <i>Eisenia fetida</i> / <i>Eisenia Andrei</i> "
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Trifluoroacetic acid, Batch code: AE C502988 00 1B99 0001, Batch No: 18921. Purity: 98.8% (analysed).
Test species:	Earthworm <i>Eisenia fetida</i> , ( <i>Eisenia fetida Andrei</i> )
Number of organism:	4 replicates per treatment group and control group, each with 10 individuals
Weight, age:	321-521 mg/worm, adults with clitellum, approximately 11 months old
Type of test, duration:	Laboratory sub-lethal test, 8 weeks (4 weeks adult mortality, 4 weeks juvenile development)

Applied concentrations:

Nominal:	0 (control), 10, 32, 100, 320 and 1000 mg test item/kg dry weight artificial soil. The test item was mixed into the soil.
Solvent:	None
Toxic standard:	Brabant Carbendazim Flowable (500 g Carbendazim/L), tested at concentrations rate 1.20, 1.68, 2.64, 4.08, 6.0 mg product/kg dws corresponding to 0.5, 0.7, 1.2, 1.7 and 2.5 mg a.s./kg dws.
Test substrate:	Artificial soil: 10 % sphagnum peat, 20 % kaolin clay, 69.5 % industrial quartz sand, 0.5 % calcium carbonate. The artificial soil was prepared by mixing the dry components intensely in a

	laboratory mixer. Then, the soil was moistened with deionised water to reach a water content of 59% of the maximum water holding capacity.
Substrate/vessel:	The plastic boxes (length x width x height <i>ca.</i> 18.3 cm x 13.6 cm x 6 cm, area approximately 189.75 cm <sup>2</sup> ) were used as test vessels. Each test vessel contained an amount of approximately 500 g dry weight artificial soil. The height of the soil layer in the containers was 5-6 cm.
Temperature:	19-21 °C
Light regime:	16 hours light/8 hours dark. Light intensity: 480-790 Lux
Water content:	Test start: 32.3-34.6 % (equivalent to 54.7-58.6 % of WHC) Test end: 33.9-35.7 % (equivalent of 57.5-60.5 % of WHC)
pH:	Test start :5.7-6.0 Test end: 5.6-5.8
Feeding:	Air-dried and finely ground cattle manure; feeding interval was weekly during the first 4 weeks, weekly amount of manure (5 g) depended on the feeding activity. The off spring were fed only once at the start of the second week.
Test parameters:	Temperature was recorded continuously during the whole test period. The moisture content of the artificial soil were determined once a week. The pH measurements were check at the start and of the main test for each concentration. Mortality of adults (assessed after 28 days), mean body weight of adults (measured at day 0 and after 28 days), morphological and behavioural changes of adults (observed at day 28) number of juvenile earthworms (counted after 8 weeks) . Cumulative amount of food added to each test container during the test period.
Statistic:	The endpoints were mortality, change of biomass (difference in fresh weight of surviving worms between test start and four weeks after treatment) and reproduction (the number of juveniles present). The reproduction of the surviving test organisms per test vessel at the end of the study was compared to the control values. Mortality data were analysed for significance by using the Fisher-exact test (two-sided, $\alpha = 0.05$ ). Body weight change and reproduction data were tested for normal distribution and homogeneity of variance using the Kolmogoroff-Smimov test and the Cochran test. As data of body weight changes and reproduction were normally distributed and homogeneous, the Dunnett test was used (multiple comparison, two-sided for weight and one sided smaller for reproduction, $\alpha = 0.05$ ). The software used to perform the statistical analysis was ToxRatPro, version 2.09.

## Findings:

The data on food addition was not statistically analyzed since 100% consumption in all the occasions.

A mortality of 5% was observed in the control and at test concentration of 100 mg test item/kg soil. The lower mortality 2.5% was observed at test concentration of 32 mg test item/kg soil.

The mortality in the test item treated group was not significantly different compared to the control (Fisher exact test,  $\alpha = 0.05$ ) and is not considered to be treatment related since at the two highest concentrations no mortality was observed.

The body weight changes of the test item treated groups were not significantly different compared to the control up to and including the concentration of 320 mg test item/kg soil (Dunnett test,  $\alpha = 0.05$ , two sided).

At 1000 mg test item/kg soil the body weights showed a weight increase of 28.4% which, however, was statistically significantly lower compared to the control (Dunnett test,  $\alpha = 0.05$ , two sided).

The reproduction rates were not significantly different compared to the control in any test item treated groups (Dunnett test,  $\alpha = 0.05$ , one sided smaller).

No behavioural abnormalities were observed and all worms did burrow into the soil within 15 min after introduction.

There was no significant difference in food consumption in treatments up to and including 1000 mg item/kg soil dry weight, when compared to the control.

All the treatment groups recorded complete consumption of food during the four weeks of exposure.

Effects on mortality and changes in body weight of the adults after an exposure period of 28 days and the number of offspring per test vessel after 56 days are shown in the Table B.9.4.1.5-1 below:

**Table B. 9.4.1.5-1: Effects on mortality and reproduction of *Eisenia fetida* in a sub-chronic test.**

Exposure	<i>Eisenia fetida</i>					
	Trifluoroacetic acid (TFA)					
	(mg test item/kg sdw )					
Exposure	Control	10	32	100	320	1000
Mortality of adult earthworms [%] after 28 days (Standard deviation)	5 (±5.8 )	0 <sup>ns 1</sup> (±0 )	2.5 <sup>ns1</sup> (±5.0 )	5 <sup>ns1</sup> (±5.8 )	0 <sup>ns1</sup> (±0 )	0 <sup>ns1</sup> (±0 )
Mean change of body weight of the adults from day 0 to day 28 [%] (Standard deviation)	+42.7 (±4.5 )	+36.3 <sup>ns2</sup> (±6.0 )	+39 <sup>ns2</sup> (±3.3)	+34.2 <sup>ns2</sup> (±8.1 )	+35.9 <sup>ns2</sup> (±8.5 )	+28.4 <sup>*s2</sup> (±5.7)
Mean number of offspring per test vessel after 56 days (Standard deviation)	291 (±58)	307 <sup>ns3</sup> (±89)	377 <sup>ns3</sup> (±31)	304 <sup>ns3</sup> (± 97)	322 <sup>ns3</sup> (± 28)	309 <sup>ns3</sup> (± 20)
% reproduction compared to control	100	105.7 <sup>ns3</sup>	129.7 <sup>ns3</sup>	104.6 <sup>ns3</sup>	110.7 <sup>ns3</sup>	106.2 <sup>ns3</sup>

n.s.<sup>1</sup> Mean value not statistically significant different compared to the control (p ≥ 0.05, Fisher exact test, two-sided, α = 0.05)

n.s.<sup>2</sup> Mean value not statistically significant different compared to the control (p ≥ 0.05, Dunnett test, two-sided, α = 0.05)

n.s.<sup>3</sup> Mean value not statistically significant different compared to the control (p ≥ 0.05, Dunnett test, one sided smaller, α = 0.05)

\*s.<sup>2</sup> Mean value statistically significant different compared to the control (p < 0.05, Dunnett test, two-sided, α = 0.05)

Toxic standard:

The most recent toxic standard - Carbendazim test (IBACON Project 21341022) showed statistical significant effects on reproduction at a concentration of 1.1 mg carbendazim/kg soil dw. The EC<sub>50</sub> value for reproduction was calculated as 1.25 mg carbendazim/kg soil dw.

#### Conclusion:

NOEC<sub>reproduction</sub> = 1000 mg test item/kg soil dw

LOEC<sub>reproduction</sub> > 1000 mg test item/kg soil dw

NOEC<sub>growth</sub> = 320 mg test item/kg soil dw

LOEC<sub>growth</sub> = 320 mg test item/kg soil dw

In accordance with the new data requirement ( Commission Regulation EU No 283/2013), the EC<sub>10</sub>, EC<sub>20</sub>, values should be calculated. In this case no adverse effects on reproduction were observed in this chronic earthworm study. Therefore, it is not possible to calculate an EC<sub>x</sub> value. No additional calculation is required.

**RMS comments:**

The earthworm reproduction study was conducted according to the test guidelines ISO 11268-2: 1998 (E) and BBA (1994).

Taking into account the validity criteria given in the test guideline OECD 222 (2004) the study is considered acceptable.

The mortality of adults in the control was below 10% (being: 5%). The number of juveniles per control replicate was greater than 30 (being 246-375 juveniles per replicate).

The coefficient of variation of reproduction in the control was  $\leq 30\%$  (being 19.8%).

The following deviation from the OECD 222 (2004) test guideline was noted:

- Only four control replicates were used in the reported study instead of eight as recommended by OECD 222 guideline.

**Agreed endpoints:**

NOEC<sub>reproduction</sub> = 1000 mg test item/kg soil dw

LOEC<sub>reproduction</sub>: > 1000 mg test item/kg soil dw

NOEC<sub>growth</sub> = 320 mg test item/kg soil dw

LOEC<sub>growth</sub> = 320 mg test item/kg soil dw

**B.9.4.1.6. Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474): Effects on survival, growth and reproduction on the earthworm *Eisenia fetida* tested in artificial**

<b>Reference:</b>	Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474): Effects on survival, growth and reproduction on the earthworm <i>Eisenia fetida</i> tested in artificial soil.
<b>Author(s), year:</b>	Kratz, M. A.; 2012
<b>Report/Doc. number:</b>	Report No, KRA-RG-R-131/12, Reference BCS No M-436340-01-1
<b>Guideline(s):</b>	ISO 11268-2: 1998 (E) and OECD 222: April 13, 2004
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474); Batch code: BCS-CU62474-01-01; purity: 99.4 % w/w.
Test species:	Earthworm <i>Eisenia fetida</i> ( <i>Eisenia fetida</i> Andrei)
Number of organism:	8 replicates per treatment group and control group, each with 10 individuals
Weight, age:	300-500 mg/worm, adults with clitellum, approximately 5 months old.
Type of test, duration:	Laboratory sub-lethal test, 8 weeks (4 weeks adult mortality, 4 weeks juvenile development)

Applied concentrations:

Nominal:	0 (control, quartz sand), 100 mg test item/kg dry soil. The test item was mixed into the soil.
Solvent:	None
Toxic standard:	Derosal flüssig (BAS 346F, 360 g carbendazin/L), tested at concentrations of 1.25, 2.5 and 5 mg a.s./kg soil dw
Test substrate:	Artificial soil, 5 % sphagnum peat, 20 % kaolin clay, 73.82 % industrial quartz sand, 0.18 % calcium carbonate, 1% food (dried ground cow manure). The artificial soil was prepared by mixing the dry components intensely in a laboratory mixer.
Substrate/vessel:	The plastic boxes (length x width x height <i>ca.</i> 16 cm x 12cm x 6 cm, area approximately 200 cm <sup>2</sup> ) were used as test vessels. Each test vessel contained an amount of approximately 500 g dry weight artificial soil to obtain a depth of approximately 5 cm soil in the test vessels.
Temperature:	18-22 °C
Light regime:	16 hours light/8 hours dark. Light intensity: 518 Lux at day 0, 535Lux at day 28 and 483 Lux at day 56 of the study.
Water content:	Test start: 25.73-26% (corresponding to 54.22-55.01 % of WHC)

---

pH:	Test end: 23.82-24.10% (corresponding to 48.96-49.72 % of WHC) Test start: 5.62-5.68 Test end: 6.71-6.74
Feeding:	Air-dried and finely ground cow manure; feeding interval was weekly during the first 4 weeks, weekly amount of manure (5 g) depended on the feeding activity. The off spring were fed only once at the start of the second week.
Test parameters:	Temperature was recorded continuously during the whole test period. The moisture content of the artificial soil were determined once a week. The pH measurements were determined prior the test and checked at the start and the end of test for all treatment levels. Mortality of adults (assessed after 28 days), mean body weight of adults (measured at day 0 and after 28 days), morphological and behavioural changes of adults (observed at day 28) number of juvenile earthworms (counted after 8 weeks). Cumulative amount of food added to each test container during the test period.
Statistic:	The endpoints were mortality, change of biomass (difference in fresh weight of surviving worms between test start and four weeks after treatment) and reproduction (the number of juveniles present). The reproduction of the surviving test organisms per test vessel at the end of the study was compared to the control values. Changes in body weight values of the surviving test organisms of the treatment groups during the test period were compared to the values of the control group. The homogeneity of variances of the data was checked by Cochran's test. The normal distribution of the data was tested by Kolmogorov-Smirnov test. The normality hypothesis of the data was not accepted. Therefore the data were transformed. Since the normality hypothesis of the transformed data was accepted a Student-t test was applied. The reproduction of the surviving test organisms per test vessel at the end of the study was compared to the control values. The homogeneity of variances of the data was checked by Cochran's test. The homogeneity hypothesis was even after transformation rejected. The normal distribution of the data was tested by Kolmogorov-Smirnov test. The normality hypothesis of the data was accepted. Therefore data were statistically evaluated by means of a Welch-t test for inhomogeneous variances was used. The statistical software package ToxRatPro Version 2.10 was used for the calculation. The data on food addition was not statistically analyzed since 100% consumption in all the occasions.

## Findings:

After 28 days of exposure no worms died in the control group and no mortality was observed at any test item concentration.

Statistically significant different values for the growth relative to the control were not observed.

No statistically significant different values for the number of juveniles per test vessel relative to the control were recorded.

There was no significant difference in food consumption at 100 mg item/kg soil dry weight, when compared to the control.

All the treatment groups recorded complete consumption of food during the four weeks of exposure.

In all tested concentrations, no statistically significant different values for the growth relative to the control were observed (Williams multiple sequential t-test, two-sided,  $\alpha = 0.05$ ).

Effects on mortality and changes in body weight of the adults after an exposure period of 28 days and the number of off spring per test vessel after 56 days are shown in the Table B. 9.4.1.6-1 below:

**Table B. 9.4.1.6-1: Effects on mortality and reproduction of *Eisenia fetida* in a sub-chronic test.**

	<b><i>Eisenia fetida</i></b>	
	<b>Trifluoroethanesulfonic acid (mg test item/kg dry soil)</b>	
Exposure	Control	100
Mortality of adult earthworms [%] after 28 days	0	0
Mean change of body weight of the adults from day 0 to day 28 [%] (Standard deviation)	21.68 ( $\pm 4.34$ )	24.24 <sup>ns1</sup> ( $\pm 4.12$ )
Mean number of offspring per test vessel after 56 days (Standard deviation)	322.5 ( $\pm 20.2$ )	312.9 <sup>ns2</sup> ( $\pm 58.9$ )
% reproduction compared to control	-	97.0

n.s.<sup>1</sup> Mean value not statistically significant different compared to the control ((Student-t test for homogeneous variances, two-sided,  $\alpha = 0.05$ )

n.s.<sup>2</sup> Mean value not statistically significant different compared to the control (Welch-t test for inhomogeneous variances, one-sided smaller,  $\alpha = 0.05$ )

## Toxic standard:

In positive control (Carbendazim) the number of juveniles was significantly reduced at test concentrations:

2.5 – 5.0 mg test item/kg soil dw. There were no observed juveniles in highest test concentration and were 44.5 juveniles at concentration at 2.5 mg item/kg dry weight after 8 weeks of test duration. The mean number of juveniles in the control was 322.8.

The EC<sub>50</sub> for reproduction was calculated to be 1.66 mg a.s./kg dry weight with 95 % confidence limits between 1.62 – 1.69 mg a.s./kg soil dw.

**Conclusion:**

$\text{NOEC}_{\text{growth}} \geq 100 \text{ mg test item/kg soil dw}$

$\text{LOEC}_{\text{growth}} > 100 \text{ mg test item/kg soil dw}$

$\text{NOEC}_{\text{reproduction}} \geq 100 \text{ mg test item/kg soil dw}$

$\text{LOEC}_{\text{reproduction}} > 100 \text{ mg test item/kg soil dw}$

$\text{EC}_{50} > 100 \text{ mg test item/kg soil dw}$

$\text{LOEC} > 100 \text{ mg test item/kg soil dw}$

In accordance with the new data requirement ( Commission Regulation EU No 283/2013), the  $\text{EC}_{10}$ ,  $\text{EC}_{20}$ , values should be calculated. In this case no adverse effects on reproduction were observed in this chronic earthworm study. Therefore, it is not possible to calculate an  $\text{EC}_x$  value. No additional calculations are required.

**RMS comments:**

The earthworm reproduction study was conducted according to test guideline OECD 222 (2004). Taking into account the validity criteria given in the test guideline OECD 222 (2004) the study is considered acceptable.

The mortality of adults in the control was below 10% (being: 0%). The number of juveniles per control replicate was greater than 30 (being 294-365 juveniles per replicate).

The coefficient of variation of reproduction in the control was  $\leq 30\%$  (being 6.3%).

Some deviation to the OECD guidelines was identified.

-The pH values at the end of test were at range 6.71-6.74, which are above the recommendations given in the OECD 222 guidelines (5.5-6.0).

Since all validity criteria was met, this deviation is considered to have no impact on the study result.

Therefore, the study is considered acceptable.

**Agreed endpoint:**

$\text{NOEC}_{\text{growth}} \geq 100 \text{ mg test item/kg soil dw}$

$\text{LOEC}_{\text{growth}} > 100 \text{ mg test item/kg soil dw}$

$\text{NOEC}_{\text{reproduction}} = 100 \text{ mg test item /kg soil dw}$

$\text{LOEC}_{\text{reproduction}} > 100 \text{ mg test item /kg soil dw}$

$\text{EC}_{50} > 100 \text{ mg test item/kg soil dw}$

**B.9.4.1.7. Flufenacet-thiadone (AE 1258593, BCS-AA 41715): Effects on survival, growth and reproduction on the earthworm *Eisenia fetida* tested in artificial soil.**

<b>Reference:</b>	Flufenacet-thiadone (AE 1258593, BCS-AA 41715): Effects on survival, growth and reproduction on the earthworm <i>Eisenia fetida</i> tested in artificial soil.
<b>Author(s), year:</b>	Kratz M.A. 2012
<b>Report/Doc. number:</b>	Report No, KRA-RG-R-136/12, Reference BCS No M-442579-01-1
<b>Guideline(s):</b>	ISO 11268-2: 1998 (E) and OECD 222: April 13, 2004
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet-thiadone (AE 1258593, BCS-AA 41715); Batch Code: AE 1258593-01-01; purity: 98.6 % w/w
Test species:	Earthworm <i>Eisenia fetida</i> ( <i>Eisenia fetida Andrei</i> )
Number of organism:	8 replicates per control group and 4 replicates per treatment group and, each with 10 individuals
Weight, age:	310-510 mg/worm, adults with clitellum, 5 months old
Type of test, duration:	Laboratory sub-lethal test, 8 weeks (4 weeks adult mortality, 4 weeks juvenile development)
Applied concentrations:	
Nominal:	0 (control, quartz sand), 1, 1.8, 3.2, 5.6, and 10.0 mg test item/kg dry weight artificial soil. The test item was mixed into the soil.
Solvent:	None
Toxic standard:	Derosal flüssig (BAS 346F, 360.7 g carbendazin./l), tested at concentrations of 1.25, 2.5 and 5 mg a.s./kg dws.
Test substrate:	Artificial soil, 5 % sphagnum peat, 20 % kaolin clay, 73.8 % industrial quartz sand, 0.2 % calcium carbonate, 1% dried ground cow manure (food). The artificial soil was prepared by mixing the dry components intensely in a laboratory mixer.
Substrate/vassel:	Non-re-usable plastic boxes (length x width x height <i>ca.</i> 16.5 cm x 12 cm x 6 cm, area approximately 200 cm <sup>2</sup> ) were used as test vessels. Each test vessel contained an amount of approximately 500 g dry weight artificial soil to obtain a depth of approximately 5 cm soil in the test vessels.
Temperature:	18-22 °C
Light regime:	16 hours light/8 hours dark. Light intensity: 501 Lux at day 0, 513 Lux at day 28 and 546 Lux at day 56 of the study.
Water content:	Test start: 25.20-26.20 % (corresponding to 55.50 -58.48% of WHC)

---

	Test end: 26.17-27.87 % (corresponding to 58.41-63.66% of WHC)
pH:	Prior the test: 6.12 Test end: 5.84- 6.74
Feeding:	Air-dried and finely ground cow manure; feeding interval was weekly during the first 4 weeks, weekly amount of manure (5 g) depended on the feeding activity. The off spring were fed only once at the start of the second week.
Test parameters:	Temperature was recorded continuously during the whole test period. The moisture content of the artificial soil were determined prior to test start, at day application Day 0 and day 56 of the test. The pH measurements were made prior the test and the end on Day 56 of the study. Mortality of adults (assessed after 28 days), mean body weight of adults (measured at day 0 and after 28 days), morphological and behavioural changes of adults (observed at day 28) number of juvenile earthworms (counted after 8 weeks) .
<u>Statistic:</u>	The endpoints were mortality, change of biomass (difference in fresh weight of surviving worms between test start and four weeks after treatment) and reproduction (the number of juveniles present). Changes in body weight values of the surviving test organisms of the treatment groups during the test period were compared to the values of the control group. The homogeneity of variances of the data was checked by Cochran's test. The normal distribution of the data was tested by Kolmogorov-Smirnov test. The normality hypothesis of the data was accepted. The data were statistically evaluated by means of a Williams multiple sequential t-test. The reproduction of the surviving test organisms per test vessel at the end of the study was compared to the control values. The homogeneity of variances of the data was checked by Cochran's test. The homogeneity hypothesis was rejected. The normal distribution of the data was tested by Kolmogorov-Smirnov test. The normality hypothesis of the data was accepted. A meaningful statistical calculation of the homogeneity of variances with transformed data was not possible. Therefore, the data were statistically evaluated by using the Welch-t test for inhomogeneous variances with Bonferroni-Holm adjustment. EC <sub>50</sub> could not be calculated. The statistical software package ToxRatPro Version 2.10@ was used for the calculation.
Findings:	After 28 days of exposure no worms died in the control group and no mortality was observed at all test item concentrations. No statistically significant different values for the growth relative to the control were observed (results of a Williams multiple sequential t-test, two-sided, $\alpha =$

0.05.) up and including the highest treatment rate 10.0 mg test item/kg soil dw. Statistically significant different values for the number of juveniles per test vessel relative to the control were observed at the two highest test concentrations 5.6 and 10.0 mg test item/kg dry weight artificial soil (Welch-T test for inhomogeneous variances with Bonferroni-Holm adjustment, one-sided smaller,  $\alpha = 0.05$ ).

Effects on mortality and changes in body weight of the adults after an exposure period of 28 days and the number of offspring per test vessel after 56 days are shown in the Table B.9.4.1.7-1 below:

**B.9.4.1.7-1: Effects on mortality and reproduction of *Eisenia fetida* in a sub-chronic test.**

	<b><i>Eisenia fetida</i></b>					
	<b>Flufenacet-thiadone (AE 1258593, BCS-AA 41715)</b>					
	<b>(mg test item/kg dry soil)</b>					
Exposure	Control	1.0	1.8	3.2	5.6	10.0
Mortality of adult earthworms [%] after 28 days	0	0	0	0	0	0
Mean change of body weight of the adults from day 0 to day 28 [%] (Standard deviation)	29.55 (± 4.05)	29.66 <sup>ns</sup> (±5.80 )	37.90 <sup>ns</sup> (±8.51)	40.34 <sup>ns</sup> (± 5.97)	36.40 <sup>ns</sup> (± 11.77)	36.80 <sup>ns</sup> (±3.32)
Mean number of offspring per test vessel after 56 days (Standard deviation)	340.1 (± 33.8)	324.5 (±43.1)	338.3 (±72.4)	303 (±3.6)	274.8** (±16.5 )	271** (20.1)
% reproduction compared to control	100	95.4	99.4	89.1	80.8	79.7

<sup>ns</sup> No statistical significance compared to the control (Williams Multiple Sequential t-test, two-sided,  $\alpha = 0.05$ )

<sup>\*\*</sup> Statistical significance compared to the control (Welch-t test for inhomogeneous variance with Bonferroni-Holm adjustment, one-sided smaller,  $\alpha = 0.05$ , p)

Toxic standard:

In positive control (Carbendazim) the number of juveniles was significantly reduced at all test concentrations (1.25, 2.5 and 5.0 mg a.s./kg dws). There were no observed juveniles in highest test concentration.

In the lower test concentration - 2.5 mg a.s./kg dws - 23.55 juveniles were recorded after 8 weeks of test duration. The mean number of juveniles in the control was 337.13.

The EC<sub>50</sub> for reproduction was calculated to be 1.66 mg a.s./kg soil dw.

The results of the reference test item indicated that the test system was sensitive to the reference test item.

#### Conclusion:

NOEC<sub>reproduction</sub> = 3.2 mg test item/kg soil dw

LOEC<sub>reproduction</sub> = 5.6 mg test item/kg soil dw

NOEC<sub>growth</sub> ≥ 10 mg test item/kg soil dw

LOEC<sub>growth</sub> > 10 mg test item/kg soil dw

In accordance with the new data requirements (Commission Regulation EU No 283/2013), the EC<sub>10</sub> and EC<sub>20</sub> values should be calculated.

A valid EC<sub>x</sub> calculation was not possible due to the lack of a significant concentration/response function. As at the NOEC = 3.2 mg/kg the difference in number of juveniles compared to control is about 10% this endpoint should be appropriate for being used in the risk assessment. The non-valid EC<sub>10</sub> calculation provided an EC<sub>10</sub> of about 3.32 to 3.38, depending on the model used. However, the Applicant not include them, as the fit is not significant and no significant concentration response functions could be calculated. EC<sub>10</sub> would be higher than the NOEC, so, less critical.

**Comment RMS:**

The earthworm reproduction study was conducted according to the test guideline OECD 222 (2004). Taking into account the validity criteria given in the test guideline OECD 222 (2004) the study is considered acceptable.

The mortality of adults in the control was below 10% (being: 0%). The number of juveniles per control replicate was greater than 30 (being 295-389 juveniles per replicate).

Mean change in growth of the adult earthworms in the control during the exposure period of four weeks  $\geq 20\%$  (being 29.55 %) The coefficient of variation of reproduction in the control was  $\leq 30\%$  (being 9.9%).

The following deviation from OECD 222 (2004) test guideline was identified:

-The pH value at the end of test was 6.74 (the range between 5.5 and 6.5 is recommended by OECD 222 test guideline).

Since all validity criteria was met, this deviation is considered to have no impact on the study result.

Therefore, the study is considered acceptable.

**Agreed endpoints:**

NOEC<sub>reproduction</sub> = 3.2 mg test item/kg soil dw

NOEC<sub>growth</sub>  $\geq 10$  mg test item/kg soil dw

LOEC<sub>reproduction</sub> = 5.6 mg test item/kg soil dw

LOEC<sub>growth</sub>  $> 10$  mg test item/kg soil dw

**B.9.4.1.9.****Field studies****Flufenacet SC 500****B.9.4.1.9. Flufenacet SC 500: effect on the earthworm fauna of a grassland area within one year**

<b>Reference:</b>	Flufenacet SC 500: effect on the earthworm fauna of a grassland area within one year.
<b>Author(s), year:</b>	Leicher, T., 2008
<b>Report/Doc.</b>	Study NO: E 311 3301 – 2. Reference BCS NO: M-307211-01-1
<b>Guideline(s):</b>	BBA, Part VI, 2 - 3 (January 1994):
<b>ISO Guideline</b>	CD 11268-3 (E), Soil Quality - Part 3: (1999).
<b>GLP:</b>	Yes

**Material and methods**

Test substance:	Flufenacet SC 500, Batch No.: EFKF000175, content of a.s.: 499.9 g/L, density: 1.999 g/l.
Test site:	The effect of Flufenacet 500 SC (Flufenacet 499.9 g/l) on earthworm populations was investigated in Monheim, East Germany under field conditions after one spray application on grassland at dose level of 1.2 l formulation/ha on May 22 2007 with water treated control and reference product Carbendazin 500 SC % w/w, 500 g Carbendazin/ha).
Field story	Four weeks before the application (14 April 2007), 7 weeks (10 July 2007) and 16 weeks (11 September 2007) after the application 5 l of Glyphos /ha was used in order to distinguish between possible direct effects of Flufenacet 500 SC on earthworms from in direct effects caused by its impact on the plant cover the test site.

The summary of field application, activities and earthworm sampling is summarized Table **B.9.4.1.9-1** below:

**Table B.9.4.1.9-1: Flufenacet application.**

Date	Activities	Earthworm sapling
April 19, 2007		pre-sampling
April 24, 2007	Application of Glyphos	
May 11, 2007	The soil surface was carefully crushed and flattened	
<b>May 22 , 2007</b>	<b>Application of flufenacet and carbendazim</b>	
July 10, 2007	Application of Glyphos	
July 23, 2007	Removal of weeds	
July 25, 2007	Removal of weeds	<u>x</u>
September 11, 2007	Application of Glyphos	
October 30, 2007		<u>x</u>
April 22, 2008		<u>x</u>

The study area was used as grassland for many years and has not been treated with pesticides for at least 5 years (in the year 2002) .

#### Soil type/substrate

Samples of the top 0 - 10 cm soil were taken at randomly selected places of the study area. The samples were combined, air dried and homogeneously mixed after removal of stones, roots and larger particles and subsequently sieved at 2 mm (= mixed sample).

The soil parameters are shown in the Table B.9.4.1.9-2 below.

**Table B.9.4.1.9-2: Soil parameters**

Test parameter	Results
pH	6.39
Water holding capacity	51.8
%C <sub>org</sub>	1.60
%C <sub>anor</sub>	0.05
%N	0.166
%P (mg/kg)	555
% sand	63.6
%silt	27.0
%clay	9.4
Classification according to DIN	Loamy sand

#### **Climatic condition**

The climatic data on the day of the application and 3 days afterwards are summarized in the Table below:

**Table B.9.4.1.9-3: Daily climatic condition from the day of application onwards for 6 day**

Date	Temperature (°C) (mean values per day)	Sunshine (h) per day	Precipitation (mm) per day
May 22, 2007	18.82	6.23	14.5
May 23, 2007	19.52	7.38	0
May 24, 2007	21.48	9.7	0
May 25, 2007	20.73	7.92	0
May 26, 2007	16.68	1.83	13.6
May 27, 2007	14.85	0.62	5.3
May 28, 2007	11.43	0	5.4

The mean monthly weather conditions (May 2007 – April 2008) are summarized in the Table below:

**Table B.9.4.1.9-4: Monthly climatic conditions <sup>1</sup> (mean values).**

Month	Temperature		Cumulated precipitation		Sunshine	
	°C	Compared to long - term*)	mm	Compared to long -term*)	hours	Compared to long-term*)
May, 2007 *)	15.13	13.9	104.7	61.6	148.50	187.3
June, 2007 *)	18.10	16.6	70.2	77.5	109.21	171.2
July, 2007 *)	17.85	18.7	102.9	71.3	138.43	185.6
August, 2007 *)	17.06	18.4	133.2	70.0	167.17	184.3
September, 2007 *)	13.69	14.9	43.3	61.6	118.43	134.1
October, 2007**)	11.43	10.8	41.6	61.4	132.49	99.4
November, 2007**)	7.32	6.3	95.9	62.1	29.01	54.6
December, 2007**)	4.84	3.6	98.6	69.4	55.56	36.9
January, 2008*)	6.99	2.8	69.0	60.8	42.42	46.0
February, 2008*)	6.01	3.4	47.7	47.3	131.00	70.2
March, 2008*)	5.79	6.1	92.3	58.0	72.90	103.6
April, 2008*)	8.84	9.3	68.3	55.3	114.33	151.8

<sup>1</sup> Laacher Hof Experimental Station of the BAYER CropScience closed to study area

\*) mean of 1966 - 2006

\*\*) mean of 1966 – 2005

#### Application

Flufenacet 500 SC treatment, the untreated control and the reference product were assigned to four replicate plots (12 plots total) in a randomized block design. On the test site, 10 x 10 m plots were established. Applications were made in 400 L water/ha on 22 May 2007 r. using boom sprayer. Nominal application rates of Flufenacet 500 SC was 1.2 L formulation/ha equals 14.388 g of formulated product per plot (100 m<sup>2</sup>) and Carbendazim 500 SC was applied at rate 1 x 8000 g s.a. /ha.

Within three days after application 14.5 mm of precipitation was measured

#### Earthworm sampling

Samplings were conducted on 19.04. 2007 (pre-test, 5 weeks before application), 25 July 2007 (nine weeks after treatment), 30 October 2007 (5 months after treatment) and on 22 April 2008 (11 months after treatment).

From each plot earthworms were sampled (4 areas of 0.25 m<sup>2</sup> within the inner 6 x 6 m area of each plot) by formalin extraction. To investigate the efficiency of the formalin-sampling method during the study, some samples were evaluated by hand-sorting. At each sampling date, three samples (four samples for the pre-sampling) were selected to determine the number of earthworms which were left in the soil 30 minutes after formalin

application. Immediately after finishing the formalin incubation, the soil of these samples was dug and hand-sorted to a depth of about 30 cm.

The efficiency of the sampling methods was different at the four sampling times (pre-sampling, 9 weeks, 5 months and 11 months after the application). At the pre-sampling, 66 % of the worms were detected by formalin and 34 % by hand-sorting. Nine weeks after the application, 56 % of the worms were detected by formalin extraction and 44 % by hand-sorting. Especially for *Apocorrectodea caliginosa* the extraction efficiency was in one sample (sample 3) with only 13 % very low. Five months after the application 79 % of worms were detected by formalin and 21 % by hand-sorting. Eleven months after the application 71 % of worms were detected by formalin and 29 % by hand-sorting.

Adult earthworms and juveniles were identified and their numbers and weights were recorded. In addition to the above, surface monitoring for direct mortality effects on earthworms was performed the first three days after application.

#### Chemical soil analysis

Overall 5 soil samples were taken per treatment replicate and from untreated control areas on the day of application to analytical verify application rate.

Soil samples were analysed for the determination of the residues of Flufenacet in soil by HPLC-MS/MS analysis. LOQ was 4 µg/kg.

On the treated plots Flufenacet was detected on average in concentration of 0.438 mg/kg soil dry weight assuming a soil depth of 10 cm and soil density of 1.5 g/cm<sup>3</sup>. This is equivalent to 110 % of the nominal application rate of 1.2 l Flufenacet SC500/ha resulting in nominal concentration of 399 Flufenacet mg/kg dry weight soil.

#### Statistical analysis

The results of the sampling were statistically evaluated by the Wilcoxon, Mann and Whitney U-test ( $p = 0.05$ , two sided). The statistic software used was ToxRatPro Version 2.09.

### Findings

#### Soil analysis

The application of 1.2 l Flufenacet SC 500/ha should lead to a concentration of Flufenacet of 0.399 mg Flufenacet/kg dry weight soil, considering a soil depth of 10 cm and a soil density of 1.5 g/cm.

The analytical results revealed an average content of 0.438 mg Flufenacet/kg dry weight soil corresponding to 110% of the nominal application rate on the treated plots.

The measured soil concentrations were within the range of 50-150% indicated by Kula et al (2006) as the acceptable range.

Meteorology	<p>The application of Flufenacet 500 SC was performed on May 22, 2007. The climatic data on the day of application and 3 days afterwards are summarized in the Table B.9.4.1.9-3</p> <p>Compared to the long term mean of 39 or 40 years (Table B.9.4.1.9-4:), the temperatures were higher (6 - 150 % higher) from May, June 2007 and October 2007 to February 2008. In July to September 2007 and March to April 2008, the temperatures were lower (5 - 8%), compared to the long term mean of 39 or 40 years. Sunshine was lower (8-47%) from May to September, November 2007 and January, March, April 2008, except for October, December 2007 and February 2008, when sunshine was higher (33 – 87%). There was more precipitation (1-90%) compared to the long term mean of 39 or 40 years, only in June, September, October 2007 (9- 32%) the precipitation was lower.</p>
Earthworm surface searches:	<p>During earthworm searches 1, 2 and 3 days after application, two dead earthworms were found on the soil surface in the control (on the 1<sup>st</sup> and 3<sup>th</sup> day after application) and one dead (in the 1<sup>st</sup> day after application) at Flufenacet 500 SC treatment at rate 1.2 L/ha. Only one alive earthworm was found after application rate of 8 kg carbendazim/ha.</p>
Abundance and biomass:	<p>The pre-sampling (five weeks before application) of earthworms revealed sufficiently high earthworm populations (196 individuals/m<sup>2</sup>) across all plots.</p> <p>During pre-sampling the species such as <i>Lumbricus terrestris</i>, <i>Lumbricus rubellus</i>, <i>Lumbricus castaneus</i>, <i>Aporrectodea caliginosa</i> were found.</p> <p>Anecic, endogeic and epigeic species represented 30.4% and 1.2 % and 68.4 % of the total adult earthworm, five weeks before application. The dominant anecic species was <i>Lumbricus terrestris</i> (16% of total earthworm, 30% of total adult earthworms). The dominant endogeic species was <i>Aporrectodea caliginosa</i> (0.63% of total earthworms, 1.2% of total adult). The dominant epigeic species was <i>Lumbricus castaneus</i> (32% of total earthworms, 61% of adult) followed by <i>Lumbricus rubellus</i> (3.84% of total earthworm, 7.2% of adults). The juvenile:adult ratio was 0.87 (equivalent to 53% adults).</p> <p>During the study the six different species of earthworms was found in the test plots represented by: <i>Lumbricus terrestris</i>, <i>Lumbricus rubellus</i>, <i>Lumbricus castaneus</i>, <i>Aporrectodea caliginosa</i>, <i>Aporrectodea terrestris longa</i> and <i>Aporrectodea chlorotica</i>. Because of similar external taxonomic characters of the immature (juvenile) epigeic worms, a classification to the</p>

individual species level was sometimes not possible. Therefore *L. rubellus* and *L. castaneus* were combined and are presented as "total epigeic species" and for the category "adult and juvenile" and for the category "juvenile". The class "epigeic species" may also contain a small percentage of juvenile *L. terrestris*, which, based on their size, could not be classified as *L. terrestris*.

In this field study effects are investigated for the following categories: adults and juvenile earthworm, adult earthworm, juvenile earthworm, for dominant species and for species according to ecological classes as follows:

- total anecic species (comprising *Lumbricus terrestris*, *Aporrectodea terrestris longa*)
- total of endogeic species comprising *Allolobophora chlorotica*, *Aporrectodea caliginosa*)
- epigeic species (comprising *Lumbricus rubellus*, *Lumbricus castaneus*)
- total earthworms (of all species).

Table B.9.4.1.9-5. Results of pre-sampling trials. Means and standard deviation from 16 plots per 0.25 m<sup>2</sup>.

	<i>Lumbricus terrestris</i>	<i>L. rubellus/L. castaneus</i>	<i>Aporrectodea caliginosa</i>
	<b>numbers</b>		
adult & juvenile	13.69 ± 5.59	33.88 ± 18.47	1.38 ± 1.96
adult	7.94 ± 4.82	L. rub. 1.88 ± 1.63 L. cast. 15.94 ± 12.26	0.31 ± 0.87
juvenile	5.75 ± 3.04	16.06 ± 8.10	1.06 ± 1.65
	<b>biomass [g]</b>		
adult & juvenile	25.03 ± 13.68	3.70 ± 1.89	0.55 ± 1.05
adult	19.31 ± 12.86	L. rub. 0.50 ± 0.44 L. cast. 1.31 ± 1.00	0.33 ± 0.90
juvenile	5.71 ± 3.95	1.89 ± 1.12	0.22 ± 0.48
	<b>total of anecic species</b>	<b>total of endogeic species</b>	<b>total of epigeic species</b>
	<b>numbers</b>		
adult & juvenile	13.69 ± 5.59	1.38 ± 1.96	33.88 ± 18.47
adult	7.94 ± 4.82	0.31 ± 0.87	17.81 ± 12.63
juvenile	5.75 ± 3.04	1.06 ± 1.65	16.06 ± 8.10
	<b>biomass [g]</b>		
adult & juvenile	25.03 ± 13.68	0.55 ± 1.05	3.70 ± 1.89
adult	19.31 ± 12.86	0.33 ± 0.90	1.81 ± 1.15
juvenile	5.71 ± 3.95	0.22 ± 0.48	1.89 ± 1.12
	<b>total earthworms</b>		
	<b>numbers</b>		
adult & juvenile	48.94 ± 20.02		
adult	26.06 ± 14.29		
juvenile	22.88 ± 9.41		
	<b>biomass [g]</b>		
adult & juvenile	29.28 ± 13.62		
adult	21.45 ± 13.13		
juvenile	7.83 ± 4.24		

The changes in numbers and biomass earthworms after application of Flufenacet 500 SC are presented in the Tables below:

Adult and juvenile earthworms, changes in numbers and biomass:

**Table B.9.4.1.9-6:** Data for category “adult and juvenile” and for the species classes “total”, “total anecic”, “total endogeic” and “total epigeic” earthworms. The values are replicate means (n = 4) and standard deviations per 0.25 m<sup>2</sup>. Values between parentheses are relative differences to the control in %:

Treatment group	9 weeks after the application			5 months after the application			11 months after the application		
	Numbers (n) / replicate								
	Total earthworms								
Control	28.31 ± 3.46			40.88 ± 2.99			76.50 ± 14.86		
Flufenacet	20.75 ± 3.69		(-27%) *	39.81 ± 8.61		(-3%)	76.19 ± 5.54		(0%)
Carbendazim	13.88 ± 2.92		(-51%) *	40.88 ± 8.61		(0%)	54.00 ± 5.07		(-29%)*
	Total of anecic earthworms								
Control	10.06 ± 1.55			25.25 ± 2.35			17.06 ± 3.13		
Flufenacet	9.63 ± 1.16		(-4%)	24.56 ± 3.64		(-3%)	19.75 ± 1.34		(+16%)
Carbendazim	3.38 ± 1.05		(-66%) *	20.06 ± 2.49		(-21%) *	15.63 ± 4.09		(-8%)
	Total of endogeic earthworms								
Control	8.81 ± 3.99			5.44 ± 2.81			53.13 ± 13.68		
Flufenacet	5.13 ± 2.72		(-42%)	6.81 ± 4.52		(+25%)	48.88 ± 7.11		(-8%)
Carbendazim	2.81 ± 1.71		(-68%) *	9.69 ± 2.38		(+78%)	26.69 ± 4.93		(-50%)*
	Total of epigeic earthworms								
Control	9.44 ± 1.48			10.19 ± 3.45			6.31 ± 2.15		
Flufenacet	6.00 ± 1.15		(-36%) *	8.44 ± 1.13		(-17%)	7.56 ± 2.68		(+20%)
Carbendazim	7.69 ± 3.15		(-19%)	11.13 ± 7.74		(+9%)	11.69 ± 3.78		(+85%)
	Biomass (g) / replicate								
	Total earthworms								
Control	18.21 ± 3.31			36.46 ± 9.78			44.79 ± 5.64		
Flufenacet	15.83 ± 4.86		(-13%)	36.45 ± 4.32		(0%)	47.64 ± 2.47		(+6%)
Carbendazim	5.74 ± 1.25		(-68%) *	28.34 ± 6.23		(-22%)	32.84 ± 2.67		(-27%)*
	Total of anecic earthworms								
Control	16.42 ± 3.08			33.34 ± 9.81			24.01 ± 4.22		
Flufenacet	15.13 ± 4.75		(-8%)	32.54 ± 2.54		(-2%)	28.00 ± 3.47		(+17%)
Carbendazim	4.39 ± 1.34		(-73%) *	18.96 ± 4.74		(-43%)	16.11 ± 4.44		(-33%)
	Total of endogeic earthworms								
Control	1.12 ± 0.54			2.09 ± 0.98			20.23 ± 4.36		
Flufenacet	0.40 ± 0.23		(-64%) *	2.86 ± 1.48		(+37%)	18.86 ± 2.96		(-7%)
Carbendazim	0.70 ± 0.53		(-38%)	7.11 ± 2.03		(+241%) *	15.19 ± 1.63		(-25%)
	Total of epigeic earthworms								
Control	0.67 ± 0.26			1.04 ± 0.31			0.55 ± 0.24		
Flufenacet	0.29 ± 0.26		(-56%) *	1.06 ± 0.50		(+2%)	0.78 ± 0.12		(+41%)
Carbendazim	0.66 ± 0.28		(-2%)	2.27 ± 1.54		(+119%) *	1.54 ± 0.27		(+180%)*

\* Statistically significant difference compared to control (Wilcoxon, Mann and Withney U-Test, p = 0.05)

An application of 1.2 l product/ha, Flufenacet SC 500 has no statistically significant effect on the parameters “numbers” and “biomass” of all tested categories earthworms five and 11 months after the application, indicating no effect of Flufenacet on the earthworm community.

However nine weeks after application for the category “total earthworms” a statistically significant reduction in number of -27% and a statistically insignificant reduction of the biomass of -13 % were observed. The group of anecic earthworms was not affected on Flufenacet treated plots nine weeks after application (Numbers -4 %;

biomass -8%). The ecological groups of endogeic (Number -42%; biomass -64 %) and epigeic (Number -36 %; biomass -56%) earthworms were reduced on Flufenacet treated plots nine weeks after application.

The reduction of total (adult&juvenile) endogeic and total epigeic classess, nine weeks after application of Flufenacet 500 SC study director explained by influence of Flufenacet 500 SC acting as herbicide on the vegetation of the treated plots. Although all plots were treated with Glyphos before start of the test, untreated plots showed a regrowing of weeds. Especially in the dry summer period this strong influence on the water regime of the soil thereby affecting the habitat of the endo- and epigeic earthworms could be observed.

Therefore, this variation could be not considered as compound related effect but rather a secondary effect of the herbicide Flufenacet 500 SC on the earthworm community.

An application of toxic reference at 8000 g carbendazin/ha had statistically significant effect > 50% on the parameter “number” for category total earthworm.

#### Adult earthworms changes in numbers and biomass:

**Table B.9.4.1.9-7: Data for category “adult” and for the species classes “total”, “total anecic”, “total endogeic” and “total epigeic” earthworms. The values are replicate means (n = 4) and standard deviations per 0.25 m<sup>2</sup>. Values between parentheses are relative differences to the control in %:**

Treatment group	9 weeks after the application		5 months after the application		11 months after the application	
	Numbers (n) / replicate					
	Total earthworms					
Control	5.25 ± 1.46		14.88 ± 3.11		20.94 ± 6.62	
Flufenacet	4.56 ± 1.61	(-13%)	14.81 ± 1.36	(0%)	21.63 ± 2.90	(+3%)
Carbendazim	2.00 ± 0.61	(-62%) *	19.25 ± 6.00	(+29%)	20.13 ± 2.22	(-4%)
	Total of anecic earthworms					
Control	4.94 ± 1.03		10.44 ± 3.07		7.38 ± 2.39	
Flufenacet	4.56 ± 1.61	(-8%)	10.31 ± 0.90	(-1%)	8.63 ± 1.11	(+17%)
Carbendazim	1.44 ± 0.69	(-71%) *	5.00 ± 1.06	(-52%) *	3.63 ± 1.74	(-51%) *
	Total of endogeic earthworms					
Control	0.13 ± 0.16		1.88 ± 1.70		12.38 ± 5.04	
Flufenacet	0 ± 0	(-100%)	2.44 ± 1.18	(+30%)	10.56 ± 2.13	(-15%)
Carbendazim	0.31 ± 0.38	(+150%)	7.38 ± 2.66	(+293%)*	11.94 ± 1.82	(-4%)
	Total of epigeic earthworms					
Control	0.19 ± 0.24		2.56 ± 1.74		1.19 ± 0.69	
Flufenacet	0 ± 0	(-100%)	2.06 ± 0.97	(-20%)	2.44 ± 0.92	(+105%)
Carbendazim	0.25 ± 0.20	(+33%)	6.88 ± 6.47	(+168%)	4.56 ± 1.60	(+284%) *
	Biomass (g) / replicate					
	Total earthworms					
Control	11.20 ± 2.28		25.63 ± 9.26		24.50 ± 4.72	
Flufenacet	10.10 ± 3.96	(-10%)	25.40 ± 3.63	(-1%)	26.19 ± 3.71	(+7%)
Carbendazim	3.11 ± 1.08	(-72%) *	18.48 ± 3.99	(-28%)	18.02 ± 2.86	(-26%)
	Total of anecic earthworms					
Control	11.05 ± 2.06		24.29 ± 9.07		16.51 ± 4.05	
Flufenacet	10.10 ± 3.96	(-9%)	23.44 ± 3.28	(-3%)	19.42 ± 2.94	(+18%)
Carbendazim	2.83 ± 1.20	(-74%) *	10.77 ± 3.30	(-56%) *	7.93 ± 4.17	(-52%)*
	Total of endogeic earthworms					
Control	0.08 ± 0.16		0.82 ± 0.62		7.78 ± 3.38	
Flufenacet	0 ± 0	(-100%)	1.43 ± 0.61	(+74%)	6.35 ± 1.33	(-18%)
Carbendazim	0.19 ± 0.24	(+136%)	5.83 ± 2.07	(+610%) *	9.05 ± 1.68	(+16%)
	Total of epigeic earthworms					
Control	0.07 ± 0.12		0.53 ± 0.32		0.21 ± 0.18	
Flufenacet	0 ± 0	(-100%)	0.54 ± 0.37	(+2%)	0.42 ± 0.04	(+103%)
Carbendazim	0.09 ± 0.11	(+19%)	1.89 ± 1.49	(+259%) *	1.04 ± 0.16	(+401%) *

\*Statistically significant difference compared to control (Wilcoxon, Mann and Withney U-Test, p= 0.05)

Five and 11 months after application of Flufenacet SC 500 at rate 1.2 L/ha there was not statistically significant difference on the parameters “numbers” and “biomass” of the categories “total”, “total anecic”, “total endogeic” and “total epigeic” adult earthworms, compared to control plots.

Nine weeks after application also not statistically significant differences between Flufenacet 500 SC and control plots were found for anecic earthworm.

The number of earthworms identified in the categories epigeic and endogeic were too low to perform an appropriate statistical analysis of the data.

An application of 8000 g carbendazin/ha had statistically significant effects >50% on the parameter “number” of total adult earthworm.

#### Adult earthworms change in numbers and biomass - species level

In the tested area *Lumbricus terrestris* was identified as a dominant anecic species and *Aporrectodea caliginosa* as a dominant endogeic species. *Lumbricus rubellus* and *Lumbricus castaneus* represented epigeic species in the tested plots ( see Table B.9.4.1.9-8).

Nine weeks after application, three species were observed in the control plots: the most dominant species-were *Lumbricus terrestris* (21% of total earthworms, 94% of adult earthworms), followed by *Lumbricus rubellus* (0.80% of total, 4% of adult earthworms), and *Aporrectodea caliginosa* (0.55% of total earthworms and 2.5 % of adult earthworms).

Nine weeks after application of Flufenacet 500 SC, mainly adults of *Lumbricus terrestris* species were observed in the treated plots. Not statistically significant reduction in number (-8%) and in biomass (-9%) compared to the control was recorded for earthworm species tested.

The low abundances of endogeic adult earthworm species represent by *Aporrectodea caliginosa*, as well as a low number of epigeic adult earthworm represented by *Lumbricus rubellus* in the control plots did not permit to perform a statistic analysis.

Five months after application of Flufenacet 500 SC a relative reduction (not statistically significant) in number compared to control for both of epigeic species *Lumbricus castaneus* and *L.rubellus* (-27% and -7% , respectively) were recorded.

For anecic species *L. terrestris* no change in numbers and biomass compared to the control was noted.

In the case of endogeic species - *Aporrectodea caliginosa* an increase in numbers (+81%) and biomass (+103%) in comparison to the control was observed.

Low abundances of other species such as *A.chlorotica*, *A. terresis longa* in the control plots did not permit to perform a statistic analysis.

11 months after application of Flufenacet 500 SC, the abundance in the control plots was dominated by the two species: *Aporrectodea caliginosa* followed by *Lumbricus terrestris*

*A.longa* (anecic species), *A. chlorotica* (endogeic species) were identified in still a relatively low numbers in the control and treatment plots, which did not permit to performed a statistical analysis.

After application of Flufenacet 500 SC for anecic species (*L.terrestris*) and epigeic species (*L.rubellus.*, *L.cast.*) an increase in numbers ( +22, +67 and +123%, respectively) and biomass (was +22, +83, +133, respectively) observed as compared to control.

At the same time, the relative reduction in numbers (-15%) and biomass (-11%), not statistically significant, was observed for *Aporrectodea caliginosa*.

**Table B.9.4.1.9-8: Abundance (mean number, n=4/ 0.25 m<sup>2</sup>) and biomass (g) of adult species of earthworms after the application of Flufenacet SC 500 and relative change in number and biomass in comparison to control (%).**

	A.terrestri s longa	Lumbricus terrestris	Lumbricus rubellus	Lumbricus castaneaus	Aporrectodea caliginosa	A. chlorotica	Sum Anecic	Sum Epigeic	Sum Endogeic	Sum of total adults	Sum of total adults+ juvenile
	Anecic		Epigeic		Endogeic						
Pre-sampling 5 weeks before application											
Control plots	0.0	7.94±4.82	1.88±1.63	15.94±12.6	0.31±0.87	0.0	7.94±4.82	17.81±123	0.31±0.7	26.06±14.9	48.94±202
9 weeks after application <sup>ns</sup>											
Control	0.00	4.94±1.03 (11.05±2.06)	0.19±0.24 (0.07±0.12)	0.00	0.13±0.25 (0.08±0.16)	0.00	4.94±1.03	0.19±0.24	0.13±0.5	5.25±1.46	23.31±3.4 6
Flufenact	0.00	4.56±161 (10.05±3.96)	0.00 -	0.00 -	0.00 -	0.00	4.56±1.61	0.00	0.00	4.56±1.61	20.75±3.9
Relative change in number (%)	-	8	-100	-	-100	-					
Relative change in biomass (%)	-	-9	-100	-	-100	-					
5 months after application <sup>ns</sup>											
Control	0.50±0.20 (0.79±0.32)	9.94±3.18 (23.50±9.36)	0.94±0.69 (0.33±0.25)	1.63±1.44 (0.20±0.18)	1.0±0.54 (0.63±0.37)	0.88±1.27 (0.19±0.30)	10.44±3.07	2.56±1.74	1.88±1.0	14.88±3.11	40.88±2. 99
Flufenaet	0.38±0.32 (0.51±0.42)	9.94±0.63 (22.92±3.18)	0.88±0.78 (0.39±0.38)	1.19±0.77 ( 0.15± 0.09)	1.81±0.94 (1.28 ±0.53)	0.6±0.43 (0.15±0.11)	10.31±0.90	2.06±0.97	2.44±1.8	14.81±1.36	39.81±8.6 1
Relative change in number (%)	-25 <sup>a</sup>	0	-7	-27	+81	-29 <sup>a</sup>					
Relative change in biomass (%)	-35a	-2	+17	-22	+103	-23 <sup>a</sup>					

**Table B.9.4.1.9-8 continued. Abundance (mean number, n=4/ 0.25 m<sup>2</sup>) and biomass (g) of adult species of earthworms after the application of Flufenacet SC 500 and relative change in number and biomass in comparison to control (%).**

	A.terrestris longa	Lumbricus terrestris	Lumbricus rubellus	Lumbricus castaneus	Aporrectodea caliginosa	Aporrectodea chlorotica	Sum Anecic	Sum Epigeic	Sum Endogeic	Sum of total adults	Sum of total adults+ juvenile
	Anecic		Epigeic		Endogeic						
11 month after application <sup>ns</sup>											
Control	0.56±0.31  (1.06±0.81)	6.81±2.4  (15.44±3.81)	0.38±0.60  (0.13±0.20)	0.81±0.83  (0.08±0.11)	12.25±5.22  (7.76±3.41)	0.13±0.25  (0.02±0.04)	7.38±29	1.19±0.69	12.38±5.04	20.94±6.62	76.50±14.6
Flufenacet	0.31±0.63  (0.58±1.1)	8.31±1.9  (18.84±3.04)	1.06±0.81  (0.23±0.16)	1.8±1.14  (0.10±0.15)	10.44±2.22  (6.32±1.34)	0.13±0.14  (0.03±0.03)	8.±1.11	2.44±0.92	10.56±2.13	21.63±2.90	76.19±5.54
Relative change in number (%)	-44 <sup>a</sup>	+22	+67	+123	-15	0 <sup>a</sup>					
Relative change in biomass (%)	-46 <sup>a</sup>	+22	+83	+133	-18	+33 <sup>a</sup>					

Value in parentheses – biomass (g), without parentheses – mean number of individ./0.25m<sup>2</sup>, ( n=4)

<sup>a</sup> Because of the very low abundance of this species, these data have not been statistically evaluated.

+ increase compared to control

- decrease compared to control

ns- no statistically significant difference in number and biomass compared to control (Wilcoxon, Mann and Withney U-Test, p= 0.05)

Juvenile worms; changes in numbers and biomass:**Table B.9.4.1.9-9:** Data for category “juvenile” and for the species classes “total”, “total anecic”, “total endogeic” and “total epigeic” earthworms. The values are replicate means (n = 4) and standard deviations per 0.25 m<sup>2</sup>. Values between parentheses are relative differences to the control in %:

Treatment group	9 weeks after the application		5 months after the application		11 months after the application	
	Numbers (n) / replicate					
	Total earthworms					
Control	23.06 ± 2.92		26.00 ± 2.39		55.56 ± 9.64	
Flufenacet	16.19 ± 3.36	(30%)*	25.00 ± 7.43	(-4%)	54.56 ± 7.63	(-2%)
Carbendazim	11.88 ±2.66	(49%) *	21.63 ± 3.00	(17%)	33.88 ± 4.09	(-39%)*
	Total of anecic earthworms					
Control	5.13 ± 0.60		14.81 ± 1.31		9.69 ± 1.14	
Flufenacet	5.06 ± 0.63	(-1%)	14.25 ± 3.52	(-4%)	11.13 ± 1.05	(+15%)
Carbendazim	1.94 ± 0.47	(-62%) *	15.06 ± 1.60	(+2%)	12.00 ± 3.33	(+24%)
	Total of endogeic earthworms					
Control	8.69 ± 4.05		3.56 ± 1.39		40.75 ± 9.43	
Flufenacet	5.13 ± 2.72	(-41%)	4.38 ± 3.62	(+23%)	38.31 ± 7.80	(-6%)
Carbendazim	2.50 ± 1.34	(-71%) *	2.31 ± 1.71	(-35%)	14.75 ± 3.38	(-64%)*
	Total of epigeic earthworms					
Control	9.25 ±1.34		7.63 ± 1.76		5.13 ± 2.05	
Flufenacet	6.00 ±1.15	(-35%) *	6.38 ± 0.72	(-16%)	5.13 ± 2.11	(0%)
Carbendazim	7.44 ± 3.11	(-20%)	4.25 ± 1.66	(-44%)*	7.13 ± 2.24	(+39%)
	Biomass (g) / replicate					
	Total earthworms					
Control	7.01 ±1.07		10.83 ± 1.15		20.30 ± 2.16	
Flufenacet	5.73 ±1.40	(-18%)	11.05 ± 1.99	(+2%)	21.44 ± 2.07	(+6%)
Carbendazim	2.63 ±0.31	(-62%) *	9.86 ± 2.41	(-9%)	14.82 ± 1.54	(-27%)*
	Total of anecic earthworms					
Control	5.38 ±1.20		9.05 ± 1.31		7.50 ± 1.16	
Flufenacet	5.03 ±1.20	(-6%)	9.10 ± 1.69	(+1%)	8.58 ± 1.40	(+14%)
Carbendazim	1.56 ±0.18	(-71%) *	8.19 ± 1.68	(-9%)	8.18 ± 1.16	(+9%)
	Total of endogeic earthworms					
Control	1.04 ±0.61		1.27 ± 0.56		12.45 ± 1.29	
Flufenacet	0.40 ±0.23	(-61%)	1.43 ± 1.00	(+13%)	12.51 ± 3.24	(0%)
Carbendazim	0.50 ±0.30	(-55%)	1.35 ± 0.97	(+6%)	6.24 ± 1.05	(-50%)*
	Total of epigeic earthworms					
Control	0.60 ±0.16		0.51 ± 0.09		0.34 ± 0.18	
Flufenacet	0.29 ±0.07	(-51%) *	0.52 ± 0.20	(+1%)	0.36 ± 0.09	(+4%)
Carbendazim	0.57 ±0.23	(-5%)	0.38 ± 0.13	(-26%)	0.50 ± 0.15	(+46%)*

\* Statistically significant difference compared to control (Wilcoxon, Mann and Withney U-Test, p = 0.05)

An application of 1.2 L product/ha, Flufenacet SC 500 has no statistically significant effect on the parameters “numbers” and “biomass” of the categories “total”, “total anecic”, “total endogeic” and “total epigeic” juvenile earthworms five and 11 months after the application.

Nine weeks after application also no statistically significant differences in number and biomass between Flufenacet and control plots for the categories “total anecic” and “total endogeic” were found. For the category “total” the number of earthworm was reduced by 30 % and for the group of “total epigeic” earthworms the biomass was reduced by 51 %. Possible explanation according study director for this observation is the influence of Flufenacet 500 SC acting as herbicide on the vegetation of the treated plots. Although all plots were treated with Glyphos before start of the test, untreated plots showed a regrowing of weeds. Especially in the dry summer period this strong influence on the water regime of the soil thereby affecting the habitat of the endo- and epigeic earthworms could be observed. Therefore, this variation could be not considered as compound related effect but rather a secondary effect of the herbicide Flufenacet 500 SC on the earthworm community.

In addition an application of 8000 g carbendazin/ha has statistically significant effects closed to 50% (being 49%) on the parameter “number” for category total earthworm.

Summary of relative changes of number and biomass for two most abundant species are presented in the tables below:

**Table B.9.4.1.9-10: Changes in numbers and biomass of *L. terrestris*. Data is presented for the species *L. terrestris*. The values are replicate means (n = 4) and standard deviations per 0.25 m<sup>2</sup>. Values between parentheses are relative differences to the control in %:**

Treatment group	9 weeks after the application	5 months after the application	11 months after the application
<b>Number</b>			
<b>Relative changes of number of <i>L. terrestris</i> in the study plots (from replicate means)</b>			
<b>Adult &amp; juvenile <i>L. terrestris</i></b>			
Control	10.06 ± 1.55	23.44 ± 2.66	15.75 ± 3.39
Flufenacet	9.63 ± 1.16 (-4%)	23.63 ± 3.56 (-3%)	18.69 ± 1.76 (+19%)
Carbendazim	3.38 1.05 (-66%) *	18.63 ± 2.05 (-24%) *	14.00 ± 3.40 (-11%)
<b>Adult <i>L. terrestris</i></b>			
Control	4.94 ± 1.03	9.94 ± 3.18	6.81 ± 2.54
Flufenacet	4.56 ± 1.61 (-8%)	9.94 ± 0.63 (0%)	8.31 ± 1.39 (+22%)
Carbendazim	1.44 ± 0.63 (-71%) *	4.38 ± 1.05 (-56%) *	2.94 ± 1.25 (-57%) *
<b>Juvenile <i>L. terrestris</i></b>			
Control	5.13 ± 0.60	14.50 ± 1.55	8.94 ± 1.51
Flufenacet	5.06 ± 0.63 (-1%)	13.69 ± 3.51 (-6%)	10.38 ± 0.88 (+16%)
Carbendazim	1.94 ± 0.47 (-62%) *	14.25 ± 1.08 (-2%)	11.06 ± 3.20 (+24%)
<b>Biomass</b>			
<b>Relative changes of number of <i>L. terrestris</i> in the study plots</b>			
<b>Adult &amp; juvenile <i>L. terrestris</i></b>			
Control	16.42 ± 3.08	32.20 ± 10.10	21.80 ± 3.46
Flufenacet	15.13 ± 4.75 (-8%)	31.20 ± 2.60 (-3%)	26.37 ± 3.96 (+21%)
Carbendazim	4.39 1.34 (-73%) *	16.60 ± 3.96 (-48%) *	13.21 ± 2.92 (-39%) *
<b>Adult <i>L. terrestris</i></b>			
Control	11.05 ± 2.06	23.50 ± 9.36	15.44 ± 3.81
Flufenacet	10.10 ± 3.96 (-9%)	22.92 ± 3.18 (-2%)	18.84 ± 3.04 (+22%)
Carbendazim	2.83 ± 1.20 (-74%) *	9.58 ± 3.08 (-59%) *	6.59 ± 3.15 (+57%) *
<b>Juvenile <i>L. terrestris</i></b>			
Control	5.38 ± 1.20	8.70 ± 1.35	6.35 ± 1.23
Flufenacet	5.03 ± 1.20 (-6%)	8.28 ± 1.38 (-5%)	7.53 ± 1.04 (+19%)
Carbendazim	1.56 ± 0.18 (-71%) *	7.02 ± 1.09 (-19%)	6.63 ± 1.20 (+4%)

\*Statistically significant difference compared to control (Wilcoxon, Mann and Withney U-Test, p= 0.05)

**Table B.9.4.1.9-11: Changes in numbers and biomass of *A. caliginosa* .Data is presented for the species *A. caliginosa*. The values are replicate means (n = 4) and standard deviations per 0.25 m<sup>2</sup>. Values between parentheses are relative differences to the control in %:**

Treatment group	9 weeks after the application	5 months after the application	11 months after the application
<b>Number</b>			
<b>Relative changes of number of <i>A. caliginosa</i> in the study plots (from replicate means)</b>			
<b>Adult &amp; juvenile <i>A. caliginosa</i></b>			
Control	8.50 ± 4.25	4.31 ± 1.61	52.88 ± 14.14
Flufenacet	5.13 ± 2.72 (-40%)	5.88 ± 3.68 (+36%)	48.56 ± 7.05 (-8%)
Carbendazim	2.81 ± 1.71 (-67%) *	9.38 ± 2.40 (+117%) *	26.63 ± 4.99 (-50%) *
<b>Adult <i>A. Caliginosa</i></b>			
Control	0.13 ± 0.25	1.00 ± 0.54	12.25 ± 5.22
Flufenacet	0 (-100%)	1.81 ± 0.94 (+81%)	10.44 ± 2.22 (-15%)
Carbendazim	0.31 ± 0.38 (+150%)	7.13 ± 2.62 (+613%) *	11.88 ± 1.90 (-3%)
<b>Juvenile <i>A. Caliginosa</i></b>			
Control	8.38 ± 4.31	3.31 ± 1.25	40.63 ± 9.66
Flufenacet	5.13 ± 2.72 (-39%)	4.06 ± 3.18 (+23%)	38.13 ± 7.70 (-6%)
Carbendazim	2.50 ± 1.34 (-70%) *	2.25 ± 1.79 (-32%)	14.75 ± 3.38 (-64%) *
<b>Biomass</b>			
<b>Relative changes of number of <i>A. caliginosa</i> in the study plots (from replicate means)</b>			
<b>Adult &amp; juvenile <i>A. caliginosa</i></b>			
Control	1.07 ± 1.07	1.88 ± 0.83	20.19 ± 4.42
Flufenacet	0.40 ± 0.23 (-62%) *	2.69 ± 1.35 (+43%)	18.81 ± 2.94 (-7%)
Carbendazim	0.70 ± 0.53 (-35%)	7.03 ± 2.02 (+275%)	15.17 ± 1.65 (-25%)
<b>Adult <i>A. Caliginosa</i></b>			
Control	0.08 ± 0.16	0.63 ± 0.37	7.76 ± 3.41
Flufenacet	0 (-100%)	1.28 ± 0.53 (+103%)	6.32 ± 1.34 (-18%)
Carbendazim	0.19 ± 0.24 (+136%)	5.76 ± 2.06 (+811%) *	9.03 ± 1.70 (+16%)
<b>Juvenile <i>A. Caliginosa</i></b>			
Control	0.98 ± 0.65	1.24 ± 0.56	12.43 ± 1.32
Flufenacet	0.40 ± 0.23 (-59%) *	1.41 ± 0.98 (+13%)	12.49 ± 3.22 (0%)
Carbendazim	0.50 ± 0.30 (-49%)	1.28 ± 0.91 (+3%)	6.14 ± 0.97 (-51%) *

\*Statistically significant difference compared to control (Wilcoxon, Mann and Withney U-Test, p= 0.05)

*Lumbricus terrestris:*

Nine weeks after application of 1.2 l Flufenacet 500 SC, there was no reduction in biomass and number for anecic species *L. terrestris* for all categories (total adults, total juvenile and adult+juvenile).

*A. calliginosa*

At the same time, nine weeks after application of 1.2 l Flufenacet 500 SC/ha, for endogeic species *A. calliginosa*, a statistically significant reduction in biomass for category adult+juvenile (-62%) and category juvenile (-59%) was recorded.

However, five and 11 months after application of 1.2 L Flufenacet 500 SC /ha, there was no difference between treated and control plots.

Summary of effects:

Nine weeks after application of Flufenacet SC 500 at rate 1.2 L/ha, a relative reduction of adult & juvenile earthworms -27 % (number, significant) and -13 % (biomass) was observed respectively.

For total juvenile category statistical significant relative reduction was -30 % (number) and not statistical significant relative reduction in biomass was (-18%).

For total adults category not statistical significant relative reduction in number (-13%) and biomass (-10%) was determined. Five months after application of Flufenacet SC 500 at rate 1.2 L/ha a relative reduction in the total number of juvenile and adult earthworms was -3 % and no change in the biomass compared to control plots was observed.

After 11 months after application of Flufenacet 500 SC at rate 1.2 L/ha changes of a relative abundance of adult & juvenile earthworms relative to control was 0% (number) and +6% (biomass).

**Changes in numbers and biomass for juvenile & adult earthworms**

**Table B.9.4.1.9-12: The values are replicate means (n = 4) and standard deviations per 0.25 m<sup>2</sup>. Values between parentheses are relative differences to the control in %:**

Treatment group	9 weeks after the application		5 months after the application		11 months after the application	
	Relative number of juvenile & adult earthworms in the study plots (from replicate means)					
	Total earthworms					
Control	28.31 ± 3.46		40.88 ± 2.99		76.50 ± 14.86	
Flufenacet	20.75 ± 3.69	(-27%) *	39.81 ± 8.61	(-3%)	76.19 ± 5.54	(0%)
Carbendazim	13.88 ± 2.92	(-51%) *	40.88 ± 8.61	(0%)	54.00 ± 5.07	(-29%) *
	Relative changes of biomass of juvenile & adult earthworms in the study plots (from replicate means)					
Control	18.21 ± 3.31		36.46 ± 9.78		44.79 ± 5.64	
Flufenacet	15.83 ± 4.86	(-13%)	36.45 ± 4.32	(0%)	47.64 ± 2.47	(+6%)
Carbendazim	5.74 ± 1.25	(-68%) *	28.34 ± 6.23	(-22%)	32.84 ± 2.67	(-27%) *

\* Significant difference from control according to the U-test, two sided at the significance level alpha = 0.05 (U-test from Wilcoxon, Mann and Whitney after SACHS 1978).

Conclusion:

In the study the statistically significant reduction in numbers in category adult & juvenile earthworms (-27 %) and in category total juvenile (-30 %) was observed only nine weeks after application of Flufenacet 500 SC of 1.2 L/ha.

At the same time for total adults category not statistical significant relative reduction in number (-13%) and biomass (-10%) was determined.

Five months after application of Flufenacet SC 500 at rate 1.2 L/ha a relative reduction in the total number of juvenile and adult earthworms was -3 % and no change in the biomass compared to control plots was observed. After 11 months after application of Flufenacet 500 SC at rate 1.2 L/ha changes of a relative abundance of adult & juvenile earthworms relative to control was 0% (number) and +6% (biomass).

Based on the study results, it can be concluded that there was no long term adverse effects from application of 1.2 L Flufenacet 500 SC/ha for population of earthworm.

Therefore, NOAER from this study is determined to be 1.2 L Flufenacet 500 SC/ha.

**Comments RMS:**

The study was performed according to the guidelines BBA, Part VI, 2 - 3 (January 1994), ISO Guideline CD 11268-3 (E), (1999).

The following validity criteria given in the recommendation Kula et al. (2006) should be met during the study:

**- Earthworm abundance (average) should be > 60 earthworms/m<sup>2</sup> (for arable and 100 earthworm /m<sup>2</sup> for grassland at test initiation for all treatment groups.**

The abundance of earthworms at the study site was determined 5 weeks before application and was 196 earthworms/m<sup>2</sup>.

However, it should be noted that pre sampling period was longer ( 5 weeks) than recommended by Kula (2006).

Nine weeks after the application the mean number of earthworms in the control plots, sampled 113 earthworms/m<sup>2</sup>, five months after the application 164 earthworms/m<sup>2</sup> and eleven months after the application 306 earthworms/m<sup>2</sup>, respectively.

**- Representative, regional specific earthworm species of different ecological life forms (i.e. endogeics and anecics) have to be present in sufficiently high numbers of at least 10 individuals per m<sup>2</sup> or at least 10 % of total adult earthworm abundance.**

- Five weeks before application in the control plots *Lumbricus terrestris* was identified as the most dominant anecic species - 32 ind/m<sup>2</sup>. This species was also observed in the control 9 weeks after application (20 ind/m<sup>2</sup>). The most abundant epigeic species in the pre sampling period *L. castaneus* was recorded in 63 ind/m<sup>2</sup> but this species was not seen 9 weeks after application in the control plots.

The endogeic species –*A. calliginosa* was observed in very low numbers in the pre sampling period as in 9 weeks after application of Flufenacet 500 SC in the control plots. However, taking into consideration the category total adult & juvenile the number of endogeic species achieved 34 ind./m<sup>2</sup> in the control plots.

Generally, two dominant groups of earthworm were recorded during the study:

The other species were observed in low numbers at the test start.

**- Reduction of the earthworm abundance by the toxic reference item should be > 50 % in comparison to the control plots for at least one post-treatment.**

The treatment with the reference substance - Carbendazim showed strong effects nine weeks after the application on the earthworm community (adult & juveniles) in comparison with the control. The reference item applied at a rate of 8 kg carbendazim/ha did decrease the abundance of earthworms by -51% (nine weeks after application). The reference item treatment confirmed the sensitivity of the earth worm population under the specific experimental conditions and the validity of the study.

The study is considered acceptable.

**Agreed endpoint:**

**NOAER=1.2 L Flufenacet 500 SC/ha corresponding to 600 g flufenacet/ha and 0.438 mg a.s./kg soil dw, measured value**

The results of this study was not included in the risk assessment because the most sensitive species to flufenacet - *Octolasion lacteum*, identified as such in another field study for representative formulation was not tested.

**Therefore, the NOAER value of 0.438 mg flufenacet/kg dws was not used in the risk assesment.**

#### **B.9.4.1.10. Additional information**

For the first EU approval of the active substance flufenacet acute earthworm studies were submitted addressing the risk to earthworm. According to the current date requirements (Regulation No 283/2013) acute toxicity studies are no longer required. Nevertheless, the study summaries from the DAR are included in the RAR as additional information.

Heimbach, 1995

Active substance:

Methods

*The acute toxicity of flufenacet (purity: 96.8%) to the earthworm, Eisenia candida was investigated in an artificial soil under laboratory condition. Earthworm were introduced to soil treated with flufenacet at seven different rates: 10, 32, 56, 100, 178, 336 and 1000 mg a.s./kg dws ( nominal) corresponding to 9.7, 31 ,54 ,96.8, 172.7, 325g and 970 mg a.s./kg dws (corrected purity).*

*Four replicates were used for each treatment and for each replicate ten worms were weighted together and placed on the top of the soil in a 1 l glass container. The containers were incubated for two weeks at 20±1 °C and constant light (400-800 Lux). After 7 and 14 days of exposure the worms were examined and counted, and mortalities were recorded. At the end of the test the worms were weighted again.*

*Results:*

*An increased mortality (90 and 100%) compared to the control (0%) was found at the two highest test concentrations. At lower test concentrations the mortality were such as 3 ±5 % at rate 9.7 mg a.s./kg dws and 18±17 mg a.s./kg dws at rate of 172.7 mg a.s./kg dws. Abnormalities, e.g. changes in behavior, were not observed than earthworm seemd to be slightly hardened at the end of the test concentrations of 172.7 and 306.5 mg a.s./kg dws.*

A treatment related decrease in weight was observed at the lowest test concentration of 9.7 mg a.s./kg dws. Calculated 14 days LC<sub>50</sub>-value (with 95% confidence limits) was 218.8 mg a.s. (201 - 238) mg flufenacet/kg soil, respectively and NOEC was determined to be < 9.7 mg a.s./kg dws.

Comments:

The study was conducted in compliance with GLP and the OECD guideline 207.

Metabolites:

Heimbach, 1999

Methods

The acute toxicity of FOE 5043-sulfonic Na-salt (purity: 74%) to the earthworm, *Eisenia candida* was investigated in an artificial soil under laboratory condition. Earthworms were introduced to soil treated with FOE 5043-sulfonic Na-salt at five different rates: 3.2, 19, 32, 100 and 316 mg FOE 5043-sulfonic Na-salt /kg dws. Four replicates were used for each treatment and for each replicate ten worms were weighted together and placed on the top of the soil in a 1.5 L glass container. The containers were incubated for two weeks at 20±2 °C and constant light (400-800 Lux). After 7 and 14 days of exposure the worms were examined and counted, and mortalities were recorded. At the end of the test the worms were weighted again.

Results:

No mortality was detected during the test in any treatment and all earthworms were health.

On day 14, the LC<sub>50</sub> and LOEC values for FOE 5043-sulfonic acid Na-salt were estimated to be higher than 1000 mg test item/kg dry soil. The NOEC was established at 1000 mg FOE 5043-sulfonic acid Na-salt/kg dry

Comments:

The study was conducted in compliance with GLP and the OECD guideline 207.

Heimabach, 1999

Methods

The acute toxicity of FOE 5043-oxalate (purity: 99.8%) to the earthworm, *Eisenia candida* was investigated in an artificial soil under laboratory condition. Earthworms were introduced to soil treated with FOE 5043-oxalate at five different rates: 3.2, 10, 32, 100 and 316 mg FOE 5043-oxalate /kg dws.

Four replicates were used for each treatment and for each replicate ten worms were weighted together and placed on the top of the soil in a 1.5 L glass container. The containers were incubated for two weeks at 20±2 °C and constant light (400-800 Lux). After 7 and 14 days of exposure the worms were examined and counted, and mortalities were recorded. At the end of the test the worms were weighted again.

Results:

No mortality was detected during the test in any treatment and all earthworms were health.

On day 14, the LC<sub>50</sub> and LOEC values for FOE 5043-oxalate were estimated to be higher than 1000 mg test item/kg dry soil. The NOEC was established at 1000 mg FOE 5043-oxalate/kg dry soil, since no statistically significant difference was observed between the tested treatments.

Comments:

The study was conducted in compliance with GLP and the OECD guideline 207.

**B.9.4.2. Effects on non-target soil meso- and macrofauna (other than earthworms)**

According to the data requirements on active substances (Regulation 283/2013), the risk to soil dwelling organisms has to be addressed (1) if a risk to non-target arthropods was identified or (2) if the product is applied to the bare soil (pre-emergence). The examination of the intended uses pattern in cereals (use at BBCH 00-22) the risk to soil meso- and macrofauna from exposure to the active substance and its major soil metabolites has to be addressed. Hence, laboratory studies with the soil organisms *Folsomia candida* and *Hypoaspis aculeifer* were submitted for active substance and following soil metabolites: FOE oxalate, FOE sulfonic acid-Na-salt, FOE-methylsulfone, TFA, FOE5043-trifluoroethanesulfonic acid and FOE-Thiadone. The study summaries for studies with the active substance flufenacet and the soil metabolites are provided in the Table below. The study summaries for the studies with representative formulation DFF+FFA SC 600 are given in the Vol.3.B9 ( CP).

**Table B.9.4.2-1: Reproductive toxicity data of flufenacet and metabolites to the other non target macro-organism.**

Test substance	Test species	Endpoint	Reference
Flufenacet	<i>Folsomia candida</i>	NOEC=31.5* mg a.s./kg dws	Frommholz (2010) M-363896-01-1
	<i>Hypoaspis aculeifer</i>	NOEC=281* mg a.s./kg dws	Kratz (2013) M-455214-01-1
FOE -oxalate	<i>Folsomia candida</i>	NOEC≥100 mg /kg dws	Frommholz (2010) M-393634-01-1
	<i>Hypoaspis aculeifer</i>	NOEC≥100 mg/kg dws	Kratz (2010) M-393634-01-1
FOE -sulfonic acid-Na-salt	<i>Folsomia candida</i>	NOEC≥100 mg /kg dws	Frommholz (2010) M-396039-01-1
	<i>Hypoaspis aculeifer</i>	NOEC≥ 100 mg./kg dws	Kratz (2013) M-455654-01-1
FOE methylsulfone	<i>Folsomia candida</i>	NOEC≥100 mg /kg dws	Frommholz (2010) M-392345-01-1
	<i>Hypoaspis aculeifer</i>	NOEC =500 mg /kg dws	Kratz (2009) M-357707-01
TFA	<i>Folsomia candida</i>	NOEC ≥100 mg /kg dws	Frommholz (2012) M-436127-01-1
	<i>Hypoaspis aculeifer</i>	NOEC ≥100 mg/kg dws	Kratz (2012) M-436128-01-1
FOE 5043-trifluoroethane sulfonic acid	<i>Folsomia candida</i>	NOEC≥100 mg/kg dws	Frommholz (2012) M-436128-01-1
	<i>Hypoaspis aculeifer</i>	NOEC ≥100 mg /kg dws	Kratz (2012) M-436315-01-1
FOE-Thiadone	<i>Folsomia candida</i>	NOEC=1.8 mg/kg dws	Frommholz (2012) M-440372-01-1
	<i>Hypoaspis aculeifer</i>	NOEC=32 mg/kg dws EC <sub>10</sub> =28 mg/kg dws	Kratz (2012) M-442897-01-1

\* Endpoints corrected to allow for log P<sub>ow</sub> > 2

pm Pure metabolite

dws Dry weight soil

**B.9.4.2.1. Flufenacet a.s.: Influence on the reproduction of the collembola species Folsomia candida tested in artificial soil with 5% peat.**

<b>Reference:</b>	Flufenacet a.s.: Influence on the reproduction of the collembola species Folsomia candida tested in artificial soil with 5% peat.
<b>Author(s), year:</b>	Frommholz U., 2010
<b>Report/Doc. number:</b>	Report No: E 314 3782-8, Reference BCS No. M-363896-01-1
<b>Guideline(s): I</b>	ISO 11267 (1999)
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet (FOE 5043), Batch No.: K664078, purity 97.5 % w/w (analysed)
Test species:	Collembola, Folsomia candida
Number of organism:	5 replicates per control and treatment group, each with 10 individuals. 1 additional replicate for control and one for treatment group to check the pH and water content of the test substrate after 28 days.
Life stage, age:	Juveniles/adults 10-12 days old
Type of test, duration:	Laboratory sub-lethal limit test, 28 days
Applied concentrations:	Nominal: 0 (control, quartz sand), 32, 63, 125, 250, 500 mg a.s./kg soil dw The test item was mixed into the soil.
Solvent:	None
Toxic standard:	Betosip (a.s. Phenmedipham 153 g/L) tested at concentrations of 50 to 200 mg Betosip/kg soil dw (corresponding to 7.6, 15.2 and 30.4 mg a.s./kg soil dw respectively).

Test condition:

Test substrate:	Artificial soil: 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 74.8 % fine quartz sand, 0.2% calcium carbonate ( for the adjustment to pH to 6 ±0.5).
Substrate/test vessel:	Test containers were glass vessels (volume 140 ml, diameter 5 cm at the bottom, height 7 cm). Each test vessel contained 30 g wet weight artificial soil. The test vessels were covered with glass lids to prevent the collembolans from escaping but allowing aeration during the test period.
Temperature	20±2 °C
Light regime	16 hours light/8 hours dark. Light intensity: 574-591 Lux
Water content:	Test start: 18.51-19.86% (corresponding to 46.12-50.32 % of WHC) Test end: 16.85-17.84% (corresponding to 41.16-44.08 % of WHC)
pH	Test start: 5.88 –6.0 Test end: 5.79-5.81

Feeding:	Directly after the addition of the collembolans, they were fed with granulated dry yeast (one spatula tip). Feeding was also done 14 days after test start, if necessary. Approximately 2 mg per test vessel was added per feeding date.
Test parameters:	pH and water content were determined at test start and test end. Water content maintenance was checked weekly after application. Mortality of adults, behavioral effects and number of juvenile Collembola were assessed after 28 days.
Statistic:	Data of reproduction were tested for normal distribution and homogeneity of variance using Kolmogorov-Smirnov-Test and Cochran's -Test ( $\alpha = 0.05$ ) respectively. Data of reproduction were normally distributed but homogeneity of variances was not given. Therefore, Holms Bonferroni U-test (one-sided-smaller, $\alpha = 0.05$ ) was used to determine NOEC and LOEC values. The software used to perform the statistical analysis was ToxRat Pro 2.09
Findings:	The highest mortality rate of 12 % was found in the test with 32 and 500 mg test item/kg soil dw. In the control 8% of mortality was recorded. Concerning the number of juveniles statistical analysis revealed significant differences between the control and the treatment groups with 125, 250 and 500 mg test item/kg artificial soil dw. No abnormal behavior was observed with the surviving Collembola.

**Table B. 9.4.2.1-1: Effects on mortality and reproduction of Folsomia candida in a sub-chronic test.**

Exposure	Flufenacet (mg test item/ kg soil dw)					
	Control	32	63	125	250	500
Mortality of adult Collembola [%] after 28 days	8	12	2	8	8	12
Mean number of offspring per test vessel after 28 days ( $\pm$ SD)	1050 ( $\pm$ 71)	973( $\pm$ 156)	1181( $\pm$ 153)	665( $\pm$ 262)	301 ( $\pm$ 57)	156( $\pm$ 17)
% reproduction compared to control		93	113	63 *	29 *	15 *

$\pm$ SD Standard deviation

\* Statistically significantly different compared to control (Dunnett's Test, one-sided-smaller,  $\alpha = 0.05$ )

#### Toxic standard:

The toxic standard Betosip (Phenmedipham, 153 g/L) in the most recent test caused mortality of adult Collembola at levels 4 %, 6 % and 60 % at test concentrations 50, 100 and 200 mg Betosip/kg soil dw, respectively.

In all treatment groups the number of juveniles were statistically significant reduced in comparison to the control.  $NOEC_{\text{reproduction}}$  was calculated to be < 50 mg Betosip (7.6 mg a.s.)/kg soil dw and the  $LOEC_{\text{reproduction}}$ : 50 mg Betosip (7.6 mg a.s.)/kg soil dw.

Conclusion:

$NOEC_{\text{reproduction}} = 63 \text{ mg flufenacet /kg soil dw}$

$LOEC_{\text{reproduction}} = 125 \text{ mg flufenacet kg soil dw}$

In accordance with the new data requirement (Commission Regulation EU No 283/2013), the  $EC_{10}$ ,  $EC_{20}$ ,  $EC_{50}$  values should be calculated. Please provided relevant calculations of  $EC_{10}$ ,  $EC_{20}$  and  $EC_{50}$  values.

**Comments RMS:**

The Collembola reproduction study was conducted according to the test guideline ISO 11267 (1999) .

Taking into account the validity criteria given in the test guideline OECD 232 (2009), the study was considered acceptable. The mean mortality of the adults in the control was below 20 % (being 8%). The mean number of juveniles per control replicate was greater than 100 (being 1050 per replicate). The coefficient of variation of reproduction in the control was <30 (being 6.7%).

**Agreed endpoints:**

$NOEC_{\text{reproduction}} = 63 \text{ mg flufenacet/kg soil dw}$

$LOEC_{\text{reproduction}} = 125 \text{ mg flufenacet/kg soil dw}$

**B.9.4.2.2. Flufenacet a.s.: Influence on mortality and reproduction of the soil mite species****Hypoaspis aculeifer tested in artificial soil.**

<b>Reference:</b>	Flufenacet a.s.: Influence on mortality and reproduction of the soil mite species <i>Hypoaspis aculeifer</i> tested in artificial soil.
<b>Author(s), year:</b>	Kratz., M.A., 2013
<b>Report/Doc. number:</b>	Study No: E 428 4460-8, Reference BCS No: M-455214-01-1
<b>Guideline(s):</b>	OECD 226 (2008)
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet Batch ID: AE F133402- 01-18, Purity 98.18% w/w (analysed)
Test species:	Predatory mites, <i>Hypoaspis aculeifer</i>
Number of organism:	8 replicates per control and 4 replicates per each treatment group, each with 10 individuals
Life stage, age:	Adult females of <i>Hypoaspis aculeifer</i>
Type of test, duration	Laboratory sub-lethal limit test, 14 days

Applied concentrations:

	0 (control, quartz sand ), 100, 178, 316, 562 and 1000 mg test item/kg sdw The test item was applied by mixing a test item-quartz sand-mixture into the artificial soil.
Toxic standard:	Dimethoate 440 EC tested at concentration of 1.0, 1.8, 3.2, 5.6 and 10.0 mg dimethoate/kg soil dw
Test substrate:	Artificial soil, 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 75 % fine quartz sand, 0.2% calcium carbonate (for the adjustment to pH to 6 ±0.5)
Substrate/Test vessel	Test containers were glass vessels (volume 140 ml, diameter 5 cm at the bottom, height 7 cm). Each test vessel contained 20 g wet weight artificial soil. The test vessels were covered with glass lids to prevent the <i>Hypoapsis</i> from escaping but allowing aeration during the test period.
Temperature	20 ± 2 °C
Light regime:	16 hours light/8 hours dark. Light intensity: 620-18 Lux.
Water content:	Test start: 18.64-20.85 % (equivalent to 43.74-50.31 % of WHC) Test end: 19.06- 20.72% (equivalent to 44.96-49.91 % of WHC)
pH:	Test start 5.46-5.54 Test end: 5.52-5.77
Feeding:	Feeding was done before test and directly at start, 3, 7, and 10 days after test start. The predatory mites were fed cheese mites ( <i>Tyrophagus putrescentiae</i> ). Between 43 and 99 mg food per test vessel was added per feeding date.

Test parameters:	<p>pH were determined at test start and test end.</p> <p>Water content maintenance was checked weekly after application.</p> <p>Mortality of adults, effects and number of juveniles were assessed after 14 days.</p>
Statistic:	<p>The LC<sub>10</sub>, 20, 50 values were determined by Probit analysis.</p> <p>Determination of mean number of juveniles for control and treatment and the percent values for treatment in comparison to the untreated control were calculated. Mean number of juveniles for control and treatment and the percent values for treatment in comparison to the untreated control were calculated. For the determination of normal distribution and homogeneity of variance Kolmogoroff-Smirnov Test and Cochran-Test (<math>\alpha = 0.05</math>), respectively were used.</p> <p>Data of reproduction were normally distributed and homogeneity of variances was given. Therefore William's-t test (one-sided-smaller, <math>\alpha = 0.05</math>) was used to determine NOEC and LOEC values.</p>
Findings:	<p>The mean mortality rate of <i>Hypoaspis aculeifer</i> of 3.8 % was found in the control. Concerning the number of juveniles statistical analysis revealed a significant difference between control and the highest treatment group of 1000 mg test item/kg soil dw.</p>

#### B.9.4.2.2-1: Effects on mortality and reproduction of *Hypoaspis aculeifer* in a sub-chronic test.

D.3.4.2.2-1: Effects on mortality and reproduction of Hypoaspis aculeifer in a sub-chronic test.

	Flufenacet (mg a.s./kg soil dw)					
Exposure	Control	100	178	316	562	1000
Mortality of adult [%] after 14 days	3.8	15	0.0	10	12.5	20
Mean number of offspring per test vessel after 14 days (±SD)	272.3 (±56.1)	311.5 (±65.4)	322.8 (±5.7)	294.5 (±52.0)	265.5 (±30.6)	198.3 (±31.5)
% reproduction compared to control		114.4	118.5	108.2	97.5	72.8*
LC <sub>10,20,50</sub> /EC <sub>10,20,50</sub> <sup>1</sup>		Adult mortality				Reproduction
LC <sub>10</sub> /EC <sub>10</sub>		-				751.21
LC <sub>20</sub> /EC <sub>20</sub>		-				905.60
LC <sub>50</sub> /EC <sub>50</sub>		-				1294.90
NOEC <sub>reproduction</sub> = 562 mg test item/kg soil dw						
LOEC <sub>reproduction</sub> =1000 mg test item/kg soil dw						

\* Statistically significant difference compared to control (William's-t.-test one sided smaller;  $\alpha=0.0$ )

<sup>1</sup> Probit analysis (95 % confidence limits could not be determined due to mathematical reasons)

**Toxic standard:**

The most recent test with the reference item Dimethoate 440 EC was performed at test concentrations 1.0, 1.8, 3.2, 5.6 and 10.0 mg dimethoate/kg soil dw. Dimethoate showed a  $LC_{50}$  of 4.32 mg a.s./kg (95 % CI: 4.31-4.32 mg a.s./kg) for mortality of the adult mites.

The reproduction of the soil mites was not significantly reduced in comparison to the control up to 3.2 mg a.s./kg soil dw. Therefore, the NOEC was calculated to be 3.2 mg a.s./kg, the LOEC was 5.6 mg a.s./kg.

Dimethoate EC 400E G showed a  $EC_{50}$  of 5.67 mg a.s./kg (95 % CI: 5.58 mg a.s./kg to 5.79 mg a.s./kg) for reproduction.

**Conclusion:**

$LC_{50} > 1000$  mg test item/kg soil dw

$NOEC_{\text{reproduction}} = 562$  mg test item/kg soil dw

$LOEC_{\text{reproduction}} = 1000$  mg test item/kg soil dw

$EC_{10\text{reproduction}} = 751.21$  test item/kg soil dw

$EC_{20\text{reproduction}} = 905.60$  test item/kg soil dw

$EC_{50\text{reproduction}} = 1294.90$  mg test item/kg soil dw

**Comments RMS:**

Predatory mite production study was conducted according to the OECD test guideline 226 (2008).

Taking into account the validity criteria given in the test guideline OECD 226 (208), the study is considered acceptable.

The mean mortality of the adults in the control were below 20 % (being 3.8% per replicate).

The mean number of juveniles per control was greater than 50 (being 272.3 per replicate).

The coefficient of variation of reproduction in the control were < 30 (being 20.6%).

Therefore, the study is considered acceptable.

**Agreed endpoints:**

$NOEC_{\text{reproduction}} = 562$  mg test item /kg soil dw

$LOEC_{\text{reproduction}} = 1000$  mg test item/kg soil dw

$EC_{10\text{reproduction}} = 751.21$  mg test item/kg soil dw

$EC_{20\text{reproduction}} = 905.60$  mg test item/kg soil dw

$LC_{50} > 1000$  mg test item/kg soil dw

**B.9.4.2.3. Flufenacet–oxalate: Influence on the reproduction of the collembolan species****Folsomia candida tested in artificial soil.**


---

<b>Reference:</b>	Flufenacet–oxalate: Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil.
<b>Author(s), year:</b>	Frommholz U., 2010
<b>Report/Doc. number:</b>	Report No: E 314 3927-9, Report FRM-COLL-95/10, Reference BCS No. M-394712-01-1
<b>Guideline(s):</b>	OECD 232 adopted, September 07, 2009
<b>GLP:</b>	Yes

---

Material and methods:

Test substance	Flufenacet-oxalate, Batch code: BCS-AB 16305-01-01, Purity: 95.3 % w/w (analysed)
Test species:	Collembola, Folsomia candida
Number of organism:	8 replicates per control and treatment group, each with 10 individuals. One additional replicate per treatment and one per control to check the pH and water content of the test substrate after 28 days.
Life stage, age:	Juveniles/Adults, 11-12 days old
Type of test, duration:	Laboratory sub-lethal limit test, 28 days
Applied concentrations:	Nominal: 0 (control, quartz sand), 100 mg test item/kg soil dw. The test item was applied by mixing a test item-quartz sand-mixture into the artificial soil.
Solvent:	None
Toxic standard:	Boric acid tested at concentrations of 44, 67, 100, 150 and 225 mg/kg soil dw
Test substrate:	Artificial soil, 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 74.8 % fine quartz sand, 0.2% calcium carbonate ( for the adjustment to pH to 6 ±0.5.
Substrate/Test vessel:	Test containers were glass vessels (volume 140 ml, diameter 5 cm at the bottom, height 7 cm). Each test vessel contained 30 g wet weight artificial soil. The test vessels were covered with glass lids to prevent the collembolans from escaping but allowing aeration during the test period.
Temperature:	20±2 °C
Light regime:	16 hours light/8 hours dark. Light intensity: 584-605 Lux
Water content:	Test start: 19.47-20.04% (equivalent to 50.49-51.47 % of WHC) Test end: 19.30-19.44 % (equivalent of 49.11-49.56 % of WHC)
pH:	Test start: 6.57-6.58 Test end: 6.45-6.48

Feeding:	Directly after the addition of the collembolans, they were fed with granulated dry yeast. Feeding was also done 14 days after test start. Approximately 2 mg (one spatula tip) per test vessel was added per feeding date.
Test parameters:	pH and water content were determined at test start and test end. Water content maintenance was checked weekly after application. Mortality of adults, behavioral effects and number of juvenile Collembola were assessed after 28 days.
Statistic:	Data of reproduction were tested for normal distribution and homogeneity of variance using Kolmogorov - Smirnov-Test and Cochran's -Test ( $\alpha = 0.05$ ) respectively. Data of reproduction were normally distributed and homogeneity of variances was given. Therefore Student-t test (one-sided-smaller, $\alpha = 0.05$ ) was used to determine NOEC and LOEC values. The software used to perform the statistical analysis was ToxRat Pro 2.09.
Findings:	No mortality was observed after 28 days of exposure at the control group and at the tested concentration of 100 mg test item/ kg soil dw. An $LC_{50}$ could not be calculated and is considered to be >100 mg test item/kg soil dw. No statistically significant different values for the number of juveniles per test vessel relative to the control were observed at the tested concentration of 100 mg FOE 5043-oxalate/kg soil dw. No abnormal behavior was observed with the surviving Collembola.

**Table B. 9.4.2.3-1: Effects on mortality and reproduction of *Folsomia candida* in a sub-chronic test.**

	Control	FOE 5043-oxalate (mg test item/kg soil dw)
Exposure	-	100
Mortality of adult Collembola [%] after 28 days(±SD)	3.8 (±0.5)	12.5 (±0.9)
Mean number of offspring per test vessel after 28 days (±SD)	1450 (±75.9)	1486.9 (±115.7)
% reproduction compared to control		102.5 <sup>ns</sup>

ns Not statistically significant compared to control (Student-t test, one sided smaller,  $\alpha = 0.05$ )

**Toxic standard:**

In the most recent study, the  $EC_{50}$  of 96 mg/ kg soil dw for the reference item boric acid was determined.

The  $NOEC_{\text{reproduction}}$  was calculated to be 44 mg Boric acid/kg soil dw and the  $LOEC_{\text{reproduction}}$  was 67 mg Boric acid/kg soil dw.

Conclusion:

NOEC<sub>reproduction</sub> ≥ 100 mg test item/kg soil dw

LOEC<sub>reproduction</sub> > 100 mg test item/kg soil dw

**Comments RMS:**

The Collembola reproduction study was conducted according to the OECD test guideline 232 (2009).

Taking into account the validity criteria given in the test guideline OECD 232 (2009) the study is considered acceptable.

The mean mortality of the adults in the control was below 20 % (being 3.8%). The mean number of juveniles per control replicate was greater than 100 (being 1450, mean with ±76 (SD), per replicate). The coefficient of variation of reproduction in the control was < 30 (being 5.2%).

The following deviation from recommendations given in the test OECD 232 guideline was recorded:

- At the start of the test the pH values for the control and the treatment group were slight above the recommended value of 6 ±0.5 (being 6.57-6.58).

Indicated deviation is, however considered as having no impact on the study results, since all validity criteria were met. The study is thus considered acceptable.

**Agreed endpoints:**

NOEC<sub>reproduction</sub> ≥ 100 mg test item/kg soil dw

LOEC<sub>reproduction</sub> > 100 mg test item/kg soil dw

**Table B. 9.4.2.4. Flufenacet-oxalate: Influence on mortality and reproduction on the soil mite species *Hypoaspis aculeifer* tested in artificial soil with 5 % peat.**

<b>Reference:</b>	Flufenacet-oxalate: Influence on mortality and reproduction on the soil mite species <i>Hypoaspis aculeifer</i> tested in artificial soil with 5 % peat.
<b>Author(s), year:</b>	Kratz. M.A., 2010
<b>Report/Doc. number:</b>	Study No:EBFOL171, Reference BCS No: M-393634-01-1.
<b>Guideline(s):</b>	OECD 226 from October 03, 2008:
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet-oxalate, Batch code: BCS-AB16305-01-01, Purity 95.3 % w/w. (analysed).
Test species:	Predatory mites, <i>Hypoaspis aculeifer</i> .
Number of organism:	8 replicates per control and 8 replicates per treatment group, 2 additional replicates per treatment and control to check the pH and water content of the test substrate after 14 days, each with 10 individuals.

Life stage, age:	Adults females
Type of test, duration	Laboratory sub-lethal limit test, 14 days
Applied concentrations:	Nominal: 0 (control, quartz), 100 mg item/kg soil dw. The test item was applied by mixing a test item-quartz sand-mixture into the artificial soil.
Solvent:	None
Toxic standard:	Dimethoate EC 400, 411.7 g/L (analysed) tested at concentration of .0, 1.8, 3.2, 5.6 and 10.0 mg dimethoate/ kg soil dw.
Test substrate	Artificial soil, 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 74.8 % fine quartz sand, 0.2% calcium carbonate ( for the adjustment to ph to $6 \pm 0.5$ ).
Substrate/Test vessel:	Test containers were glass vessels (volume 140 ml, diameter 5 cm at the bottom, height 7 cm). Each test vessel contained 20 g dry weight soil /test container. The test vessels were covered with glass lids to prevent the <i>hypoaspis aculeifer</i> escaping but allowing aeration during the test period.
Temperature:	$20 \pm 2$ °C
Light regime:	16 hours light/8 hours dark. Light intensity ranged from 599-620 Lux.
Water content:	Test start: 19.74-20.04% (equivalent to 50.49- 51.47 of WHC) Test end: 20.24-20.32% (equivalent to 52.10-52.37of WHC)
pH:	Test start: 6.57-6.58 Test end: 6.39-6.41
Feeding:	Feeding was done before test and directly at start, 3, 7, and 10 days after test start. The predatory mites were fed cheese mites ( <i>Tyrophagus putrescentiae</i> ).
Test parameters:	pH and water content were determined at test start and test end. Water content maintenance was checked weekly after application. Mortality of adults, differences in morphology, behavioural effects and number of juveniles were assessed after 14 days.
Statistic:	Data of reproduction were tested for normal distribution and homogeneity of variance using Kolmogoroff-Smirnov Test and Cochran-Test ( $\alpha = 0.05$ ), respectively. Data of reproduction were normally distributed and homogeneity of variances was given. Therefore Student-t test (one-sided smaller, $\alpha = 0.05$ ) was used to determine NOEC and LOEC values. The software used to perform the statistical analysis was ToxRat Pro 2.10.
Findings:	The mortality rate of 7.5 % was found in the control and 15% in the test with 100 mg test item/kg artificial soil dw. Concerning the number of juveniles statistical analysis (Student-t test, one-sided smaller, $\alpha = 0.05$ ) revealed no significant differences between the control and treatment.

**Table B. 9.4.2.4-1: Effects on mortality and reproduction of *Hypoaspis aculeifer* in a sub-chronic test.**

Exposure	Flufenacet-oxalate (mg test item/kg soil dw)	
	Control	100
Mortality of adult [%] after 14 days	7.5	15
Mean number of offspring per test vessel after 14 days (±SD)	288.1(±55.3)	248.1 (±53.5) <sup>ns</sup>
% reproduction compared to control		86.1 <sup>ns</sup>

ns No statistical significance (Student-t test one-sided smaller,  $\alpha = 0.05$ )

#### Toxic standard:

In the most recent test with the reference item Dimethoate 440EC, analysed in the separately report, tested at concentrations of 0, 1.8, 3.2, 5.6 and 10.0 mg dimethoate/ kg soil dw, a  $LC_{50}$  of 4.2 mg a.s./kg (95 % CI: 3.6 - 5.0 mg a.s./ kg soil dw) for mortality of the adult mites and the  $NOEC_{\text{reproduction}}$  of 3.2 mg a.s./ kg soil dw were determined. In addition, a  $EC_{50}$  of 5.7 mg a.s./ kg soil dw (95 % CI: 5.7-5.8 mg a.s./kg dry weight) for reproduction was found.

#### Conclusion:

$NOEC_{\text{reproduction}} \geq 100$  mg test item/kg soil dw

$LOEC_{\text{reproduction}} > 100$  mg test item/kg soil dw

Based on the point 5(123) of RT, the  $EC_{10}$  and  $EC_{20}$  values should be calculated. In this case, the  $EC_x$  value cannot be calculated for this *Hypoaspis aculeifer* study since it was conducted as limit test with only one test rate.

#### **Comments RMS:**

Predatory mite production study was conducted according to the OECD test guideline 226 (2008).

Taking into account the validity criteria given in the test guideline OECD 226 (2008) the study is considered acceptable.

The mean mortality of the adults in the control was below 20 % (being 7.5%). The mean number of juveniles per control replicate was greater than 50 (being 288.1 per replicate).

The coefficient of variation of reproduction in the control was  $< 30$  (being 19.2%).

The study is considered acceptable.

#### **Agreed endpoints:**

$NOEC_{\text{reproduction}} \geq 100$  mg test item/kg soil dw

$LOEC_{\text{reproduction}} > 100$  mg test item/kg soil dw

---

**B.9.4.2.5. Flufenacet-sulfonic acid Na-salt: Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil.**


---

<b>Reference:</b>	Flufenacet-sulfonic acid Na-salt: Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil.
<b>Author(s), year:</b>	Frommholz U., 2010
<b>Report/Doc. number:</b>	Study No: E 314 3920-2, Reference BCS No. M-396039-01-1
<b>Guideline(s):</b>	OECD 232 adopted, September 07, 2009
<b>GLP:</b>	Yes

---

Material and methods:

<u>Test substance:</u>	Flufenacet-sulfonic acid Na-salt (AE 0841914), Batch code: AE F 0841914-01-03, Purity 92.4 % w/w (analysed).
Test species:	Collembola, Folsomia candida
Number of organism:	8 replicates per control and treatment group, each with 10 individuals. 2 additional replicates per treatment and control group to check the pH and water content of the test substrate after 28 days.
Life stage, age:	Juveniles/Adults, 10-12 days old
Type of test, duration:	Laboratory sub-lethal limit test, 28 days
Applied concentrations:	Nominal: 0 (control quartz sand ), 100 mg test item/kg soil dw. The test item was applied by mixing a test item-quartz sand-mixture into the artificial soil.
Solvent:	None
Toxic standard:	Boric acid tested at concentrations of 44, 67, 100, 150 and 225 mg/kg soil dw
Test substrate:	Artificial soil, 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 74.8 % fine quartz sand, 0.2% calcium carbonate (for the adjustment to pH to 6 ±0.5.
Substrate/Test vessel:	Test containers were glass vessels (volume 140 ml, diameter 5 cm at the bottom, height 7 cm). Each test vessel contained 30 g wet weight artificial soil. The test vessels were covered with glass lids to prevent the collembolans from escaping but allowing aeration during the test period.
Temperature:	20±2 °C
Light regime:	16 hours light/8 hours dark. Light intensity ranged from 56 to 639 Lux.
Water content:	Test start: 19.46-19.63% (equivalent to 49.60-50.14% of WHC) Test end: 17.55-17.72% (equivalent to 43.72-44.23% of WHC)
pH	Test start: 6.32 -6.35 Test end: 6.31

Feeding:	Directly after the addition of the collembolans, they were fed with granulated dry yeast. Feeding was also done 14 days after test start. Approximately 2 mg (one spatula tip) per test vessel was added per feeding date.
Test parameters:	pH and water content were determined at test start and test end. Water content maintenance was checked weekly after application. Mortality of adults, behavioral effects and number of juvenile Collembola were assessed after 28 days.
Statistic:	Data of reproduction were tested for normal distribution and homogeneity of variance using Kolmogorov - Smirnov-Test and Cochran's-Test ( $\alpha = 0.05$ ) respectively. Data of reproduction were normally distributed and homogeneity of variances was given. Therefore Student-t test (one-sided-smaller, $\alpha = 0.05$ ) was used to determine NOEC and LOEC values. The software used to perform the statistical analysis was ToxRat Pro 2.09
Findings:	The mean mortality in the control and treated group were 12.5% and 8.8%, respectively. No statistically significant different values for the number of juveniles per test vessel relative to the control were observed at the tested concentration of 100 mg Flufenacet-sulfonic acid Na-salt/kg kg soil dw. No abnormal behavior was observed with the surviving Collembola.

**Table B. 9.4.2.5-1: Effects on mortality and reproduction of Folsomia candida in a sub-chronic test.**

	Control	Flufenacet-sulfonic acid Na-salt (mg test item/kg soil dw)
Exposure	-	100
Mortality of adult Collembola [%] after 28 days ( $\pm$ SD)	12.5 ( $\pm$ 0.7)	8.8 ( $\pm$ 0.6)
Mean number of offspring per test vessel after 28 days ( $\pm$ SD)	1282.8 ( $\pm$ 106.7)	1381.6 ( $\pm$ 62.9)
% reproduction compared to control		107.7 <sup>ns</sup>

ns Not statistically significant compared to control (Student-t test, one sided smaller,  $\alpha = 0.05$ )

**Toxic standard:**

In the most recent study the  $EC_{50}$  of 96 mg/ kg soil dw for the reference item boric acid was determined. The  $NOEC_{\text{reproduction}}$  was calculated to be 44 mg Boric acid/kg soil dw and accordingly the  $LOEC_{\text{reproduction}}$  was 67 mg Boric acid/kg soil dw.

**Conclusion:**

$NOEC_{\text{reproduction}} \geq 100$  mg test item/kg soil dw

$LOEC_{\text{reproduction}} > 100$  mg test item/kg soil dw

Based on the point 5(123) of RT, the EC<sub>10</sub> and EC<sub>20</sub> values should be calculated. In this case, the EC<sub>x</sub> value cannot be calculated for this *Folsomia candida* study since it was conducted as limit test with only one test rate and the difference in the reproduction performance between the control and the treatment group was <10%.

#### Comments RMS:

The Collembola reproduction study was conducted according to the OECD test guideline 232 (2009).

Taking into account the validity criteria given in the test guideline OECD 232 (2009) the study is considered acceptable. The mean mortality of the adults in the control was below 20 % (being 12.5%). The mean number of juveniles per control replicate was greater than 100 (being 1282.8 mean with  $\pm 106.7$  (SD), per replicate). The coefficient of variation of reproduction in the control was <30 (being 8.3 %).

#### Agreed endpoints:

NOEC<sub>reproduction</sub>  $\geq$  100 mg test item/kg soil dw

LOEC<sub>reproduction</sub> > 100 mg test item/kg soil dw

#### 9.4.2.6. Flufenacet-sulfonic acid Na-salt (BCS-AZ23374): Influence on mortality and reproduction of the soil mite species *Hypoaspis aculeifer* tested in artificial soil.

<b>Reference:</b>	Flufenacet-sulfonic acid Na-salt (BCS-AZ23374): Influence on mortality and reproduction of the soil mite species <i>Hypoaspis aculeifer</i> tested in artificial soil.
<b>Author(s), year:</b>	Kratz. M-A., 2013
<b>Report/Doc. number:</b>	Study No: E 428 4485-5, Reference BCS No: M-455654-01-1.
<b>Guideline(s):</b>	OECD 226 from October 03, 2008:
<b>GLP:</b>	Yes

#### Material and methods:

Test substance:	Flufenacet-sulfonic acid Na-salt (BCS-AZ23374): Batch code: AE 0841914-01-05, Purity: 93.4 % w/w (analysed)
Test species:	Predatory mites, <i>Hypoaspis aculeifer</i>
Number of organism:	8 replicates per control and 8 replicates per treatment group each with 10 individuals. 2 additional replicates per treatment and control to check the pH and water content of the test substrate after 14 days.
Life stage, age:	Adults females

Type of test, duration	Laboratory sub-lethal limit test, 14 days
Applied concentrations:	Nominal: 0 (control, quartz sand ), 100 mg pure metabolite/kg soil dw (corresponding to 107 mg test item)/kg soil dw The test item was applied by mixing a test item-quartz sand-mixture into the artificial soil.
Solvent:	None
Toxic standard:	Dimethoate EC 400, tested at concentrations of 0, 1.8, 3.2, 5.6 and 10.0 mg dimethoate/kg soil dw.
Test substrate	Artificial soil, 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 75 % fine quartz sand, calcium carbonate ( for the adjustment to pH to 6 $\pm$ 0.5).
Substrate/Test vessel	Test containers were glass vessels (volume 140 ml, diameter 5 cm at the bottom, height 7 cm). Each test vessel contained 20 $\pm$ 1 g soil dw /test container. The test vessels were covered with glass lids to prevent the <i>Hypoaspis aculeifer</i> escaping but allowing aeration during the test period.
Temperature:	20 $\pm$ 2 °C
Light regime:	16 hours light/8 hours dark. Light intensity: 585-718 Lux.
Water content:	Test start: 19.04-19.58% (equivalent to 44.92-46.50% of WHC) Test end: 20.65-20.72 (equivalent to 49.71-49.91 % of WHC)
pH	Test start: 5.46-5.51 Test end: 5.55-5.56
Feeding:	Feeding was done before test and directly at start, 3, 7, and 10 days after test start. The predatory mites were fed cheese mites ( <i>Tyrophagus putrescentiae</i> ). Between 43 and 99 mg food per test vessel was added per feeding date.
Test parameters:	pH and water content were determined at test start and test end. Water content maintenance was checked weekly after application. Mortality of adults, differences in morphology, behavioural effects and number of juveniles were assessed after 14 days.
<u>Statistic:</u>	Mean number of juveniles for control and treatment in percent values for treatment in comparison to the untreated control were calculated. For the determination of normal distribution and homogeneity of variance Kolmogoroff-Smirnov Test and Cochran-Test ( $\alpha = 0.05$ ), respectively were used. Data of reproduction were normally distributed and homogeneity of variances was given. Therefore Student-t test (one-sided-smaller, $\alpha = 0.05$ ) was used to determine NOEC and LOEC values. The software used to perform the statistical analysis was ToxRat Professional 2.10.

Findings:

Effects on mortality and the number of offspring per test vessel after 14 days are shown in the table below:

**Table B.9.4.2.6-1: Effects on mortality and reproduction of *Hypoaspis aculeifer* in a sub-chronic test.**

Exposure	Flufenacet-sulfonic acid Na-salt (BCS-AZ23374): (mg pure metabolite)/kg soil dw	
	Control	100
Mortality of adult [%] after 14 days (±SD)	3.8 (±0.7)	6.3 (±1.4)
Mean number of offspring per test vessel after 14 days (±SD)	272.3 (±56.1)	264.9 (±50.6) <sup>ns</sup>
% reproduction compared to control	100	97.31 <sup>ns</sup>

ns No statistical significance difference compared to control (Student-t test one-sided smaller,  $\alpha = 0.05$ )

Toxic standard:

The most recent test with the reference item Dimethoate 400E G was performed at test concentrations 1.0, 1.8, 3.2, 5.6 and 10.0 mg dimethoate/kg soil dw. Dimethoate showed a  $LC_{50}$  of 4.2 mg a.s./kg (95 % CI: 3.6-5.0 mg a.s./kg soil dw) for mortality of the adult mite and  $NOEC_{reproduction}$  of 3.2 mg a.s./kg soil dw.

In addition, Dimethoate 400E G showed an  $EC_{50}$  of 5.7 mg a.s./kg soil dw (95 % CI: 5.7-5.8 mg a.s./kg soil dw) for reproduction.

#### Conclusion:

$NOEC_{reproduction} \geq 100$  mg p.m./kg soil dw

$LOEC_{reproduction} > 100$  mg p.m./kg soil dw

Based on the point 5(123) of RT, the  $EC_{10}$  and  $EC_{20}$  values should be calculated. In this case, the  $EC_x$  value cannot be calculated for this *Hypoaspis aculeifer* study since it was conducted as limit test with only one test rate and the difference in the reproduction performance between the control and the treatment group was <10%.

#### **Comments RMS:**

Predatory mite production study was conducted according to the OECD test guideline 226 (2008).

Taking into account the validity criteria given in the test guideline OECD 226 (2008) the study is considered acceptable. The mean mortality of the adults in the control was below 20 % (being 3.8%). The mean number of juveniles per control replicate was greater than 50 (being 272.3 per replicate). The coefficient of variation of reproduction in the control was < 30 (being 20.6%).

The study is considered acceptable.

#### **Agreed endpoints:**

$NOEC_{reproduction} \geq 100$  mg p.m./kg soil dw

$LOEC_{reproduction} > 100$  mg p.m./kg soil dw

---

**B.9.4.2.7. Flufenacet-methylsulfone (BCS-CO62475): Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil.**


---

<b>Reference:</b>	Flufenacet-methylsulfone (BCS-CO62475): Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil.
<b>Author(s), year:</b>	Frommholz U., 2010
<b>Report/Doc. number:</b>	Study No: E 314 3923-5, Reference BCS No. M-392345-01-1
<b>Guideline(s):</b>	OECD 232 adopted, September 07, 2009.
<b>GLP:</b>	Yes

---

Material and methods:

Test substance :	Flufenacet-methylsulfone, Batch code: BCS-CO62475-01-01, Purity: 97.6 % w/w (analysed)
Test species:	Collembola Folsomia candida
Number of organism:	8 replicates per control and treatment group, each with 10 individuals. 2 additional replicates per treatment and control to check the pH and water content of the test substrate after 28 days.
Life stage, age:	Juveniles/Adults, 10-12 days old
Type of test, duration:	Laboratory sub-lethal limit test, 28 days
Applied concentrations:	Nominal: 0 (control, quartz sand), 100 mg test item /kg soil dw. The test item was applied by mixing a test item-quartz sand-mixture into the artificial soil.
Solvent:	None
Toxic standard:	Boric acid tested at concentrations of 44, 67, 100, 150 and 225 mg a.s./kg soil dw
Test substrate	Artificial soil, 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 74.8 % fine quartz sand, 0.15% calcium carbonate (for the adjustment to pH to 6 ±0.5)
Test vessel:	Test containers were glass vessels (volume 140 ml, diameter 5 cm at the bottom, height 7 cm). Each test vessel contained 30 g wet weight artificial soil. The test vessels were covered with glass lids to prevent the collembolans from escaping but allowing aeration during the test period.
Temperature	20±2 °C
Light regime	16 hours light/8 hours dark. Light intensity: 594-615 Lux
Water content	Test start: 19.07-19.38% ( equivalent to 48.37-49.35% of WHC) Test end: 19.10% (equivalent to 48.48 of WHC)
pH	Test start: 6.33-6.41 Test end: 6.28-6.37

Feeding:	Directly after the addition of the collembolans, they were fed with granulated dry yeast. Feeding was also done 14 days after test start. Approximately 2 mg (one spatula tip) per test vessel was added per feeding date.
Test parameters:	pH and water content were determined at test start and test end. Water content maintenance was checked two weeks after application. Mortality of adults, behavioral effects and number of juvenile Collembola were assessed after 28 days.
Statistic:	Data of reproduction were tested for normal distribution and homogeneity of variance using Kolmogorov - Smirnov-Test and Cochran's-Test ( $\alpha = 0.05$ ) respectively. Data of reproduction were normally distributed and homogeneity of variances was given. Therefore Student-t test (one-sided-smaller, $\alpha = 0.05$ ) was used to determine NOEC and LOEC values. The software used to perform the statistical analysis was ToxRat Pro 2.09.
Findings:	The mortality of Folsomia candida in the control group was determined to be 3.8 % and 1.3% in the treatment group. . In the treatment group Student-t test, one-sided smaller, $\alpha = 0.05$ revealed a significant difference in reproduction compared to control. Because of the low difference of the treatment group to the control group (8.8 %) in relation to a coefficient of variation of 10.2 % concerning the number of juveniles the effect is not considered to be test item related, but is in the range of the biological variability of the test system. No abnormal behavior was observed with the surviving Collembola.

**Table B. 9.4.2.7-1: Effects on mortality and reproduction of Folsomia candida in a sub-chronic test.**

	Control	Flufenacet-methylsulfone (BCS-CO62475) (mg test item/kg soil dw)
Exposure	-	100
Mortality of adult Collembola [%] after 28 days ( $\pm$ SD)	3.8 ( $\pm$ 0.5)	1.3 ( $\pm$ 0.4)
Mean number of offspring per test vessel after 28 days ( $\pm$ SD)	1470.1 ( $\pm$ 150.4)	1340.9 ( $\pm$ 105.6)
% reproduction compared to control		91.2*

\* Significantly different compared to the control (Student T-test, one-sided-smaller,  $\alpha = 0.05$ )

Toxic standard:

In the most recent study the EC<sub>50</sub> of 96 mg/kg soil dw for the reference item boric acid was determined. The NOEC<sub>reproduction</sub> was calculated to be 44 mg Boric acid/kg soil dw and the LOEC<sub>reproduction</sub> was 67 mg Boric acid/kg soil dw.

Conclusion:

NOEC ≥ 100 mg test item/kg soil dw

LOEC > 100 mg test item/kg soil dw

Based on the point 5(123) of RT, the EC<sub>10</sub> and EC<sub>20</sub> values should be calculated. In this case, the EC<sub>x</sub> value cannot be calculated for this *Folsomia candida* study since it was conducted as limit test with only one test rate and the difference in the reproduction performance between the control and the treatment group was <10%.

**Comments RMS:**

The Collembola reproduction study was conducted according to the OECD test guideline 232 (2009). Taking into account the validity criteria given in the test guideline OECD 232 (2009) the study is considered acceptable.

The mean mortality of the adults in the control was below 20 % (being 3.8%). The mean number of juveniles per control replicate was greater than 100 (being 1470.1 per replicate). The coefficient of variation of reproduction in the control was < 30 (being 10.2%).

The study is considered acceptable.

**Agreed endpoints:**

NOEC<sub>reproduction</sub> ≥ 100 mg test item/kg soil dw

LOEC > 100 mg test item/kg soil dw

**B.9.4.2.8. Flufenacet-methylsulfone: Influence on mortality and reproduction on the soil mite species *Hypoaspis aculeifer* tested in artificial soil with 5 % peat.**

<b>Reference:</b>	Flufenacet-methylsulfone: Influence on mortality and reproduction on the soil mite species <i>Hypoaspis aculeifer</i> tested in artificial soil with 5 % peat.
<b>Author(s), year:</b>	Kratz. M. A., 2009
<b>Report/Doc. number:</b>	Study No: E 428 3685-6, Reference BCS No: M-357707-01-1
<b>Guideline(s):</b>	OECD 226 (October 03, 2008)
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet-methylsulfone, Batch Code BCS-CO62475-01-01, Purity 97.6% (analysed).
Test species:	Predatory mites, <i>Hypoaspis aculeifer</i>
Number of organism:	8 replicates per control and 4 replicates for treatment group, each with 10 individuals. 2 additional replicates per treatment and control to check the pH and water content of the test substrate after 14 days.
Life stage, age:	Adult females of <i>Hypoaspis aculeifer</i> .
Type of test, duration	Laboratory sub-lethal limit test, 14 days
Applied concentrations:	Nominal: (control, quartz sand), 63, 125, 250, 500 and 1000 mg test item/kg soil dw. The test item was applied by mixing a test item-quartz sand-mixture into the artificial soil.
Toxic standard:	Dimethoate tested at concentrations of 0.98, 1.61, 2.85, 4.99 and 8.92 mg Dimethoate/kg soil dw.
Test substrate	Artificial soil, 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 74.8 % fine quartz sand, 0.2% calcium carbonate (for the adjustment to pH to 6 ±0.5)
Test vessel	Test containers were glass vessels (volume 140 ml, diameter 5 cm at the bottom, height 7 cm). Each test vessel contained 20 g wet weight artificial soil. The test vessels were covered with glass lids to prevent the <i>Hypoapsis</i> from escaping but allowing aeration during the test period.
Temperature	20 ± 2 °C
Light regime:	16 hours light/8 hours dark. Light intensity: 582-598 Lux
Water content:	Test start: 17.90-18.35% (equivalent to 47.20-48.64% of WHC) Test end: 18.54-19.58% (equivalent to 49.25-52.70% of WHC)
pH:	Test start: 5.86-5.89 Test end: 5.53-5.69

Feeding:	Feeding was done before test and directly at start, 4, 7, and 10 days after test start. Approximately 55 mg food per test vessel was added per feeding date.
Test parameters:	pH were determined at test start and test end. Water content maintenance was checked weekly after application. Mortality of adults, effects and number of juveniles were assessed after 14 days.
Statistic:	Determination of mean number of juveniles for control and treatment and the percent values for treatment in comparison to the untreated control were calculated. For normal distribution and homogeneity of variance using Kolmogoroff-Smirnov Test and Cochran-Test ( $\alpha = 0.05$ ), respectively were used. Data of reproduction were normally distributed but homogeneity of variances was not given. Therefore Student t-test for homogeneous variances (one-sided smaller, $\alpha = 0.05$ ) was used to determine NOEC and LOEC values.
Findings:	The mean mortality rate of <i>Hypoaspis aculeifer</i> of 3.8 % was found in the control and 5.0% in at test concentrations: 63, 250 and 1000 mg test item/kg soil dw Concerning the number of juveniles statistical analysis (Williams Test, one-sided smaller, $\alpha = 0.05$ ) revealed significant differences between the control and 1000 mg test item/kg kg soil dw.

Effects on mortality and the number of offspring per test vessel after 14 days are shown in the table below:

**Table B.9.4.2.8-1: Effects on mortality and reproduction of *Hypoaspis aculeifer* in a sub-chronic test.**

Exposure	Flufenacet-methylsulfone (mg test item/kg soil dw)					
	Control	63	125	250	500	1000
Mortality of adult [%] after 14 days	3.8	5.0	0.0	5.0	0.0	5.0
Mean number of offspring per test vessel after 14 days ( $\pm$ SD)	355.5 ( $\pm$ 31.1)	370.8 ( $\pm$ 22.8)	387.0 ( $\pm$ 28.2)	390.5 ( $\pm$ 16.8)	374.5 ( $\pm$ 24.6)	304.3* ( $\pm$ 10.9)
% reproduction compared to control		104.3	108.9	109.8	105.3	85.6*

\* Statistical differences between control and treatments group (Williams-t.-test one sided smaller;  $\alpha=0.05$ )

Toxic standard:

In most recent test with the reference item Dimethoate analysed in separately report, tested at concentrations of 0.98, 1.61, 2.85, 4.99 and 8.92 mg Dimethoate/kg soil dw, showed a LC<sub>50</sub> of 3.86 mg a.s./kg (95 % CI: 3.34 -

4.45 mg a.s./kg soil dw) for mortality of the adult mites. The  $\text{NOEC}_{\text{reproduction}}$  of 1.61 mg a.s./kg soil dw and  $\text{LOEC}_{\text{reproduction}}$  of 2.85 mg a.s./kg soil dw were found.

Dimethoate showed an  $\text{EC}_{50}$  of 5.45 mg a.s./kg dw (95 %CI: 4.59 - 6.53 mg a.s./kg dw) for reproduction.

**Conclusion:**

$\text{NOEC}_{\text{reproduction}} = 500 \text{ mg test item/kg soil dw}$

$\text{LOEC}_{\text{reproduction}} = 1000 \text{ mg test item/kg soil dw}$

In accordance with the new data requirement (Commission Regulation EU No 283/2013), the  $\text{EC}_{10}$ ,  $\text{EC}_{20}$ ,  $\text{EC}_{50}$  values should be calculated.

A valid  $\text{EC}_x$  calculation was not possible due to the lack of a significant concentration/response function. As the difference to the control at the  $\text{NOEC}=500 \text{ mg/kg}$  is 105.3% compared to the control, it can be reasonably assumed that the  $\text{EC}_{10}$  is higher than the  $\text{NOEC}$ , thus, less critical. Therefore the  $\text{NOEC}$  can be considered as the relevant endpoint in the risk assessment as a worst case approach.

**Comments RMS:**

Predatory mite production study was conducted according to the OECD test guideline 226 (2008).

Taking into account the validity criteria given in the test guideline OECD 226 (2008) the study is considered acceptable

The mean mortality of the adults in the control were below 20 % (being 3.8% per replicate). The mean number of juveniles per control was greater than 50 (being 355.5 per replicate). The coefficient of variation of reproduction in the control were <30 (being 8.8%).

**Agreed endpoint:**

$\text{NOEC}_{\text{reproduction}} = 500 \text{ mg test item/kg soil dw}$

$\text{LOEC}_{\text{reproduction}} = 1000 \text{ mg test item/kg soil dw}$

**B.9.4.2.9. Trifluoroacetic acid Na-salt (BCS-AZ56567): Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil.**

<b>Reference:</b>	Trifluoroacetic acid Na-salt (BCS-AZ56567): Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil.
<b>Author(s), year:</b>	Frommholz U., 2012
<b>Report/Doc. number:</b>	Study No: E 314 4345-4, Reference BCS No. :M-436127-01-1
<b>Guideline(s):</b>	OECD 232 adopted, September 07, 2009
<b>GLP:</b>	Yes

Material and methods:

Test substance	Trifluoroacetic acid Na-salt (BCS-AZ56567, Batch code: AE 1046319-01-01, Purity: 95.1 % w/w (analyzed )
Test species:	Collembola, Folsomia candida
Number of organism:	8 replicates per control and treatment group, each with 10 individuals. 2 additional replicates per treatment and control to check the pH and water content of the test substrate after 28 days
Life stage, age:	Juveniles/Adults, 11-12 days old
Type of test, duration	Laboratory sub-lethal limit test, 28 days
Applied concentrations:	Nominal: 0 (control, quartz sand), 100 mg test item/kg soil dw. The test item was applied by mixing a test item-quartz sand-mixture into the artificial soil.
Solvent:	None
Toxic standard	Boric acid tested at concentrations of 44, 67, 100, 150 and 225 mg/kg soil dw
Test substrate	Artificial soil, 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 74.8 % fine quartz sand (sort F36, particle size 0.2-0.05 mm=91.50%), 0.2% calcium carbonate (for the adjustment to pH to 6 ±0.5.
Test vessel	Test containers were glass vessels (volume 140 ml, diameter 5 cm at the bottom, height 7 cm). Each test vessel contained 30 g w ±1 wet weight artificial soil. The test vessels were covered with glass lids to prevent the collembolans from escaping but allowing aeration during the test period
Temperature:	20±2 °C
Light regime:	16 hours light/8 hours dark. Light intensity ranged from 597-688 Lux.
Water content	Test start:20.93-21.10% ( equivalent to 46.6% of WHC) Test end: 20.98-21.48 % (equivalent to 46.53-48.22% of WHC)
pH	Test start:5.56-5.79 Test end: 5.82-6.21

Feeding:	Directly after the addition of the collembolans, they were fed with granulated dry yeast. Feeding was also done 14 days after test start. Approximately 2 mg (one spatula tip) per test vessel was added per feeding date.
Test parameters:	pH and water content were determined at test start and test end. Water content maintenance was checked weekly after application. Mortality of adults, behavioral effects and number of juvenile Collembola were assessed after 28 days.
Statistic:	Data of reproduction were tested for normal distribution and homogeneity of variance using Kolmogorov - Smirnov-Test and Cochran's-Test ( $\alpha = 0.05$ ) respectively. Data of reproduction were normally distributed and homogeneity of variances was given. Therefore Student-t test (one-sided-smaller, $\alpha = 0.05$ ) was used to determine NOEC and LOEC values. The software used to perform the statistical analysis was ToxRat Pro 2.09
Findings:	The mean mortality in the control and treated group were 16.3% and 10%, respectively. No statistically significant different values for the number of juveniles relative to the control were observed at the tested concentration of 100 mg /kg soil dw. No abnormal behavior was observed with the surviving Collembola.

**Table 9.4.2.9-1: Effects on mortality and reproduction of Folsomia candida in a sub-chronic test.**

	Trifluoroacetic acid Na-salt (BCS-AZ56567)	
	Control	(mg test item/kg soil dw)
Exposure	-	100
Mortality of adult Collembola [%] after 28 days	16.3 ( $\pm 0.7$ )	10 ( $\pm 0.8$ )
Mean number of offspring per test vessel after 28 days ( $\pm$ SD)	1132.6 ( $\pm 110.4$ )	1051.9 ( $\pm 133.4$ ) <sup>ns</sup>
% reproduction compared to control		92.9 <sup>ns</sup>

ns Not statistically significant compared to control (Student-t test, one sided smaller,  $\alpha = 0.05$ )

#### Toxic standard:

In the most recent study the EC<sub>50</sub> of 116 mg/ kg artificial soil dw for the reference item boric acid was determined. The NOEC<sub>reproduction</sub> was calculated to be 67 mg Boric acid/kg soil dw and the LOEC<sub>reproduction</sub> was 100 mg Boric acid/kg soil dw.

Conclusion:

NOEC<sub>reproduction</sub> ≥ 100 mg test item/kg soil dw

LOEC<sub>reproduction</sub> >100 mg test item/kg soil dw

Based on the point 5(123) of RT, the EC<sub>10</sub> and EC<sub>20</sub> values should be calculated. In this case, the EC<sub>x</sub> value cannot be calculated for this *Folsomia candida* study since it was conducted as limit test with only one test rate and the difference in the reproduction performance between the control and the treatment group was <10%.

**Comments RMS:**

The Collembola reproduction study was conducted according to the OECD test guideline 232 (2009). Taking into account the validity criteria given in the test guideline OECD 232 (2009) the study is considered acceptable.

The mean mortality of the adults in the control was below 20 % (being 16.3%). The mean number of juveniles per control replicate was greater than 100 (being 1132.6 with ±110.4 (SD), per replicate). The coefficient of variation of reproduction in the control was < 30 (being 9.7 %).

**Agreed endpoints:**

NOEC<sub>reproduction</sub> ≥ 100 mg test item/kg soil dw

LOEC<sub>reproduction</sub> >100 mg test item/kg soil dw

**9.4.2.10. Trifluoroacetic acid Na-salt (BCS-AZ56567): Influence on mortality and reproduction on the soil mite species *Hypoaspis aculeifer* tested in artificial soil.**

<b>Reference:</b>	Trifluoroacetic acid Na-salt (BCS-AZ56567): Influence on mortality and reproduction on the soil mite species <i>Hypoaspis aculeifer</i> tested in artificial soil.
<b>Author(s), year:</b>	Kratz, M.A., 2012
<b>Report/Doc. number:</b>	Study No: E 428 4349-4, Reference BCS No: M-436326-01-1.
<b>Guideline(s):</b>	OECD 226 (October 03, 2008)
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Trifluoroacetic acid Na-salt (BCS-AZ56567), Batch code: AE 1046319-01-01, Purity: 95.1 %w/w (analysed).
Test species:	Predatory mites, <i>Hypoaspis aculeifer</i>
Number of organism:	8 replicates per control and 8 replicates per treatment group, each with 10 individuals.

---

	2 additional replicates per treatment and control to check the pH and water content of the test substrate after 14 days
Life stage, age:	Adults females
Type of test, duration	Laboratory sub-lethal limit test, 14 days
Applied concentrations:	Nominal: 0 (control, quartz sand), 100 mg test item /kg soil dw The test item was applied by mixing a test item-quartz sand-mixture into the artificial soil.
Solvent:	None
Toxic standard:	Dimethoate tested at concentration of 0, 1.8, 3.2, 5.6 and 10.0 mg dimethoate/ kg soil dw
Test substrate	Artificial soil, 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 74.8 % fine quartz sand, 0.2% calcium carbonate ( for the adjustment to pH to 6 ±0.5).
Substrate/Test vessel	Test containers were glass vessels (volume 140 ml, diameter 5 cm at the bottom, height 7 cm). Each test vessel contained 20 g soil dw (height of artificial soil layer approximately 1.5 cm). The test vessels were covered with glass lids to prevent the <i>Hypoaspis aculeifer</i> escaping but allowing aeration during the test period.
Temperature:	20±2 °C
Light regime:	16 hours light/8 hours dark. Light intensity: 648-706 Lux.
Water content:	Test start: 5.56-5.79 Test end: 5.75
pH:	Test start:20.93-21.10% (equivalent to 46.66-47.13% of WHC) Test end: 21.23-21.40% (equivalent to 47.51-47.99% of WHC)
Feeding:	Feeding was done before test and directly at start, 3, 7, and 10 days after test start. The predatory mites were fed cheese mites ( <i>Tyrophagus putrescentiae</i> Between 52 and 107 mg food per test vessel was added per feeding date.
Test parameters:	pH and water content were determined at test start and test end. Water content maintenance was checked weekly after application. Mortality of adults and number of juveniles were assessed after 14 days.
Statistic:	The mean number of surviving adult, female <i>Hypoaspis aculeifer</i> for control and treatment were determined. Determination of the percent mortality for control and treatment were calculated. Determination of mean number of juveniles for control and treatment and the percent values for treatment in comparison to the untreated control were determined. For normal distribution and homogeneity of variance using Kolmogoroff-Smirnov Test and Cochran-Test ( $\alpha = 0.05$ ), respectively were used. Data of reproduction were normally distributed but homogeneity of variances was not

given. Therefore Student t-test for homogeneous variances (one-sided smaller,  $\alpha = 0.05$ ) was used to determine NOEC and LOEC values.

Findings: The mean mortality rate of *Hypoaspis aculeifer* of 2.5 % was found in the control and no mortality was determined in the test with 100 mg test item/kg soil dw.

Concerning the number of juveniles statistical analysis (Student-t test, one-sided smaller,  $\alpha=0.05$ ) revealed no significant differences between the control and the concentration of 100 mg test item/kg soil dw.

Effects on mortality and the number of offspring per test vessel after 14 days are shown in the table below:

**Table 9.4.2.10-1: Effects on mortality and reproduction of *Hypoaspis aculeifer* in a sub-chronic test.**

Exposure	Trifluoroacetic acid Na-salt (mg test item/ kg soil dw)	
	Control	100
Mortality of adult [%] after 14 days(±SD)	2.5 (±0.5)	0
Mean number of offspring per test vessel after 14 days (±SD)	346.5 (±23.5)	372.1 (±19.1) <sup>ns</sup>
% reproduction compared to control		107.4 <sup>ns</sup>

ns No statistically significant compared to control (Student-t test one-sided smaller,  $\alpha = 0.05$ )

#### Toxic standard

In the most recent test with the reference item Dimethoate, analysed in the separately report, tested at concentrations of 0, 1.8, 3.2, 5.6 and 10.0 mg dimethoate/ kg soil dw, a LC<sub>50</sub> of 3.894 mg a.s./kg for mortality of the adult mites and the NOEC<sub>reproduction</sub> of 3.2 mg a.s./ kg soil dw were determined. In addition, a EC<sub>50</sub> of 6.62 mg a.s./ kg soil dw for reproduction was found.

#### Conclusions:

NOEC<sub>reproduction</sub> ≥ 100 mg test item/kg soil dw

LOEC<sub>reproduction</sub> > 100 mg test item/kg soil dw

Based on the point 5(123) of RT, the EC<sub>10</sub> and EC<sub>20</sub> values should be calculated. In this case, the EC<sub>x</sub> value cannot be calculated for this *Hypoaspis aculeifer* study since it was conducted as limit test with only one test rate and the difference in the reproduction performance between the control and the treatment group was <10%.

**Comments RMS:**

Predatory mite production study was conducted according to the OECD test guideline 226 (2008).

Taking into account the validity criteria given in the test guideline OECD 226 (2008) the study is considered acceptable.

The mean mortality of the adults in the control was below 20 % (being 2.5%). The mean number of juveniles per control replicate was greater than 50 (being 346.5 per replicate). The coefficient of variation of reproduction in the control was <30 (being 6.8%).

**Agreed endpoints:**

NOEC<sub>reproduction</sub> ≥ 100 mg test item/kg soil dw

LOEC<sub>reproduction</sub> > 100 mg test item/kg soil dw

**B.9.4.2.11. Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474): Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil.**

<b>Reference:</b>	Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474): Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil.
<b>Author(s), year:</b>	Frommholz U., 2012
<b>Report/Doc. number:</b>	Study No: E 314 4272-3, Reference BCS No. M-436128-01-1
<b>Guideline(s):</b>	OECD 232 adopted, September 07, 2009
<b>GLP:</b>	No

Material and methods:

Test substance:	Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474,) Batch code: BCS-CU62474-01-01, Purity 99.4 % w/w
Test species:	Collembola, Folsomia candida
Number of organism:	8 replicates per control and treatment group, each with 10 individuals. 2 additional replicates per treatment and control to check the pH and water content of the test substrate after 28 days.
Life stage, age:	Juveniles/Adults, 11-12 days old
Type of test, duration:	Laboratory sub-lethal limit test, 28 days
Applied concentrations:	
Nominal:	0 (control, quartz sand) 100 mg test item/kg soil dw. The test item was applied by mixing a test item-quartz sand-mixture into the artificial soil.
Solvent:	None
Toxic standard:	Boric acid tested at concentrations of 44, 67, 100, 150 and 225 mg Boric acid

	/kg soil dw.
Test substrate:	Artificial soil, 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 74.8 % fine quartz sand , 0.2% calcium carbonate (for the adjustment to ph to $6 \pm 0.5$ ).
Test vessel:	Test containers were glass vessels (volume 140 ml, diameter 5 cm at the bottom, height 7 cm). Each test vessel contained 30 g wet weight artificial soil. The test vessels were covered with glass lids to prevent the collembolans from escaping but allowing aeration during the test period.
Temperature:	$20 \pm 2$ °C
Light regime:	16 hours light/8 hours dark. Light intensity ranged from 597-688 Lux.
Water content:	Test start: 20.93-21.13% (equivalent to 46.66-47.22% of WHC) Test end: 21.18-21.40% (equivalent to 47.35-47.99% of WHC)
pH:	Test start: 5.56-5.81 Test end: 5.86-6.21
Feeding:	Directly after the addition of the collembolans, they were fed with granulated dry yeast. Feeding was also done 14 days after test start. Approximately 2 mg (one spatula tip) per test vessel was added per feeding date.
Test parameters:	pH and water content were determined at test start and test end. Water content maintenance was checked weekly after application. Mortality of adults, behavioral effects and number of juvenile Collembola were assessed after 28 days.
Statistic:	Data of reproduction were tested for normal distribution and homogeneity of variance using Kolmogorov - Smirnov-Test and Cochran's-Test ( $\alpha = 0.05$ ) respectively. Data of reproduction were normally distributed and homogeneity of variances was given. Therefore Student-t test (one-sided-smaller, $\alpha = 0.05$ ) was used to determine NOEC and LOEC values.
Findings:	The software used to perform the statistical analysis was ToxRat Pro 2.09 The mean mortality in the control and treated group were 16.3% and 12.5%, respectively. No statistically significant different values for the number of juveniles per test vessel relative to the control were observed at the tested concentration of 100 mg Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474)/kg soil dw. No abnormal behavior was observed with the surviving Collembola.

**Table B.9.4.2.11-1: Effects on mortality and reproduction of Folsomia candida in a sub-chronic test.**

	Control	Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474) (mg test tem/kg soil dw)
Exposure	-	100
Mortality of adult Collembola [%] after 28 days (±SD)	16.3 (±0.7)	12.5 (±0.7)
Mean number of offspring per test vessel after 28 days (±SD)	1132.6 (±110.4)	1066.0 (±64.9)
% reproduction compared to control		94.1 <sup>ns</sup>

ns Not statistically significant compared to control (Student-t test, one sided smaller,  $\alpha = 0.05$ )

#### Toxic standard:

In the most recent study the EC<sub>50</sub> of 116 mg/ kg soil dw for the reference item boric acid was determined.

The NOEC<sub>reproduction</sub> was 67 mg Boric acid/kg soil dw and the LOEC<sub>reproduction</sub> was 100 mg Boric acid/kg soil dw.

The result are in the recommended range of the guideline (about 100 mg Boric acid/kg artificial soil dry weight).

This shows that the test organisms are sufficiently sensitive.

#### Conclusions:

NOEC<sub>reproduction</sub> ≥ 100 mg test item/kg soil dw

LOEC<sub>reproduction</sub> >100 mg test item/ kg soil dw.

Based on the point 5(123) of RT, the EC<sub>10</sub> and EC<sub>20</sub> values should be calculated. In this case, the EC<sub>x</sub> value cannot be calculated for this Folsomia candida study since it was conducted as limit test with only one test rate and the difference in the reproduction performance between the control and the treatment group was <10%.

#### Comments RMS:

The Collembola reproduction study was conducted according to the OECD test guideline 232 (2009). Taking into account the validity criteria given in the test guideline OECD 232 (2009) the study is considered acceptable.

The mean mortality of the adults in the control was below 20 % (being 16.3%). The mean number of juveniles per control replicate was greater than 100 (being 1132.6 with ±110.4 (SD), per replicate). The coefficient of variation of reproduction in the control was <30 (being 9.7 %).

#### Agreed endpoints:

NOEC<sub>reproduction</sub> ≥ 100 mg test item/kg soil dw

LOEC<sub>reproduction</sub> >100 mg test item/ kg soil dw.

---

**B.9.4.2.12. Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474): Influence on mortality and reproduction on the soil mite species *Hypoaspis aculeifer* tested in artificial soil.**


---

<b>Reference:</b>	Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474): Influence on mortality and reproduction on the soil mite species <i>Hypoaspis aculeifer</i> tested in artificial soil.
<b>Author(s), year:</b>	Kratz, M.A., 2012
<b>Report/Doc. number:</b>	Study No: E 428 4271-8, Reference BCS No: M-436315-01-1.
<b>Guideline(s):</b>	OECD 226 (October 03, 2008)
<b>GLP:</b>	Yes

---

Material and methods:

Test substance:	Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474), Batch code: BCS-CU62474-01-01, Purity: 99.4 % w/w (analysed).
Test species:	Predatory mites, <i>Hypoaspis aculeifer</i>
Number of organism:	8 replicates per control and 8 replicates per treatment group, each with 10 individuals. 2 additional replicates per treatment and control to check the pH and water content of the test substrate after 14 days
Life stage, age:	Adults females
Type of test, duration:	Laboratory sub-lethal limit test, 14 days
Applied concentrations:	Nominal: 0 (control, quartz sand), 100 mg test item /kg soil dw. The test item was applied by mixing a test item-quartz sand-mixture into the artificial soil.
Solvent:	None
Toxic standard:	Dimethoate tested at concentrations of 0, 1.8, 3.2, 5.6 and 10.0 mg dimethoate/ kg soil dw
Test substrate:	Artificial soil, 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 74.8 % fine quartz sand, 0.25% calcium carbonate (for the adjustment to pH to 6 ±0.5)
Substrate/Test vessel:	Test containers were glass vessels (volume 140 ml, diameter 5 cm at the bottom, height 7 cm). Each test vessel contained 20±1 g soil dw (height of soil layer approximately 1.5 cm). The test vessels were covered with glass lids to prevent the <i>Hypoaspis aculeifer</i> escaping but allowing aeration during the test period.
Temperature:	20±2 °C
Light regime:	16 hours light/8 hours dark. Light intensity: 648-706 Lux.
Water content:	Test start:20.93-21.13% (equivalent to 46.66-47.22% of WHC) Test end:21.18-21.40% (equivalent to 47.35-47.99% of WHC)
pH:	Test start:5.56-5.81

	Test end: 5.75-5.79
Feeding:	Feeding was done before test and directly at start, 3, 7, and 10 days after test start. The predatory mites were fed cheese mites ( <i>Tyrophagus putrescentiae</i> ). Between 49 and 107 mg food per test vessel was added per feeding date.
Test parameters:	pH and water content were determined at test start and test end. Water content maintenance was checked weekly after application. Mortality of adults and number of juveniles were assessed after 14 days.
Statistic:	The mean number of surviving adult, female <i>Hypoaspis aculeifer</i> for control and treatment were determined. Determination of the percent mortality for control and treatment were calculated. Mean number of juveniles for control and treatment and the percent values for treatment in comparison to the untreated control were also calculated. For normal distribution and homogeneity of variance using Kolmogoroff-Smirnov Test and Cochran-Test ( $\alpha = 0.05$ ), respectively were used. Data of reproduction were normally distributed but homogeneity of variances was not given. Therefore Student t-test for homogeneous variances (one-sided smaller, $\alpha = 0.05$ ) was used to determine NOEC and LOEC values.
Findings:	The mean mortality rate of 2.5 % was found in the control and 5.0% in the test with 100 mg test item/kg soil dw. Concerning the number of juveniles statistical analysis (Student-t test, one-sided smaller, $\alpha=0.05$ ) revealed no significant differences between the control and he concentration of 100 mg test item/kg dry weight soil.

Effects on mortality and the number of offspring per test vessel after 14 days are shown in the table below:

**Table B.9.4.2.12-1: Effects on mortality and reproduction of *Hypoaspis aculeifer* in a sub-chronic test.**

	<b>Flufenacet-trifluoroethanesulfonic acid Na-salt (mg test item/kg soil dw)</b>	
<b>Exposure</b>	<b>Control</b>	<b>100</b>
Mortality of adult [%] after 14 days(±SD)	2.5 (±0.7)	5.0 (±0.5)
Mean number of offspring per test vessel after 14 days (±SD)	346.5 (±23.5)	387.9 (±36.8) <sup>ns</sup>
% reproduction compared to control		119 <sup>ns</sup>

ns No statistically significance compared to control (Student-t test one-sided smaller,  $\alpha = 0.05$ )

Toxic standard:

In the most recent test with the reference item Dimethoate, analysed in the separately report, tested at concentrations of 0, 1.8, 3.2, 5.6 and 10.0 mg dimethoate/ kg soil dw, a  $LC_{50}$  of 3.894 mg a.s./kg for mortality of the adult mites and the  $NOEC_{\text{reproduction}}$  of 3.2 mg a.s./kg soil dw were determined. In addition, a  $EC_{50}$  of 6.62 mg a.s./kg soil dw for reproduction was found.

Conclusions:

$NOEC_{\text{reproduction}} \geq 100$  test item/kg soil dw

$LOEC_{\text{reproduction}} > 100$  test item/kg soil dw

Based on the point 5(123) of RT, the  $EC_{10}$  and  $EC_{20}$  values should be calculated. In this case, the  $EC_x$  value cannot be calculated for this *Hypoaspis aculeifer* study since it was conducted as limit test with only one test rate and the difference in the reproduction performance between the control and the treatment group was <10%.

**Comments RMS:**

Predatory mite production study was conducted according to the OECD test guideline 226 (2008).

Taking into account the validity criteria given in the guideline OECD 226 (2008) the study is considered acceptable.

The mean mortality of the adults in the control was below 20 % (being 2.5%). The mean number of juveniles per control replicate was greater than 50 (being 346.5 per replicate). The coefficient of variation of reproduction in the control was <30 (being 6.8%).

**Agreed endpoints:**

$NOEC_{\text{reproduction}} \geq 100$  mg test item /kg soil dw,  $LOEC_{\text{reproduction}} > 100$  test item item /kg soil dw

---

**B.9.4.2.13. Flufenacet-thiadone (BCS-AA41715): Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil.**


---

<b>Reference:</b>	Flufenacet-thiadone (BCS-AA41715): Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil.
<b>Author(s), year:</b>	Frommholz U., 2012
<b>Report/Doc. number:</b>	Study No: E 314 4344-3, Reference BCS No.:M-440372-01-1
<b>Guideline(s):</b>	OECD 232 adopted, September 07, 2009
<b>GLP:</b>	Yes

---

Material and methods:

Test substance :	Flufenacet-thiadone, (BCS-AA41715); Batch code: AE 1258593-01-01; Purity: 98.6 % w/w (analysed).
Test species:	Collembola, Folsomia candida
Number of organism:	<i>The first test run:</i> 8 replicates per control and treatment group, each with 10 individuals. 2 additional replicates per treatment and control to check the pH and water content of the test substrate after 28 days. <i>The second test run:</i> 8 replicates per control and 4 replicates per treatment groups, each with 10 individuals.
Life stage, age:	<i>The first test run:</i> Juveniles/Adults, 11-12 days old <i>The second test run:</i> Juveniles/Adults, 9 -12 days old
Type of test, duration:	Laboratory sub-lethal limit test, 28 days
<u>Applied concentrations:</u>	<i>The first test run:</i>
Nominal:	0 (control, quartz sand), 100 mg test substance/kg soil dw <i>The second test run:</i>
Nominal:	0 (control, quartz sand) and 1.0, 1.8, 3.2, 5.6 and 10 mg test substance /kg soil dw. The test item was applied by mixing a test item-quartz sand-mixture into the artificial soil ( the first and the second runs)
Solvent:	None
Toxic standard:	Boric acid tested at concentrations of 44, 67, 100, 150 and 225 mg Boric acid /kg soil dw.

---

Test substrate:	Artificial soil, 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 75 % fine quartz sand, calcium carbonate (for the adjustment to pH to $6 \pm 0.5$ )
Substrate/Test vessel:	<p>Test containers were glass vessels (volume 140 ml, diameter 5 cm at the bottom, height 7 cm). Each test vessel contained 30 g w <math>\pm 1</math> wet weight artificial soil.</p> <p>The test vessels were covered with glass lids to prevent the collembolans from escaping but allowing aeration during the test period.</p>
Temperature:	$20 \pm 2$ °C for both test runs
Light regime:	<p><i>The first test run:</i></p> <p>16 hours light/8 hours dark. Light intensity ranged from 597-688 Lux.</p> <p><i>The second test run:</i></p> <p>16 hours light/8 hours dark. Light intensity ranged from 532-673 Lux.</p>
Water content:	<p><i>The first test run:</i></p> <p>Test start: 20.90-20.93 % (equivalent to 46.57-46.66% of WHC)</p> <p>Test end: 20.89-20.94% (equivalent to 46.53-46.68 of % WHC)</p> <p><i>The second test run:</i></p> <p>Test start: 21.16-22-20% (equivalent to 47.30-50.29% of WHC)</p> <p>Test end: 20.72-23.19% (equivalent to 46.07-53.21% of WHC)</p>
pH:	<p><i>The first test run:</i></p> <p>Test start: 5.56-5.75</p> <p>Test end: 5.98-6.21</p> <p><i>The second test run:</i></p> <p>Test start: 5.85-5.94</p> <p>Test end: 5.77-5.84</p>
Feeding:	Directly after the addition of the collembolans, they were fed with granulated dry yeast. Feeding was also done 14 days after test start. Approximately 2 mg (one spatula tip) per test vessel was added per feeding date.
Test parameters:	<p>pH and water content were determined at test start and test end. Water content maintenance was checked two weeks after application.</p> <p>Mortality of adults, behavioral effects and number of juvenile Collembola were assessed after 28 days.</p>
Statistic:	<p><i>The first test run:</i></p> <p>Statistical analysis was not conducted, since adult and juvenile collembolans were not found after 28 days of exposure.</p> <p><i>The second test run:</i></p> <p>Data of reproduction were tested for normal distribution and homogeneity of variance using Kolmogorov-Smirnov-Test and Cochran's-Test (<math>\alpha = 0.05</math>) respectively. Data of reproduction were normally distributed and but</p>

homogeneity of variances was not given even after transformation. Therefore Welch-t test (one-sided-smaller,  $\alpha = 0.05$ ) was used to determine NOEC and LOEC values. The software used to perform the statistical analysis was ToxRat Professional 2.10.

#### Findings:

In the control group 16.3% (the first test run) and 6.3% (the second test run) of the adult *Folsomia candida* died.

In the first run all adult collembolans died in the treatment group with 100 mg test item/kg soil dw. In the second run the highest mortality rate of 25% was observed in the treatment group with 5.6 test item/kg soil dw.

In the first test run no juveniles were found in the treatment group with 100 mg test item/kg artificial soil dw. Concerning the number of juveniles statistical analysis (Welch's t test, one-sided, smaller,  $\alpha = 0.05$ ) revealed statistically significant difference between control and the treatment groups with 10, 5.6 and 3.2 mg test item/kg soil dw in the second test run.

**Table B. 9.4.2.13-1: Effects on mortality and reproduction of *Folsomia candida* in a sub-chronic tests.**

Exposure (mg test item /kg soil dw)	Flufenacet-thiadone (BCS-AA41715)		
	Adult mortality (%)	Mean number of juveniles ( $\pm$ SD)	Reproduction (% of control)
<b>The first test run</b>			
Control	16.3	1132.6 ( $\pm$ 110.4)	-
100	100	-	0
<b>The second test run</b>			
Control	6.3	1196.1 ( $\pm$ 126.7)	-
10	20.0	690.8 ( $\pm$ 497.1)	57.7 <sup>ns*</sup>
5.6	25.0	954.5 ( $\pm$ 126.6)	79.8*
3.2	20.0	881.3 ( $\pm$ 95.0)	73.7*
1.8	10.0	1156.3 ( $\pm$ 77.4)	96.7 <sup>n.s.</sup>
1.0	2.5	1125.0 ( $\pm$ 93.6)	94.1 <sup>n.s.</sup>

SD Standard deviation

\* Statistically significant compared to control (Welch's t-test one-sided-smaller,  $\alpha = 0.05$ )

n.s. Statistically not significant compared to control (Welch's t-test one-sided-smaller,  $\alpha = 0.05$ )

n.s.\* Statistically not significant compared to control (Welch's t-test one-sided-smaller,  $\alpha = 0.05$ ) due to high SD in this treatment group . 42.3% difference to control confirms the effect on the reproduction of juveniles.

Toxic standard:

In the most recent study the EC<sub>50</sub> of 116 mg/ kg soil dw for the reference item Boric acid was determined. The NOEC<sub>reproduction</sub> was calculated to be 67 mg Boric acid/kg soil dw and the LOEC<sub>reproduction</sub> was 67 mg Boric acid/kg soil dw.

Conclusions:

NOEC<sub>reproduction</sub> = 1.8 mg test item/kg soil dw

LOEC<sub>reproduction</sub> = 3.2 mg test item/kg soil dw

In accordance with the new data requirement (Commission Regulation EU No 283/2013), the EC<sub>10</sub>, EC<sub>20</sub>, EC<sub>50</sub> values should be calculated. A valid ECx calculation was not possible due to the lack of a significant concentration/response function. As at the NOEC = 1.8 mg/kg the difference in number of juveniles compared to control is lower than 10% this endpoint should be appropriate for being used in the risk assessment. The non-valid EC10 calculation provided an EC10 of about 1.92, to 1.87, depending on the model used. The Applicant did not include them, as the fit is not significant and no significant concentration response functions could be calculated. EC10 would be higher than the NOEC, so, less critical. Therefore the NOEC can be considered in the risk assessment as a worst case approach

**Comments RMS:**

The Collembola reproduction study was conducted according to the OECD test guideline 232 (2009). Taking into account the validity criteria given in the test guideline OECD 232 (2009) the study is considered acceptable.

The mean mortality of the adults in the control was below 20 % (being 16.3% in 1<sup>st</sup> test and 6.3% in the 2<sup>nd</sup> test, respectively).

The mean number of juveniles per control replicate was greater than 100 (being 1132.6 per replicate in the 1<sup>st</sup> test and 1196.1 in the 2<sup>nd</sup> test). The coefficient of variation of reproduction in the control was <30 (being 9.7 % and 10.6% in the 1<sup>st</sup> and 2<sup>nd</sup> test runs, respectively).

The following deviation from recommendations given in the test guideline was noted:

-The amount of % calcium carbonate added to the tested artificial soil was missing in the study protocol.

Indicated deviation is, however considered as having no impact on the study results, since all validity criteria were met.

The study is thus considered acceptable.

**Agreed endpoints:**

NOEC<sub>reproduction</sub> = 1.8 mg test item/kg soil dw

LOEC<sub>reproduction</sub> = 3.2 mg test item/kg soil dw

**B.9.4.2.14. Flufenacet-thiadone (BCS-AA41715): Influence on mortality and reproduction on the soil mite species *Hypoaspis aculeifer* tested in artificial soil.**

<b>Reference:</b>	Flufenacet-thiadone (BCS-AA41715): Influence on mortality and reproduction on the soil mite species <i>Hypoaspis aculeifer</i> tested in artificial soil.
<b>Author(s), year:</b>	Kratz, M.A., 2012
<b>Report/Doc. number:</b>	Study No: E 428 4348-3, Reference BCS No: M-442897-01-1
<b>Guideline(s):</b>	OECD 226 (October 03, 2008)
<b>GLP:</b>	Yes

Material and methods:

Test substance:	Flufenacet-thiadone (BCS-AA41715) The first Test run: Batch code: AE 1258593-01-01, Purity: 98.6 %w/w. (analysed) The second test run and the third test runs: Batch code: AE 1258593-01-01; Purity: 98.6 %w/w (analysed)
Test species:	Predatory mites, <i>Hypoaspis aculeifer</i>
Number of organism:	<u>The first test run:</u> 8 replicates per control and each treatment groups, each with 10 individuals. <u>The second and the third test runs</u> 8 replicates per control and 4 replicates per treatment groups, each with 10 individuals 3 additional replicates for measurement of pH value and moisture of the soil for each test run.
Life stage, age:	Adult females
Type of test, duration	Laboratory sub-lethal limit test, 14 days
Applied concentrations:	<u>First test run:</u>
Nominal:	0 (contro, quartz sand), 100 mg test item/kg soil dw
	<u>The second test run:</u>
Nominal:	0 (control, quartz sand), 1.0, 1.8, 3.2, 5.6 and 10 mg test item/kg soil dw.
	The third test run:
Nominal:	0 (control, quartz sand), 1, 18, 32, 56 mg test item/kg soil dw. The test item was applied by mixing a test item-quartz sand-mixture into the artificial soil (all tested runs)
Toxic standard:	Dimethoate EC400 E G tested at concentrations of 0, 1.8, 3.2, 5.6 and 10.0 mg dimethoate/ kg soil dw.
Test substrate:	<u>The first test run:</u>

Artificial soil, 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 74.7 % fine quartz sand, 0.2% calcium carbonate (for the adjustment to pH to 6 ±0.5).

#### Second and third test runs

Artificial soil, 5 % sphagnum peat, air dried and finely ground, 20 % kaolin clay, 75 % fine quartz sand, 0.2% calcium carbonate (for the adjustment to pH to 6 ±0.5).

Substrate/Test vessel:

Test containers were glass vessels (volume 140 ml, diameter 5 cm at the bottom, height 7 cm). Each test vessel contained 20 g soil dw (height of artificial soil layer approximately 1.5 cm).

The test vessels were covered with glass lids to prevent the organism from escaping but allowing aeration during the test period.

Temperature

20±2 °C

Light regime:

#### The first test run:

16 hours light/8 hours dark. Light intensity: 648-706 Lux.

#### The second test run:

16 hours light/8 hours dark. Light intensity: 608-681 Lux.

#### The third test run:

16 hours light/8 hours dark. Light intensity: 590-616 Lux.

pH

#### The first test run:

Test start: 5.56-5.75

Test end: 5.74-5.75

pH

#### The second test run:

Test start: 5.84-5.94

Test end: 5.77-6.0

#### The third: test run:

Test start: 5.83-6.01

Test end: 5.68-6.16

Water content:

#### The first test run

Test start: 20.90-20.93% (equivalent to 46.57-46.66% of WHC)

Test end: 20.97-21.40% (equivalent to 46.75-47.99% of WHC)

#### The second test run

Test start: 21.16-22.20% (equivalent to 47.30-50.29% of WHC)

Test end: 19.88-22.98% (equivalent to 43.73-52.58% of WHC)

#### The third test run

Test start: 20.99-22.57% (equivalent to 46.81-51.36% of WHC)

Test end: 20.83-22.06% (equivalent to 46.37-48.87% of WHC)

---

Feeding:	Feeding was done before test and directly at start, 3, 7, and 10 days after test start. The predatory mites were fed cheese mites ( <i>Tyrophagus putrescentiae</i> ). Between 52 and 107 mg (1 <sup>st</sup> test run), 50 and 68 mg (2 <sup>nd</sup> test run) and 61-123 mg (3 <sup>rd</sup> test run) food per test vessel were added per feeding date.
Test parameters:	pH and water content were determined at test start and test end. Water content maintenance was checked weekly after application. Mortality of adults and number of juveniles were assessed after 14 days.
Statistic:	Determination of mean number of juveniles for control and treatment and the percent values for treatment in comparison to the untreated control were calculated. <u>1<sup>st</sup> test run:</u> Since no juveniles were extracted in the 1st test run no statistical calculations were performed. <u>2<sup>nd</sup> test run:</u> For the determination of normal distribution and homogeneity of variance Kolmogoroff-Smirnov Test and Cochran-Test ( $\alpha = 0.05$ ), respectively were used. Data of reproduction were normally distributed and homogeneity of variances was given. Therefore William's t-test for homogeneous variances (one-sided smaller, $\alpha = 0.05$ ) was used to determine NOEC and LOEC values. <u>3<sup>rd</sup> test run:</u> For the determination of normal distribution and homogeneity of variance Kolmogoroff-Smirnov Test and Cochran-Test ( $\alpha = 0.05$ ), respectively were used. Data of reproduction were normally distributed but homogeneity of variances was not given even after transformation. Therefore Welch-t test for Inhomogeneous Variances with Bonferroni-Holm Adjustment (one-sided smaller, $\alpha = 0.05$ ) was used to determine NOEC and LOEC values. The software used to perform the statistical analysis was ToxRat Pro 2.10 .
<u>Findings:</u>	The mortality of the adult <i>Hypoaspis aculeifer</i> in the control groups were 2.5 % (1 <sup>st</sup> run), 3.8 % (2 <sup>nd</sup> run) and 1.3 % (3 <sup>rd</sup> run), respectively. The highest mortality rate of 100 % was observed in the treatment groups with 56 ( 3 <sup>rd</sup> test run) and 100 mg test item/kg soil dw (1 <sup>st</sup> test run).  The LC <sub>50</sub> for adult mortality was 35 mg test item/kg soil dw. The confidence limits could not be determined due to mathematical reasons or inappropriate data. In the 1 <sup>st</sup> test run no NOEC could be determined and a 2 <sup>nd</sup> test run with lower concentrations was performed. Concerning the number of juveniles statistical analysis (William's t- test, one-sided smaller, $\alpha = 0.05$ ) revealed no significant difference between

control and any treatment group. Therefore NOEC for reproduction was estimated to be  $\geq 10$  mg test item/kg dry weight soil.

The 3<sup>rd</sup> test run with concentrations lower than the 1<sup>st</sup> test run and higher than the 2<sup>nd</sup> test run was performed. In this 3<sup>rd</sup> test run the highest test concentration of 56 mg/kg test item/kg soil dw was statistically significant concerning the number of juveniles where the test concentration of 32 mg/kg test item/kg soil dw revealed no statistically significant difference compared to the control (Welch t-test for inhomogeneous variances with Bonferroni-Holm adjustment, one-sided smaller,  $\alpha = 0.05$ ).

**Table 9.4.2-14-1: Effects on mortality and reproduction of *Hypoaspis aculeifer* in a sub-chronic test (the first run test).**

	Flufenacet-thiadone (BCS-AA41715)/ (mg test item/kg soil dw)	
Exposure	Control	100*
<b>The first test run</b>		
Mortality of adult [%] after 14 days	2.5	100
Mean number of offspring per test vessel after 14 days ( $\pm$ SD)	346.5 ( $\pm$ 23.5)	0
% reproduction compared to control		nd

nd not determined

**Table: 9.4.2-14-2: Effects on mortality and reproduction of *Hypoaspis aculeifer* in a sub-chronic test (the second run test).**

	Flufenacet-thiadone (BCS-AA41715) (mg pure metabolite/kg soil dw)					
Exposure	Control	1.0	1.8	3.2	5.6	10
<b>The second test run</b>						
Mortality of adult [%] after 14 days	3.8	7.5	7.5	7.5	0.0	2.5
Mean number of offspring per test vessel after 14 days ( $\pm$ SD)	367.8 ( $\pm$ 44.6)	402.5 ( $\pm$ 22.6)	385.0 ( $\pm$ 30.7)	360.5 ( $\pm$ 59.1)	382.0 ( $\pm$ 45.7)	418.5 ( $\pm$ 12)
% reproduction compared to control		109.4 <sup>ns</sup>	104.7 <sup>ns</sup>	98 <sup>ns</sup>	103.9 <sup>ns</sup>	113.8 <sup>ns</sup>

ns No statistically compared to control (Williams-t.-test one sided smaller;  $\alpha=0.05$ )

In the most recent test with the reference item Dimethoate EC400 E G , analysed in the separately report, tested at concentrations of 0, 1.8, 3.2, 5.6 and 10.0 mg dimethoate/ kg soil dw, a LC<sub>50</sub> of 3.894 mg a.s./kg for mortality of the adult mites and the NOEC<sub>reproduction</sub> of 3.2 mg a.s./ kg soil dw were determined. In addition, a EC<sub>50</sub> of 6.62 mg a.s./ kg soil dw for reproduction was found.

**Table 9.4.2-14-3: Effects on mortality and reproduction of Hypoaspis aculeifer in a sub-chronic test ( the third run test).**

	Flufenacet-thiadone (BCS-AA41715) (mg pure metabolite/kg soil dw)			
Exposure	Control	18	32	56
The third test run				
Mortality of adult [%] after 14 days (±SD)	1.3 (±0.5)	5.9	22.5	100*
Mean number of offspring per test vessel after 14 days (±SD)	346 (±24.2)	339.8 (±17.3)	245.3 (± 95.4)	4.5 (± 4.2)*
% reproduction compared to control		98.0	70.7	1.3*
LC <sub>10</sub> , 20, 50 <sup>1</sup> /EC <sub>10</sub> , 20, 50 <sup>1</sup>		Adult mortality		Reproduction (%)
LC <sub>10</sub> /EC <sub>10</sub>		30 mg pure metabolite /kg soil dw		28
LC <sub>20</sub> /EC <sub>20</sub>		32 mg pure metabolite/ kg soil dw		30
LC <sub>50</sub> /EC <sub>50</sub>		35 mg pure metabolite/kg soil dw		36
NOEC=32 mg pure metabolite /kg soil dw				
LOEC=56 mg pure metabolite /kg soil dw				

\* Statistically significant compared to control (Welch-t.-test one sided smaller;  $\alpha=0.05$ )

1 In reference to the confidence limits for LC<sub>50</sub> and EC<sub>50</sub> values could not be determined due to mathematical reasons or inappropriate data

The most recent test with the reference item Dimethoate EC400E G, analyzed in separately report tested at concentrations 1.0, 1.8, 3.2, 5.6 and 10.0 mg dimethoate/kg soil dw. dimethoate showed a LC<sub>50</sub> of 3.894 mg a.s./kg for mortality of the adult mites. The value of NOEC was estimated to be 3.2 mg a.s./kg and a EC<sub>50</sub> of 6.62 mg a.s./kg .

**Conclusions:**

NOEC<sub>(reproduction)</sub> = 32 mg test item/kg soil dw

LOEC<sub>(reproduction)</sub> = 56 mg test item/kg soil dw

LC<sub>10 (adult mortality)</sub> = 30 mg test item/kg soil dw

LC<sub>20 (adult mortality)</sub> = 32 mg test item/kg soil dw

LC<sub>50 (adult mortality)</sub> = 35 mg test item/kg soil dw

EC<sub>10 (reproduction)</sub> = 28 mg test item/kg soil dw

EC<sub>20 (reproduction)</sub> = 30 mg test item/kg soil dw

EC<sub>50 (reproduction)</sub> = 36 mg test item/kg soil dw

**Comments RMS:**

Predatory mite production study was conducted according to the OECD test guideline 226 (2008). Taking into account the validity criteria given in the OECD 226 (2008) test guideline the study is considered acceptable.

The mean mortality of the adults in the controls were below 20 % in the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> tests run (being 2.5, 3.8, 1.3%, respectively). The mean number of juveniles per controls replicate were greater than 50 in the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> tests run (being 346.5, 367.8 and 346.8 per replicate, respectively).

The coefficient of variation of reproduction in the controls were <30 in the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> tests run (being 6.8%, 12.1% and 7%, respectively).

**Agreed endpoints:**

NOEC<sub>reproduction</sub> = 32 mg test item /kg soil dw

LOEC<sub>reproduction</sub> = 56 mg test item /kg soil dw

LC<sub>10 (adult mortality)</sub> = 30 mg test item/kg soil dw

LC<sub>20 (adult mortality)</sub> = 32 mg test item /kg soil dw

LC<sub>50 (adult mortality)</sub> = 35 mg test item/kg soil dw

EC<sub>10 (reproduction)</sub> = 28 mg test item /kg soil dw

EC<sub>20 (reproduction)</sub> = 30 mg test item/kg soil dw

EC<sub>50 (reproduction)</sub> = 36 mg test item /kg soil dw

**For the risk assessment EC<sub>10</sub>, reproduction of 28 mg test item/kg sdw rather than NOEC reproduction should be used since ~ 30% effects were observed at 32 mg test item/kg sdw.**

**B.9.5. EFFECTS ON SOIL NITROGEN TRANSFORMATION**

In the first EU approval of the active substance flufenacet nitrogen and carbon mineralization studies were submitted addressing the risk to soil micro-flora. According to the EU data requirements for active substances (Regulation 283/2013) and plant protection products (Regulation 284/2013) the impact on soil microbial activity should be evaluated, in terms of nitrogen transformation. Hence, the available studies on micro-flora respiration (carbon transformation) are given as additional information only. Toxicity study on the effects of flufenacet on nitrogen transformation performed according to BBA: VI, I-I guideline (Anderson J.P.E, 1994) was reviewed during Annex I inclusion. The endpoints obtained from this study were expressed in terms of total nitrogen formation. In accordance with 216 OECD test guideline the results from the nitrogen transformation test should be expressed in terms of nitrogen rates. Therefore, the study was re-evaluated in this RAR according to current regulatory requirements. No unacceptable effects on nitrate formation rate were found at the highest tested dose level 4.13 mg a.s./kg s. dw. In addition, studies with the soil metabolites such as: FOE oxalate, FOE sulfonic acid-Na-salt, FOE methylsulfone, TFA, FOE 5043-trifluoroethane sulfonic acid and FOE-Thiadone were conducted addressing the risk to soil micro-flora. The one study on the effects of formulation DFF+FFA SC 600 on nitrogen transformation were also performed. The study summaries for studies with the active substance flufenacet and the soil metabolites are given in the Table B.9.5.1. The study summaries for the studies with representative formulation DFF+FFA SC 600 are given in RAR, Volume 3, B.9 (CP).

**Table B. 9.5-1: Effects of flufenacet and its soil metabolites on soil nitrogen transformation.**

Test item	Test design	Tested concentration	% effect on soil nitrogen transformation rate at 28 days after treatment compared to control	Reference
Flufenacet a.s.	silty sand soils, 28 d	0.83 mg s.a./kg dws (equiv. to 0.62 kg a.s./ha)	+3.20	Anderson J.P.E (1994) M-003871-01-2 Recalculated by BCS
		4.13 mg s.a./kg dws (equiv. to 3.10 kg a.s./ha)	-0.60	
FOE oxalate	1 soil, 28 d	2.48 mg p.m./kg dws (equiv. to 1.86 kg p.m./ha)	+8	Lechelt-Kunze, 2005 M-250511-01-1
FOE sulfonic acid-Na-salt	1 soil, 28 d	3.27 mg p.m./kg dws (equiv. to 2.455 kg a.s./ha)	-8	Lechelt-Kunze, 2005 M-250265-01-1
FOE methylsulfone	1 soil, 28 d	0.60 mg p.m./kg dws (equiv. to 0.451 kg p.m./ha)	+4	Lechelt-Kunze, 2005 M-398568-01-1
		6.01 mg p.m./kg dws (equiv. to 4.51 kg p.m./ha)	-5	
TFA	1 soil, 28 d	0.32 mg p.m./kg dws (equiv. to 0.24 p.m.kg a.s./ha)	+3.1	Frommholz, 2010 M-444423-01-1
		1.60 mg p.m./kg dws (equiv. to 1.2 kg p.m./ha)	+24.4	
FOE 5043-trifluoroethane sulfonic acid	1 soil, 28 d	0.164 mg p.m./kg dws (equiv. to 0.123 kg p.m./ha)	-2.3	Schulz, 2013 M-457331-01-1
		0.820 mg p.m./kg dws (equiv. to 0.615 kg p.m./ha)	+15.4	
FOE-Thiadone	1 soil, 28 d	0.149 mg p.m./kg dws (equiv. to 0.112 kg p.m./ha)	+19.3	Schulz, 2013 M-457326-01-1
		0.749 mg p.m./kg dws (equiv. to 0.562 kg p.m./ha)	-3.2	

**B.9.5.1. Influence of FOE 5043 on Microbial Mineralization of Nitrogen in Soils.**

<b>Reference:</b>	Influence of FOE 5043 on Microbial Mineralization of Nitrogen in Soils
<b>Author(s), year:</b>	Anderson J.P.E, 1994
<b>Report/Doc. number:</b>	Report No: E 337 0885-4; Reference BCS no. M-003871-01-2
<b>(original report).</b>	
<b>Guideline(s):</b>	Guidelines for the Official Testing of Plant Protectants, Part VI, 1-1, BBA Braunschweig, Germany, March 1990 (2nd ed.), ISO/DIS 1036-6:1992,
<b>GLP:</b>	Yes

The original study Anderson I.P.E., 1994 (M-003871-01-2) presents results in terms of nitrate-nitrogen levels relative to control at sampling dates. Therefore, the raw data from the study were evaluated the Applicant according to OECD 216 (2000) data requirements in terms of nitrate formation rates expressed in mg nitrate/kg dry weight soil/day. Mean values (MV) and standard deviations (SD) of nitrate formation rates are calculated per time interval. The new statistical calculation were performed. The results were submitted in the report presented below:

<b>Reference:</b>	Evaluation of nitrate formation rates on study M-003871-01-2 according to OECD 216.
<b>Author(s), year:</b>	Ernst G., Croder S., 2016
<b>Report/Doc. number:</b>	Amedmend to the original report M-003871-01-2
<b>Guideline(s):</b>	OECD 216

The summary of the study with consideration the new statistical calculations provided by Applicant is presented below:

**Material and methods:**

Test substance	FOE 5043 technical ingredient, Batch No: 898313105, purity: 96.80%, CAS No: 142459-58-3
Test species:	Soil microflora
Type of test, duration:	Nitrogen transformation test 28 days
Applied concentrations:	0 (control, quartz sand), 0.83 and 4.13 mg test item/kg soil dw, 3 replicates per control and treatment groups.  The test item was applied at an application rate equivalent to 0.62 kg s.a.item/ha and a five fold application rate of 3.10 kg s.a./ha.  Lucerne-grass-green meal was added to soils (5 g/kg soil dw) to stimulate nitrogen transformation.
Toxic standard	ARETIT FLUSSIG® (a.s. dinoseb acetate)  In tests with the 2 agricultural soils described the equivalents of 6 L/ha

---

	and 30 L/ha had distinct influences on the microbial activity for at least 91 days.
Test substrate:	<p>Two soil types were used in the study:</p> <p><b>Soil 1</b></p> <p>Agriculturally soil (removed to a depth of 20 cm), from a field located in Germany, Laacherhof.</p> <p>No application of and plant protection chemicals since 1981.</p> <p>C<sub>ORG</sub> 0.7 %</p> <p>Carbon content of microbial biomass: 215 mg microbial C/kg soil dw (corresponding to 3.07 % of C<sub>ORG</sub>)</p> <p>Total nitrogen content: 0.1%</p> <p>Texture according to DIN 11277: 3.6 % clay, 19.2 % silt, 56.2% sand</p> <p>Soil class: silty sand</p> <p><b>Soil 2</b></p> <p>Agriculturally soil removed to a depth of 20 cm, from a field located in Germany, Hohenseh. No application of fertilizers and plant protection chemicals since 1998 and 1988, respectively.</p> <p>C<sub>ORG</sub> 2.2%,</p> <p>Carbon content of microbial biomass: 1019 mg microbial C/kg soil dw (corresponding to 4.63 % of C<sub>ORG</sub>)</p> <p>Total nitrogen content: 0.2%</p> <p>0.5% lucerne grass meal</p> <p>Texture according to DIN 11277: 11.1 % clay, 83.7% silt, 5.2% sand</p> <p><b>Soil class: loamy silt</b></p>
Test procedure:	<p>Sieved soil (2 mm) was supplemented with either 10 g ground quartz sand/kg soil dw (control) or a mixture of quartz sand and FOE 5043 (0.83 mg or 4.13 mg/kg soil dw). The samples were mixed with pulverized lucerne-grass-green meal (5000 mg/kg soil dw) and quartz sand in 4 liter aluminium containers. After mixing, soil samples equivalent to 250 g soil dw were poured into 500 ml brown glass bottles and these were closed with parafilm.</p>
Incubation:	20±2°C, darkness
pH:	<p>Soil 1:</p> <p>Test start 6.4</p> <p>Test end:6.8</p> <p>Soil 2:</p> <p>Test start: 7.2</p> <p>Test end: 7.3</p>
WHC:	<p>Soil 1: 40%</p> <p>Soil 2: 41.3%</p>

---

Test parameters:	<p>The nitrogen transformation was determined on day 0 and at intervals of 14 and 28 days after application. Samples (10 g soil dw) were extracted with 50 mL 1M KCl by agitating for 60 minutes on a horizontal shaker at approximately 150 strokes/min. Extracts, which could not be analyzed immediately, were stored in the deep freezer at <math>-20\pm 5^{\circ}\text{C}</math>. For the quantitative determination of the mineralized part of nitrogen the Autoanalyzer was used.</p>
Statistic:	<p>The mean nitrogen-content, standard deviation were calculated for each treatment group and sampling date.</p> <p>A statistical evaluation of the test results of mean nitrogen content was performed by means of a 2-sided t-test (for at 5 % significance level).</p> <p>To fulfill OECD 216 data requirement the new statistical calculations based on raw data from the original study was performed by Applicant.</p> <p>Therefore, data were evaluated according to OECD 216 (2000) in terms of nitrate formation rates expressed in mg nitrate/kg dry weight soil/day. Mean values (MV) and standard deviations (SD) of nitrate formation rates are calculated per time interval. The nitrate formation rate is compared with that in the control, and the percent deviation from the control calculated.</p> <p>Homogeneity of variances was determined by Cochran's Test, <math>\alpha = 0.05</math>. Student-t Test and Welch-t Test were performed for homogeneous and non-homogeneous variances, respectively. In the T-tests, the values of mg Nitrogen/kg dry weight soil/time interval/day from control soils and treated soils were compared.</p>
<u>Findings:</u>	<p>During 28 day study, in two soils amended with lucerne-grass-green-mea (0.5% in soil), it was found that 0.62 kg FOE 5043 T/ha (equivalent to 0.83 mg a.s./kg soil dw) had no statistical significant effects on the turnover of nitrogen (expressed as nitro formation, mean value ) in either a silty sand or a loamy silt.</p> <p>At the end of the 28 day experiments, the 5-fold overdose of FOE 5043T (equivalent to 4.14 mg a.s./kg soil dw) had caused a slight (max. 8 %) stimulation of nitrate formation in both soils.</p> <p>Between Days 0 and 14, deviations in nitrate formation rate compared to the control of +1.92 and – 45.53% were found at 0.83 and 4.13 mg a.s./kg soil dw, in soil 1 (silty sand), respectively.</p> <p>However, between Days 14 and 28, the differences compared to the control were +3.20 and - 0.6 % at 0.83 and 4.13 mg a.s./kg soil dw, respectively, clearly less than 25 %.</p>

Simultaneously, the soil nitrate formation rate, between Days 0 and 14 compared of control of -64.79 and -67.79 % were found at 0.83 and 4.13 mg a.s./kg soil dw in the soil 2 (loamy silt).

However, between Days 14-28 the differences in nitrate formation rate between the control and treatment groups were clearly less than 25 %, and were -1.33% and +10.87% at 0.83 and 4.13 mg a.s./kg soil dw, respectively.

**Table B. 9.5.1-1: Effects of Flufenacet on nitrate formation-Nitrogen mean values.**

Days	NO <sub>3</sub> -Nitrogen (mg/kg soil dw) Mean values					
	Control		Flufenacet			
			0.83 mg a.s. /kg soil dw 0.62 kg a.s./ha		4.13 mg a.s./kg soil dw 3.10 kg a.s./ha	
	Nitrate-N Content (±SD)	Replicate Variation <sup>1</sup>	Nitrate-N Content (±SD)	% difference to control <sup>2</sup>	Nitrate-N content (±SD)	% difference to control <sup>2</sup>
<b>Soil 1</b>						
Day 0	26.34±0.07	0.27	26.10±0.48	-0.9	25.79±0.59	-2
Day 14	19.47±0.26	1.34	19.09±0.27	-2	22.04±0.68*	+13.2
Day 28	36.56±1.20	3.29	36.73±0.75	+4.77	39.03±0.36*	+6.8

<sup>1</sup> % variation within control replicates (coeff. of variation, calculated as standard deviation / mean value \* 100)

<sup>2</sup> % deviation to control; + = stimulating effect; - = inhibitory effect

\* significant difference between treated and untreated soil samples (t-test with 5 % probability level).

**Table B. 9.5.1-2: Effects of Flufenacet on nitrate formation-Nitrogen mean values.**

Days	NO <sub>3</sub> -Nitrogen ( mg/ kg soil dw) Mean values					
	Control		Flufenacet			
			0.83 mg a.s. /kg soil dw 0.62 kg a.s./ha		4.13 mg a.s./kg soil dw 3.10 kg a.s./ha	
	Nitrate-N Content (±SD)	Replicate Variation <sup>1</sup>	Nitrate-N content (±SD)	% difference to control <sup>2</sup>	Nitrate-N content (±SD)	% difference to control <sup>2</sup>
<b>Soil 2</b>						
Day 0	25.19±0.16	0.64	25.15±0.13	-0.15	25.99±1.54	+3.2
Day 14	26.23±0.50	1.90	27.14±0.11	+3.46*	28.14±0.64	+7.28*
Day 28	34.44±0.78	2.26	35.24±0.29	+2.32	37.25±0.45	+8.2*

<sup>1</sup> % variation within control replicates (coeff. of variation, calculated as standard deviation / mean value \* 100)

<sup>2</sup> % deviation to control; + = stimulating effect; - = inhibitory effect

\* Significant difference between treated and untreated soil samples (t-test with 5 % probability level).

**Table B. 9.5.1-3: Effects of Flufenacet on nitrate formation-Nitrogen formation rates.**

Time Interval (days)	NO <sub>3</sub> -Nitrogen- Nitrate formation Rate (mg/kg soil dw/time interval/d) <sup>1</sup>				
	Flufenacet				
	Control	0.83 mg a.s. /kg soil dw 0.62 kg a.s./ha		4.13 mg a.s./kg soil dw 3.10 kg a.s./ha	
	Nitrate-N <sup>1</sup>	Nitrate-N <sup>1</sup>	% difference to control <sup>2</sup>	Nitrate-N <sup>1</sup>	% difference to control <sup>2</sup>
<b>Soil 1</b>					
0-14	-0.49	-0.5	+1.92 <sup>ns</sup>	-0.27	<b>-45.53*</b>
14-28	1.22	1.26	+3.20	1.21	-0.60 <sup>ns</sup>
<b>Soil 2</b>					
0-14	0.40	0.14	-64.79 <sup>nsw</sup>	0.15	-67.79 <sup>nsw</sup>
14-28	0.59	0.58	-1.33	0.65	+10.87 <sup>ns</sup>

<sup>1</sup> Rate: Nitrate-N in mg/kg soil dw/time interval/day, mean of 3 replicates

<sup>2</sup> % Deviation to control; + = stimulating effect; - = inhibitory effect

\* Statistically significant difference to the control (Student-t Test, two-sided, = 0.05)

n.s. = No statistically significant difference to the control (Student-t Test, two-sided, = 0.05)

nsw No statistically significant difference to the control (Welch-t Test for non-homogeneous variances, two-sided,  $\alpha = 0.05$ );

Reference toxic standard ARETIT FLUSSIG® in tests with the 2 agricultural soils describe above, the equivalents of 6 L/ha and 30 L/ha distinct influences on the microbial activity for at least 91 days.

#### Conclusion:

Flufenacet caused no adverse effects (difference to control < 25 %) on the soil nitrogen transformation (expressed as NO<sub>3</sub>-N production) at the end of the 28-day incubation period up to and including the highest test concentration rate of 4.13 mg a.s./kg soil dw corresponding to 3.10 kg a.s./ha.

#### **Comments RMS:**

The nitrogen transformation test was conducted according to BBA, 1992 guideline.

For the first EU peer review of the active substance flufenacet the study was considered acceptable.

According to the current test guideline OECD test guideline 216 (2000) the study is considered valid if the coefficients variation in the control for NO<sub>3</sub>-N were ≤ 15%.

In this study, the highest coefficient of variation (CV) between nitrate-N concentration in replicate control samples were 3.29 % in the soil 1 (silty sand) and 2.26 in the soil 2 (loamy silt), respectively.

However, the following deviations from the recommendations given in the current test guideline OECD 216 were noted:

- The some soil substrate parameters such as: pH, water content and water holding capacity were missing from the study protocol.

-The % of sand in one of tested soil „loamy silt” is < 50% (should be not less than 50% and no higher than 70%)

In opinion of RMS the used test soil “**silty sand (soil 1)**” is considered acceptable, however, the second test soil “loamy silt ” (soil 2) is not considered acceptable according to the test guideline (OECD 216, 2000) considering the sand content of < 50% .

**Agreed endpoints:**

Effects on soil nitrogen transformation on day 28:

+3.20 % at 0.83 mg a.s./kg dw soil corresponding to 0.62 kg a.s./ha

-0.6 % at 4.13 mg a.s./kg dw soil corresponding to 3.10 kg a.s./ha

**(Soil type: Silty sand)**

**Studies on metabolites**

**B.9.5.2. Metabolite flufenacet-oxalate hydrate: determination of effects on nitrogen transformation in soil.**

<b>Reference:</b>	Metabolite flufenacet-oxalate hydrate: determination of effects on nitrogen transformation in soil.
<b>Author(s), year.</b>	Lechelt-Kunze, C., 2005
<b>Report/Doc. number:</b>	Report No: LKC-N-45/05, Reference BCS No. M-250511-01-1
<b>Guideline(s):</b>	OECD No. 216, Adopted: 21st January 2000
<b>GLP:</b>	Yes

**Material and methods:**

Test substance	Metabolite Flufenacet-oxalate hydrate, Batch No. : 921103ELB0, purity: 99 %
Test species:	Soil microflora
Type of test, duration	Nitrogen transformation test 28 days
<u>Applied concentrations:</u>	Nominal: 0 (control, quartz sand), 2.48 mg test substance/kg soil dw, 3 replicates per control and treatment group. This rate is equivalent to 1.86 kg metabolite - Flufenacet-oxalate hydrate/ha. Lucerne-grass-green meal was added to soil (5 g/kg soil dw) to stimulate nitrogen transformation.
Toxic standard	Sodium chloride at rate 16 g NaCl/kg soil dw had distinct and long-term (> 28 days) influences on microbial mineralization of nitrogen.
Test substrate	Agriculturally soil (loamy sand), removed to a depth of 20 cm, from a field located in Laacherhof, Germany. No application of fertilizers and plant protection products since 1996 and 1981 respectively.

	<p>C<sub>ORG</sub> 1.2 %,</p> <p>pH: 6.7.</p> <p>Carbon content of microbial biomass: 515 mg/kg soil dw (corresponding to 4.3% of C<sub>ORG</sub>)</p> <p>Total nitrogen content: 0.10%</p> <p>CEC ( meq/100 g): 5.9</p> <p>Texture according to DIN 11277: Loamy sand: 6.6 % clay, 19.8 % silt, 73.6 % sand 0.5% lucerne meal.</p>
Test procedure:	<p>Sieved soil (2 mm) was treated with either 10 g ground quartz sand/kg soil dw (control) or a mixture of quartz sand (10 g/kg soil dw) and Flufenacet-Sulfonic acid Na-salt (3.27 mg metabolite/kg soil dw). The samples were mixed with pulverized lucerne-grass-green meal (5 g/kg soil dw) and quartz sand in 3 liter polyethylene containers.</p> <p>After mixing, soil samples equivalent to 300 g dry weight were poured into 500 ml brown glass bottles and these were closed with parafilm.</p>
Incubation:	20 ±2°C, darkness
pH	<p>Test start: 5.8</p> <p>Test end: 6.0-6.1</p>
WHC:	40%
Test parameters:	<p>The nitrogen transformation was determined on day 0 and at intervals of 7, 14 and 28 days after application. Samples (10 g soil dw) were extracted with 50 mL 1M KCl, mixed on a rotator at 150 strokes/min for 60 minutes, filtered and stored frozen prior to analysis. For the quantitative determination of the mineralized part of nitrogen the Autoanalyzer was used.</p>
Statistic:	<p>Homogeneity of variances was determined by F-test (significance level 5%). Depending on the results of the F-tests, the appropriate T-tests were performed.</p> <p>The statistical calculations were carried out using Microsoft Excel 97 software according to the formula's of Sachs (Sachs, 1978).</p>
Findings:	<p>No adverse effects of on nitrogen transformation in soil could be observed at test concentrations (2.48 mg test item/kg soil dw) during the 28 day experiment.</p> <p>The soil nitrate formation rate deviated from the control by less than ±25% throughout the study. Over days 7 to 14 the highest difference compared to the control was observed: 19 % at 2.48 mg/kg soil dw; clearly below the critical value of 25%.</p>

**Table B.9.5.2-1: Nitrate formation -Nitrogen mean values and CV in the control.**

Days	NO <sub>3</sub> -Nitrogen (mg kg soil dw) mean values	
	Control	
	Nitrate-N Content (±SD)	Replicate Variation <sup>1</sup>
Day 0	19.88±0.30	1.5
Day 7	5.99± 0.13	2
Day 14	12.34±0.65	5
Day 28	25.49±0.14	5

<sup>1</sup> % variation within control replicates (coeff. of variation, calculated as standard deviation / mean value \* 100)

**Table B. 9.5.2-2: Effect of Flufenacet-oxalate hydrate on nitrate formation-nitrogen formation rates.**

Days	NO <sub>3</sub> – Nitrogen Formation Rate (mg/kg soil dw /time interval/day) <sup>1</sup>		
	Control	Flufenacet-oxalate hydrate	
		2.48 mg metabolite /kg soil dw (1.86 kg metabolite/ha)	
	Nitrate-N Formation (±SD)	Nitrate-N Formation (±SD)	% difference to control <sup>2</sup>
0-7	-1.98 ±0.06	-1.91 ±0.13	-4
7-14	0.91±0.08	0.74±0.11	-19
14-28	0.94± 0.04	1.01±0.05	+8

<sup>1</sup> Rate: Nitrate-N in mg/kg soil dw/time interval/day, mean of 3 replicates

<sup>2</sup> % deviation to control; + = stimulating effect; - = inhibitory effect

#### Conclusion:

Flufenacet-oxalate hydrate caused no adverse effects on microbial nitrification processes in soil (< ±25% deviation from the control) after 28 days incubation up to and including the highest test concentration 2.48 mg pure metabolite /kg soil dw.

**Comments RMS:**

The nitrogen transformation test was conducted according to the OECD test guideline 216 (2000).

According to the test guideline the study is considered valid if the coefficients variation in the control for NO<sub>3</sub>-N were ≤ 15%.

In this study, the highest coefficient of variation (CV) for NO<sub>3</sub>-N was 5%, hence the study is considered valid.

The following deviations from the recommendations given in the current test guideline were recorded:

- Only one concentration of test item was tested in this study (minimum two test concentrations are recommended in the test guideline).
- The data of water content and water holding capacity in soil substrate (before test), were missing from the study protocol
- The data of water content at start and at the end of test was missing from the study protocol.

However, 40% WHC was declared in the study during incubation.

Since validity criteria was met, this deviations are considered as having no impact on results of the study result.

**Agreed endpoints:**

Effects on soil nitrogen transformation on day 28:

+8 % at 2.48 mg metabolite/kg soil dw equivalent to 1.86 kg metabolite/ha

**B.9.5.3. Metabolite Flufenacet-Sulfonic acid Na-salt: Determination of effects on nitrogen transformation in soil.**

<b>Reference:</b>	Metabolite Flufenacet-Sulfonic acid Na-salt: Determination of effects on nitrogen transformation in soil.
<b>Author(s), year.</b>	Lechelt-Kunze, C., 2005.
<b>Report/Doc. number:</b>	Report No: LKC-N-41/05, Reference BCS no. M-250265-01-1
<b>Guideline(s):</b>	OECD No. 216, Adopted: 21 <sup>st</sup> January 2000
<b>GLP:</b>	Yes

Material and methods:

Test substance: Metabolite Flufenacet-Sulfonic acid Na-salt, Batch No: KTS9465-3-3, purity: 99.5%.

Test species: Soil microflora

Type of test, duration: Nitrogen transformation test 28 days

Applied concentrations:

---

Nominal:	<p>0 (control, quartz sand ), 3.27 mg test item/kg soil dw, 3 replicates per control and treatment group.</p> <p>This rate is equivalent to 2.455 kg metabolite Flufenacet-Sulfonic acid Na-salt/ha. Lucerne-grass-green meal was added to soil (5 g/kg soil dw) to stimulate nitrogen transformation.</p>
Toxic standard:	<p>Sodium chloride, tested in separate study, was used as a reference standard in the tests. In tests (non-GLP) with the agricultural soil described below, 16 g NaCl/kg soil dw had distinct and long-term (&gt; 28 days) influences on microbial mineralization of nitrogen.</p>
Test substrate:	<p>Agriculturally soil (loamy sand), removed to a depth of 20 cm, from a field located in Laacherhof, Germany.</p> <p>No application of fertilizers and plant protection products since 1996 and 1981 respectively.</p> <p>C<sub>ORG</sub> 1.2 %</p> <p>pH: 6.7</p> <p>Carbon content of microbial biomass: 515 mg/kg soil dw (corresponding to 4.3% of C<sub>ORG</sub>)</p> <p>Total nitrogen content: 0.10%</p> <p>CEC (meq/100 g dws): 5.9</p> <p>Texture according to DIN 11277( loamy sand) : 6.6 % clay, 19.8 % silt, 73.6 % sand 0.5% lucerne meal</p>
Test procedure:	<p>Sieved soil (2 mm) was treated with either 10 g ground quartz sand/kg soil dw (control) or a mixture of quartz sand (10 g/kg soil dw) and Flufenacet-Sulfonic acid Na-salt (3.27 mg metabolite/kg soil dw). The samples were mixed with pulverized lucerne-grass-green meal (5 g/kg soil dw) and quartz sand in 3 liter polyethylene containers.</p> <p>After mixing, soil samples equivalent to 300 g dry weight were poured into 500 ml brown glass bottles and these were closed with parafilm.</p>
Incubation:	20 ±2°C, darkness
pH:	<p>Test start: 5.8</p> <p>Test end: 6.0</p>
WHC:	40% during all incubation period
Test parameters:	<p>The nitrogen transformation was determined on day 0 and at intervals of 7, 14 and 28 days after application. Samples (10 g soil dw) were extracted with 50 mL 1M KCl, mixed on a rotator at 150 strokes/min for 60 minutes, filtered and stored frozen prior to analysis. For the quantitative determination of the mineralized part of nitrogen the Autoanalyzer was used.</p>
Statistic:	<p>Homogeneity of variances was determined by F-test (significance level 5%). Depending on the results of the F-tests, the appropriate T-tests were</p>

performed. The statistical calculations were carried out using Microsoft Excel 97 software according to the formulas of Sachs (Sachs, 1978).

#### Findings:

The highest difference between nitrate-N rates per day of control and the five-fold treated soil samples was 16 % (7-14 days after soil treatment, Tab.). Thus, in none of the time intervals analysed and in particular after 28 days the difference in the daily nitrate-N rates exceeds the trigger value of 25 %.

Effects of Flufenacet-Sulfonic acid Na-salt on nitrogen transformation after 28 days are shown in the tables below.

**Table B.9.5.3-1: Nitrate formation-Nitrogen mean values and CV in the control.**

Days	NO <sub>3</sub> -Nitrogen (mg/kg soil dw) Mean values	
	Nitrate-N Content (±SD)	Replicate Variation <sup>1</sup>
Day 0	20.04±0.15	1
Day 7	4.79 ± 0.76	16
Day 14	9.93±0.35	3
Day 28	25.12±0.81	3

<sup>1</sup> % variation within control replicates (coeff. of variation, calculated as standard deviation / mean value \* 100)

**Table B. 9.5.3-2: Effect of Flufenacet-Sulfonic acid Na-salt on nitrate formation – nitrogen formation rates.**

Days	NO <sub>3</sub> – Nitrogen Formation Rate (mg /kg soil dw /time interval/ day) <sup>1</sup>		
			Flufenacet-Sulfonic acid Na-salt
	Control		3.27 mg metabolite /kg soil dw (2.455 kg metabolite/ha)
	Nitrate-N <sup>1</sup> (±SD)	Nitrate-N <sup>1</sup> (±SD)	% difference to control <sup>2</sup>
<b>0-7</b>	-2.18 ±0.13	-2.34±0.03	8
<b>7-14</b>	0.73±0.15	0.85±0.16	16
<b>14-28</b>	1.09± 0.08	1±0.05	-8

<sup>1</sup> Rate: Nitrate-N in mg/kg soil dw/time interval/day, mean of 3 replicates

<sup>2</sup> % deviation to control; + = stimulating effect; - = inhibitory effect

#### Conclusion:

Flufenacet-Sulfonic acid Na-salt caused no adverse effects on microbial nitrification processes in soil (< ±25% deviation from the control ) after 28 days incubation up to and including the highest test concentration 3.27 mg test item/kg soil dw.

**Comments RMS:**

The nitrogen transformation test was conducted according to the OECD test guideline 216 (2000).

According to the test guideline the study is considered valid if the coefficient variation in the control for NO<sub>3</sub>-N were  $\leq 15\%$ .

In this study, the highest coefficient of variation (CV) between nitrate-N concentration in replicate control samples was 16 (7 days after treatment), which is slight above recommended in the protocol.

Taken into consideration that at day 28 the difference between control and treatment was <25% coefficient of variation was <15 (being 3%) the study was considered valid.

The deviation from the study protocol were as follows:

- Only one concentration of test item was tested in this study (minimum two test concentrations are recommended in the guideline).
- The data of water content and water holding capacity in soil substrate before the test were missing from the study protocol.
- The data of water content at start and the end of test was missing from the study protocol.

However, 40% of WHC was declared in the study during incubation period.

Since validity criteria was met, this deviation are considered as having no impact on results of the study result.

**Agreed endpoints:**

Effects on soil nitrogen transformation on day 28:

-8 % at 3.27 mg metabolite /kg soil dw per day, equivalent to 2.455 kg metabolite/ha

#### B 9.5.4. Metabolite flufenacet-methylsulfone (BCS-CO62475): Determination of effects on nitrogen transformation in soil.

<b>Reference:</b>	Metabolite flufenacet-methylsulfone (BCS-CO62475): Determination of effects on nitrogen transformation in soil.
<b>Author(s), year:</b>	Frommholz, U.; 2010
<b>Report/Doc. number:</b>	Report No: E 337 3982-5, Reference BCS no. M-398568-01-1
<b>Guideline(s):</b>	OECD No. 216, Adopted: 21st January 2000
<b>GLP:</b>	Yes

#### Material and methods:

Test substance	Metabolite flufenacet-methylsulfone (BCS-CO62475), purity: 97.6 % w/w, Batch code: BCS-CO62475-01-01
Test species:	Soil microflora
Type of test, duration:	Nitrogen transformation test 28 days
Applied concentrations:	0 (control, quartz sand), 0.60 and 6.01 mg test item/kg soil dw, 3 replicates per control and treatment groups.  The test item was applied as requested at an application rate equivalent to 0.451 kg test item/ha and a fivefold application rate of 4.51 kg test item/ha. Lucerne-grass-green meal was added to soil (5 g/kg soil dw) to stimulate nitrogen transformation.
Toxic standard	Sodium chloride, tested in separate study, was used as a reference standard in the tests. In tests (non-GLP) with the agricultural soil described below, 16 g NaCl/kg soil dw had distinct and long-term (> 28 days) influences on microbial mineralization of nitrogen.
Test substrate	Agriculturally soil (loamy sand), removed to a depth of 20 cm, from a field located in Germany/Rheinland-Pfalz/Offenbach  No application of fertilizers and plant protection products since 2006  C <sub>ORG</sub> 1.46 %, pH: 7.15  Carbon content of microbial biomass: 275 mg microbial C/kg soil dw (corresponding to 1.88% of C <sub>ORG</sub> )  Total nitrogen content: 0.135%  WHC: 37.55 g/100 g soil dw  CEC (meq/100 g dry weight soil): 12.9  Texture according to DIN 11277 (loamy sand): 10.3 % clay, 28.3 % silt, 61.4 % sand  0.5% lucerne grass meal
Test procedure:	900 g dry weight sieved soil (2 mm) was treated with either 10 g ground quartz sand/kg soil dw (control) or a mixture of quartz sand and metabolite

	flufenacet-methylsulfone (BCS-CO62475) -0.60 mg or 6.01 mg/kg soil dw. The samples were mixed with pulverized Lucerne-grass-green meal (5 g/kg dry weight soil) and quartz sand in 3 litre polyethylene containers. After mixing, soil samples equivalent to 300 g dry weight were poured into 500 mL brown glass bottles and these were closed with Para film.
Incubation:	20±2°C, darkness
pH:	Test start : 7.03-7.04 Test end: 7.25-7.28
Water content:	Test start: 13.35 – 13.78 g/100 g soil dw (corresponding to 41.04-42.57% of WHC) Test end: 13.76-14.39 g/100 g soil dw (corresponding to 42.49-44.75 of WHC)
Test parameters:	The nitrogen transformation was determined on day 0 and at intervals of 7, 14 and 28 days after application. Samples (10 g soil dw) were extracted with 50 mL 1M KCl by agitating for 60 minutes on a horizontal shaker at approximately 150 strokes/min. Extracts, which could not be analyzed immediately were stored frozen. For the quantitative determination of the mineralized part of nitrogen the Autoanalyzer was used.
Statistic:	Homogeneity of variances was determined by Cochran's Test, $\alpha = 0.05$ . Depending on the results the appropriate T-tests were performed. In the T-test, the values of nitrate-N/kg soil dw/time interval/day from control soils and treated soils were compared. The statistical calculations were carried out using ToxRatPro 2.09 (Ratte 2006).
<u>Findings:</u>	During the 28-day test, 0.60 mg flufenacet-methylsulfone (BCS-CO62475)/kg soil dw and the 10-fold dose of the test item 6.01 mg flufenacet-methylsulfone (BCS-CO62475)/kg soil dw had no relevant influence on nitrogen transformation. In none of the time intervals analysed during the 28 day exposure the difference in the daily nitrate-N rates exceeds the trigger value of 25 %.

Effects of flufenacet-methylsulfone (BCS-CO62475) on nitrogen transformation after 28 days are shown in the tables below.

**Table B.9.5.4-1. Nitrate formation -Nitrogen mean values and CV in the control.**

Days	NO3-Nitrogen ( mg/ kg soil dw) Mean values	
	Control	
	Nitrate-N Content (±SD)	Replicate Variation <sup>1</sup>
Day 0	7.97±0.23	3
Day 7	2.28±0.26	11
Day 14	14.81±0.48	3
Day 28	31.96±1.10	3

<sup>1</sup> % variation within control replicates (coeff. of variation, calculated as standard deviation / mean value \* 100)

**Table B. 9.5.4-2. Effects of flufenacet-methylsulfone (BCS-CO62475) on nitrate formation-nitrogen formation rates.**

Time Interval (days)	NO <sub>3</sub> -Nitrogen Formation Rate mg/kg soil dw/time interval/d) <sup>1</sup>				
	Control	Flufenacet-methylsulfone (BCS-CO62475).			
		0.60 mg metabolite/kg soil dw equivalent to 0.451 kg metabolite/ha		6.01 mg metabolite /kg soil dw equivalent to 4.51 kg metabolite /ha	
	Nitrate-N <sup>1</sup> (±SD)	Nitrate-N <sup>1</sup> (±SD)	% difference to control <sup>2</sup>	Nitrate-N <sup>1</sup> (±SD)	% difference to control <sup>2</sup>
0-7	-0.81±0.05	-0.77±0.05	-5 n.s.	-0.72±0.12	-11 n.s.
7-14	1.79±0.03	1.71±0.13	-5 n.s.	1.82±0.14	+2 n.s.
14-28	1.22±0.08	1.27±0.11	+4 n.s.	1.16±0.08	-5 n.s.

<sup>1</sup> Rate: Nitrate-N in mg/kg soil dw/time interval/day, mean of 3 replicates

<sup>2</sup> % deviation to control; + = stimulating effect; - = inhibitory effect

n.s. No statistically significant difference to the control (Student-t Test, two-sided,  $\alpha = 0.05$ ),

#### Conclusion:

Flufenacet-methylsulfone (BCS-CO62475) caused no adverse effects on microbial nitrification processes in soil (< ±25% deviation from the control ) after 28 days incubation up to and including the highest test concentration 6.01 mg test item/kg soil dw.

#### **Comments RMS:**

The nitrogen transformation test was conducted according to the OECD test guideline 216 (2000).

According to the test guideline the study is considered valid if the coefficient variation in the control for NO<sub>3</sub>-N were ≤ 15%.

In this study, the highest coefficient of variation (CV) between nitrate-N concentrations in replicate control samples was 11%. Hence, the study is considered valid.

#### **Agreed endpoint:**

Effects on soil nitrogen transformation on day 28:

+4 % at rate 0.6 mg metabolite/kg soil dw corresponding to 0.451 kg metabolite/ha

-5% at rate 6.01 mg metabolite/kg soil dw corresponding to 4.51 kg metabolite/ha

**B.9.5.5. Trifluoroacetic acid Na-salt (BCS-AZ56567): Effects on the activity of soil microflora (Nitrogen transformation test).**


---

<b>Reference:</b>	Trifluoroacetic acid Na-salt (BCS-AZ56567): Effects on the activity of soil microflora (Nitrogen transformation test).
<b>Author(s), year:</b>	Schulz L., 2013.
<b>Report/Doc. number:</b>	Report No: 12 10 48 080 N, Reference BCS no. M-444423-01-1
<b>Guideline(s):</b>	OECD No. 216, Adopted: 21st January 2000
<b>GLP:</b>	Yes

---

Material and methods:

Test substance:	Trifluoroacetic acid Na-salt, Substance code: AE 1046319, BCS-code: BCS-AZ56567, Batch code: AE 1046319-01-01, analysed purity: 95.1 % w/w sodium trifluoroacetate.
Test species:	Soil microflora
Type of test, duration:	Nitrogen transformation test 28 days
Applied concentrations:	0 (control, quartz sand), 0.32 and 1.60 mg test item/kg soil dw, 3 replicates per control and treatment groups.  The test item was applied as requested at an application rate equivalent to 0.24 kg test item/ha and a fivefold application rate of 1.20 kg test item/ha.
Toxic standard:	Dinoterb
Test substrate:	Agriculturally soil (loamy sand), removed to a depth of 20 cm, from a field located in Canitz, Germany.  No application of fertilizers and plant protection products since 2003 and 1990, respectively.  C <sub>ORG</sub> 1.45 % pH: 6.5 Humus content: 2.49 %, Carbon content of microbial biomass: 32.62 mg C/100 g dry weight soil (corresponding to 2.25% of C <sub>ORG</sub> ) Total nitrogen content: 0.16% WHC: 33.45 g/100 g soil dw Water content: 11.71 g/100 g soil dw CEC:11.2 (cmol <sup>+</sup> /kg soil) Texture according to DIN 11277: 10.7 % clay, 35.5 % silt, 53.9 % sand Soil class: sandy loam Texture according to USDA: 10.7% clay, 37.9 silt, 51.5% sand Soil class: loamy sand 0.5% lucerne meal

---

Test procedure:	<p>200 g soil dw (= one sub-sample) per test vessel was weighted. The soil was mixed with 0.5 % lucerne meal by means of a hand-stirrer (the C/N ratio of the lucerne meal was 15.6/1). One additional soil sample (without lucerne meal) was used for determination of the initial NO<sub>3</sub>-N content.</p> <p>Since the water solubility was not available, the test item was thoroughly mixed with quartz meal.</p> <p>Subsequently the obtained mixture was added and mixed with the soil by means of a hand stirrer. For an optimum distribution of the test item in the soil, the test item quartz meal mixture was applied at a ratio of about 10 g per kg soil dw.</p> <p>Water was added to the soil to achieve a water content of approximately 45 % of WHC. The incubation of the prepared soil was carried out in wide mouth glass flasks (500 mL). The screw caps of the flasks used permitted an air exchange.</p>
Incubation:	19.2-21°C, darkness
Water content:	<p>Test start: 15.60 – 16.07g/100 g soil dw (46.7-48.0 % of WHC)</p> <p>Test end: 15.33 – 15.79 g/100 g soil dw (45.83-47.21 % of WHC)</p>
pH:	<p>Test start 5.9</p> <p>Test end 6.3</p>
Test parameters:	<p>The nitrogen transformation was determined on day 0 (during three hours) and at intervals of 7, 14 and 28 days after application. Samples (10 g soil dw) were extracted with 50 mL 1M KCl, mixed on a rotator at 150 rpm for 60 minutes, and stored deep-frozen prior to analysis at -20±5 °C. For the quantitative determination of the mineralized part of nitrogen the Autoanalyzer was used.</p>
Statistic:	<p>The mean nitrogen-content, standard deviation and coefficient of variation were calculated for each treatment group and sampling date.</p> <p>Furthermore the nitrogen transformation rate per time interval and the nitrogen transformation rate/time interval/day were calculated for each treatment group.</p> <p>A statistical evaluation of the test results was performed by means of a 2-sided Student-t-test (for homogeneous variances at 5 % significance level).</p>
<u>Findings:</u>	<p>No adverse effects of trifluoroacetic acid Na-salt on nitrogen transformation in soil could be observed in both test concentrations (0.32 mg/kg soil dw and 1.60 mg/kg soil dw) after 28 days. Differences from the control of +3.1% (test concentration 0.32 mg/kg soil dw) and +24.2% (test concentration 1.60 mg/kg soil dw) were measured at the end of the 28-day incubation period (time interval 14-28).</p>

Effects of Trifluoroacetic acid Na-salt, on nitrogen transformation after 28 days are shown in the table below.

**Table B.9.5.5-1: Nitrate formation -Nitrogen mean values and CV in the control.**

Days	NO <sub>3</sub> -Nitrogen ( mg/ kg soil dw) Mean values	
	Control	
	Nitrate-N Content (±SD)	Replicate Variation <sup>1</sup>
Day 0	22.97±0.55	2.4
Day 7	35.47± 0.40	1.1
Day 14	41.10±0.87	2.1
Day 28	49.63±0.35	0.7

<sup>1</sup> % variation within control replicates (coeff. of variation, calculated as standard deviation / mean value \* 100)

**Table B. 9.5.5-2: Effects of Trifluoroacetic acid Na-salt on nitrate formation-nitrogen formation rates.**

Time Interval (days)	NO <sub>3</sub> -Nitrogen Formation Rate (mg/kg soil dw/time interval/d)				
	Control	Trifluoroacetic acid Na-salt.			
		0.32 mg metabolite/kg soil dw equivalent to 0.24 kg metabolite/ha		1.60 mg test item/kg soil dw equivalent to 1.20 kg metabolite/ha	
		Nitrate-N <sup>1)</sup>	% difference to control <sup>2</sup>	Nitrate-N <sup>1)</sup>	% difference to control <sup>2</sup>
0-7	1.79±0.10	1.62±0.06	-9.1 <sup>n.s.</sup>	1.76±0.48	-1.6 <sup>n.s.</sup>
7-14	0.80±0.11	0.85±0.02	+5.3 <sup>n.s.</sup>	0.70±0.35	-13.0 <sup>n.s.</sup>
14-28	0.61±0.08	0.63±0.15	+3.1 <sup>n.s.</sup>	0.76±0.04	+24.2 <sup>*s</sup>

<sup>1</sup> Rate: Nitrate-N in mg/kg soil dw/time interval/day, mean of 3 replicates

<sup>2</sup> % deviation to control; + = stimulating effect; - = inhibitory effect

<sup>n.s.</sup> No statistically significant difference to the control (Student-t-test for homogeneous variances, 2-sided,  $p \leq 0.05$ )

<sup>\*s</sup> Statistically significantly different to control (Student-t-test for homogeneous variances, 2-sided,  $p \leq 0.05$ )

In separate study, the toxic standard Dinoterb caused an effect of +40.4%, +68.1% and +83.5% (required  $\geq 25\%$ ) on the nitrogen transformation in a field soil at the tested concentrations of 6.80 mg, 16.00 mg and 27.00 mg Dinoterb per kg soil dw, respectively, 28 days after application and thus demonstrates the sensitivity of the test system.

#### Conclusion:

Trifluoroacetic acid Na-salt caused no adverse effects (difference to control < 25 %) on the soil nitrogen transformation at the end of the 28-day incubation period up to and including of the the highest test concentration 1.60 mg test item/kg soil dw.

**Comments RMS:**

The nitrogen transformation test was conducted to the OECD test guideline 216 (2000).

According to the test guideline the study is considered valid if the coefficient variation in the control for NO<sub>3</sub>-N were ≤ 15%.

In this study, the highest coefficient of variation (CV) between nitrate-N concentration in replicate control samples was 2.4%. Therefore, the study was considered valid.

**Agreed endpoints:**

Effects on soil nitrogen transformation on day 28:

+3.1% at 0.32 mg metabolite/kg soil dw per day, equivalent to 0.24 kg metabolite/ha

+24.2% at 1.60 mg metabolite/kg soil dw equivalent to 1.20 kg metabolite/ha

**B.9.5.6. Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474): Effects on the activity of soil microflora(Nitrogen transformation test).**

<b>Reference:</b>	Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474): Effects on the activity of soil microflora (Nitrogen transformation test).
<b>Author(s), year.</b>	Schulz, L.; 2013
<b>Report/Doc. number:</b>	Report No: 13 10 48 079 N, Reference BCS no. M-457331-01-1
<b>Guideline(s):</b>	OECD No. 216, Adopted: 21st January 2000
<b>GLP:</b>	Yes

**Material and methods:**

Test substance:	Flufenacet-trifluoroethanesulfonic acid Na-salt, BCS-code: BCS-CU62474, Batch code: BCS-CU62474-01-02, analysed purity: 98.4 % w/w sodium 2,2,2-trifluoroethanesulfonate.
Test species:	Soil microflora
Type of test, duration:	Nitrogen transformation test 28 days
Applied concentrations:	0 (control, quartz sand), 0.164 and 0.820 mg test item/kg soil dw, 3 replicates per control and treatment groups. The test item was applied as requested at an application rate equivalent to 0.123 kg/ha and a fivefold application rate of 0.615 kg/ha.
Toxic standard:	Dinoterb
Test substrate:	Agriculturally soil (loamy sand), removed to a depth of 20 cm, from a field located in Canitz, Germany. No application of fertilizers and plant protection products since 2003 and 1990, respectively. C <sub>ORG</sub> 1.38 % pH: 6.5

	Humus content: 2.37%, Carbon content of microbial biomass: 35.51 mg C/100 g dry weight soil (corresponding to 2.57% of C <sub>ORG</sub> ) Total nitrogen content: 0.15% WHC: 36.61 g/100 g soil dw Water content: 7.87 g/100 g soil d.w. CEC: 8.9 (cmol <sup>+</sup> /kg soil) Texture according to DIN 11277: 10.3 % clay, 35.4 % silt, 54.3 % sand Soil class: sandy loam Texture according to USDA: 10.3% clay, 36.8 silt, 52.9% sand Soil class: loamy sand 0.5% lucerne meal
Test procedure:	200 g soil dw (= one sub-sample) per test vessel was weighed. The soil was mixed with 0.5 % (i.e. 1.0 g/200 g soil d.w.) lucerne meal by means of a hand-stirrer (the C/N ratio of the lucerne meal was 13.2/1). One additional soil sample (without lucerne meal) was used for determination of the initial NO <sub>3</sub> -N content. Since the water solubility was not available, the test item was thoroughly mixed with quartz sand. Subsequently the obtained mixture was added and mixed with the soil by means of a hand stirrer. For an optimum distribution of the test item in the soil, the test item quartz sand mixture was applied at a ratio of about 10 g per kg soil dw. Water was added to the soil to achieve a water content of approximately 45 % of WHC . The incubation of the prepared soil was carried out in wide mouth glass flasks (500 mL). The screw caps of the flasks used permitted an air exchange.
Incubation:	20-21°C, darkness
Water content:	Test start: 17.48 – 18.25 g/100 g soil dw (47.8-49.9% of WHC) Test end: 17.14 – 17.94 g/100 g soil dw (46.83-49.0 % of WHC)
pH:	Test start: 6.3 Test end: 6.3-6.4
Test parameters:	The nitrogen transformation was determined on day 0 (during three hours) and at intervals of 7, 14 and 28 days after application. Samples (10 g soil dw) were extracted with 50 mL 1M KCl, mixed on a rotator at 150 rpm for 60 minutes, centrifuged and stored frozen prior to analysis at -20±5 °C. For the quantitative determination of the mineralized part of nitrogen the Autoanalyzer was used.

**Statistic:** The mean nitrogen-content, standard deviation and coefficient of variation were calculated for each treatment group and sampling date. Furthermore the nitrogen transformation rate per time interval and the nitrogen transformation rate/time interval/day were calculated for each treatment group.

A statistical evaluation of the test results was performed by means of a 2-sided Student-t-test (for homogeneous variances at 5 % significance level).

**Findings:** The test item flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474) caused a temporary inhibition of the daily nitrate rate at the tested concentration of 0.820 mg/kg soil dw at time interval 7-14 days after application.

However, no adverse effects of flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474) on nitrogen transformation in soil could be observed at both tested concentrations (0.164 mg and 0.820 mg test item/kg soil dw) at the end of the 28-day experiment. Differences from the control of -2.3 % (test concentration 0.164 mg/kg soil dw) and +15.4 % (test concentration 0.820 mg/kg soil dw) were measured at the end of the 28- day incubation period (time interval 14-28).

**Table B.9.5.6-1: Nitrate formation -Nitrogen mean values and CV in the control.**

Days	NO3-Nitrogen ( mg/ kg soil dw) Mean values	
	Control	
	Nitrate-N Content (±SD)	Replicate Variation <sup>1</sup>
Day 0	14.20±0.17	1.23
Day 7	42.60± 0.89	2.1
Day 14	52.03±1.07	2.1
Day 28	69.10±0.44	0.6

<sup>1</sup> % variation within control replicates (coeff. of variation, calculated as standard deviation / mean value \* 100)

**Table B.9.5.6-2: Effects of Trifluoroethanesulfonic acid Na-salt (BCS-CU62474) on nitrate formation-nitrogen formation rates.**

NO <sub>3</sub> -Nitrogen- Nitrate formation Rate (mg/kg soil dw/time interval/d) <sup>1</sup>					
Time Interval (days)	Control	Trifluoroethanesulfonic acid Na-salt (BCS-CU62474)			
		0.164 mg metabolite /kg soil dw equivalent to 0.123 kg metabolite/ ha		0.820 mg metabolite/kg soil dw equivalent to 0.615 kg meatbolite/ha	
	Nitrate-N <sup>1</sup>	Nitrate-N <sup>1</sup>	% difference to control <sup>2</sup>	Nitrate-N <sup>1</sup>	% difference to control <sup>2</sup>
0-7	4.06±0.12	3.72±0.14	-8.2 *s.	3.82±0.26	-5.8 n.s.
7-14	1.35±0.16	1.40±0.10	+4.2 n.s.	0.96±0.11	-29.0 *s.
14-28	1.22±0.09	1.19±0.13	-2.3 n.s.	1.41±0.08	+15.4 n.s.

1 Rate: Nitrate-N in mg/kg soil dw/time interval/day, mean of 3 replicates

2 % deviation to control; + = stimulating effect; - = inhibitory effect

n.s. No statistically significant difference to the control (Student-t-test for homogeneous variances, 2-sided,  $p \leq 0.05$ )

\*s. Statistically significantly different to control (Student-t-test for homogeneous variances, 2-sided,  $p \leq 0.05$ )

In the most recent test with the toxic standard, Dinoterb caused an effect of +33.7 % and +42.6 % (required  $\geq 25$  %) on the nitrogen transformation in a field soil at the tested concentrations of 16.00 mg and 27.00 mg Dinoterb per kg soil dw, respectively, 28 days after application and thus demonstrates the sensitivity of the test system.

#### Conclusions:

Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474) caused no adverse effects (difference to control  $< 25$  %) on the soil nitrogen transformation (expressed as NO<sub>3</sub>-N production) at the end of the 28-day incubation period up and including the highest test concentration 0.820 mg metabolite/kg soil dw.

#### **Comments RMS:**

The nitrogen transformation test was conducted to the OECD test guideline 216 (2000).

According to the test guideline the study is considered valid if the coefficient variation in the control for NO<sub>3</sub>-N were  $\leq 15\%$ .

In this study, the highest coefficient of variation (CV) between nitrate-N concentration in replicate control samples was 2.1 %. Hence, the study was considered valid.

#### **Agreed endpoints:**

Effects on soil nitrogen transformation on day 28 :

-2.3 % at rate 0.164 mg metabolite/kg soil dw corresponding to 0.123 kg metabolite/ha

+15.4% at rate 0.820 mg metabolite/kg soil dw corresponding to 0.615 kg metabolite/ha

**B.9.5.7. Flufenacet-thiadone (BCS-AA41715): Effects on the activity of soil microflora (Nitrogen transformation test).**

<b>Reference:</b>	Flufenacet-thiadone (BCS-AA41715): Effects on the activity of soil microflora (Nitrogen transformation test).
<b>Author(s), year:</b>	Schulz, N.; 2013
<b>Report/Doc. number:</b>	Report No: 13 10 48 078 N, Reference BCS no. M-457326-01-1
<b>Guideline(s):</b>	OECD No. 216, Adopted: 21st January 2000
<b>GLP:</b>	Yes

Material and methods:

Test substance	Flufenacet-thiadone, BCS-code: BCS-AA41715, Batch code: AE 1258593-01-01, analysed purity: 98.6 % w/w 5-(trifluoromethyl)-1,3,4-thiadiazol-2(3H)-one.
Test species:	Soil microflora
Type of test, duration:	Nitrogen transformation test 28 days
Applied concentrations:	0 (control, quartz sand), 0.149 and 0.749 mg test item/kg soil dw, 3 replicates per control and treatment groups. The test item was applied as requested at an application rate equivalent to 0.112 kg test item/ha and a fivefold application rate of 0.562 kg test item/ha.
Toxic standard	Dinoterb
Test substrate	Agriculturally soil (loamy sand), removed to a depth of 20 cm from a field located in Canitz, Germany. No application of fertilizers and plant protection products since 2003 and 1990, respectively. C <sub>ORG</sub> 1.38 %, pH: 6.5 Humus content: 2.37% Carbon content of microbial biomass: 35.51 mg C/100 g dry weight soil (corresponding to 2.57% of C <sub>ORG</sub> ) Total nitrogen content: 0.15% WHC: 36.61 g/100 g soil dw Water content: 7.87 g/100 g soil d.w. CEC:8.9 (cmol <sup>+</sup> /kg soil) Texture according to DIN 11277: 10.3 % clay, 35.4 % silt, 54.3 % sand Soil class: sandy loam Texture according to USDA: 10.3% clay, 36.8 silt, 52.9% sand Soil class: loamy sand 0.5% lucerne meal

Test procedure:	<p>200 g soil dw (=one sub-sample) per test vessel was weighed. The soil was mixed with 0.5 % lucerne meal by means of a hand-stirrer (the C/N ratio of the lucerne meal was 13.2/1). One additional soil sample (without lucerne meal) was used for determination of the initial NO<sub>3</sub>-N content.</p> <p>Since the water solubility was not available, the test item was thoroughly mixed with quartz sand.</p> <p>Subsequently the obtained mixture was added and mixed with the soil by means of a hand-stirrer. For an optimum distribution of the test item in the soil, the test item quartz sand mixture was applied at a ratio of about 10 g per kilogram soil dw. Water was added to the soil to achieve a water content of approximately 45 % of WHC.</p>
Incubation:	20-21°C, darkness
Water content	<p>Test start: 17.39 – 18.10 g/100 g soil dw (47.51-49.45 % of WHC)</p> <p>Test end: 17.06 – 17.76 g/100 g soil dw (46.61-48.52 % of WHC)</p>
pH	<p>Test start : 6.3</p> <p>Test end: 6.3-6.4</p>
Test parameters:	<p>The nitrogen transformation was determined on day 0 (during three hours) and at intervals of 7, 14 and 28 days after application. Samples (10 g soil dw) were extracted with 50 mL 1M KCl, mixed on a rotator at 150 rpm for 60 minutes, centrifuged and stored frozen prior to analysis at -20±5 °C. For the quantitative determination of the mineralized part of nitrogen the Autoanalyzer was used.</p>
Statistic:	<p>The mean nitrogen-content, standard deviation and coefficient of variation were calculated for each treatment group and sampling date.</p> <p>Furthermore the nitrogen transformation rate per time interval and the nitrogen transformation rate/time interval/day were calculated for each treatment group.</p> <p>A statistical evaluation of the test results was performed by means of a 2-sided Student-t-test (for homogeneous variances at 5 % significance level).</p>
Findings:	<p>The test item Flufenacet-thiadone (BCS-AA41715) caused a temporary inhibition of the daily nitrate rate at the tested concentration of 0.149 mg/kg at time interval 7-14 days after application.</p> <p>However, no adverse effects of Flufenacet-thiadone (BCS-AA41715) on nitrogen transformation in soil could be observed at both test concentrations (0.149 mg/kg soil dw and 0.749 mg/kg soil dw) at the end of the 28- day experiment.</p>

Differences from the control of +19.83 % (test concentration 0.149 mg/kg soil dw) and -3.2 % (test concentration 0.749 mg/kg soil dw) were measured at the end of the 28-day incubation period (time interval 14-28).

**Table B. 9.5.7-1: Nitrate formation -Nitrogen mean values and CV in the control.**

Days	NO <sub>3</sub> -Nitrogen Formation Rate ( mg/ kg soil dw) Mean values	
	Control	
	Nitrate-N Content (±SD)	Replicate Variation <sup>1</sup>
Day 0	13.73±0.38	2.8
Day 7	40.63± 0.32	0.8
Day 14	50.47±1.50	3
Day 28	67.37±0.90	1.3

<sup>1</sup> % variation within control replicates (coeff. of variation, calculated as standard deviation / mean value \* 100)

**Table B. 9.5.7-2: Effects of Flufenacet-thiadone (BCS-AA41715) on nitrate formation-Nitrogen formation rates.**

NO <sub>3</sub> -Nitrogen- Nitrate formation Rate mg/kg soil dw/time interval/d) <sup>1</sup>					
Time Interval (days)	Control	Flufenacet-thiadone (BCS-AA41715)			
		0.149 mg metabolite/kg soil dw equivalent to 0.112 kg metabolite/ha		0.749 mg metabolite/kg soil dw equivalent to 0.562 kg metabolite/ha	
	Nitrate-N <sup>1</sup> (±SD)	Nitrate-N <sup>1</sup> (±SD)	% difference to control <sup>2</sup>	Nitrate-N <sup>1</sup> (±SD)	% difference to control <sup>2</sup>
0-7	3.84±0.05	3.86±0.24	+0.52 <sup>n.s.</sup>	4.19±0.15	+9.1 <sup>*s.</sup>
7-14	1.40±0.20	1.03±0.08	-26.42 <sup>*s.</sup>	1.20±0.09	-14.6 <sup>n.s.</sup>
14-28	1.21±0.11	1.45±0.22	+19.83 <sup>n.s.</sup>	1.17±0.15	-3.2 <sup>n.s.</sup>

<sup>1</sup> Rate: Nitrate-N in mg/kg soil dw/time interval/day, mean of 3 replicates

<sup>2</sup> % deviation to control; + = stimulating effect; - = inhibitory effect

n.s. = No statistically significant difference to the control (Student-t-test for homogeneous variances, 2-sided, p ≤ 0.05)

\*s. = statistically significantly different to control (Student-t-test for homogeneous variances, 2-sided, p ≤ 0.05)

In the most recent test with the toxic standard Dinoterb caused an effect of +33.7 % and +42.6 % (required ≥ 25 %) on the nitrogen transformation in a field soil at the tested concentrations of 16.00 mg and 27.00 mg Dinoterb per kg soil dw, respectively, 28 days after application and thus demonstrates the sensitivity of the test system.

#### Conclusion:

Flufenacet-thiadone (BCS-AA41715) caused no adverse effects (difference to control < 25 %, OECD 216) on the soil nitrogen transformation (expressed as NO<sub>3</sub>-N production) at the end of the 28-day incubation period up and including the test concentrations of 0.149 mg and 0.749 mg metabolite/kg soil dw.

**Comments RMS:**

The nitrogen transformation test was conducted to the OECD test guideline 216 (2000).

According to the test guideline the study is considered valid if the coefficient variation in the control for NO<sub>3</sub>-N were ≤ 15%.

In this study, the highest coefficient of variation (CV) between nitrate-N concentration in replicate control samples was 3%. Therefore, the study was considered valid.

**Agreed endpoints:**

Effects on soil nitrogen transformation on day 28 :

+19.83% at rate 0.149 mg metabolite/kg soil dw corresponding to 0.112 kg metabolite/ha

- 3.2% at rate 0.749 mg metabolite/kg soil dw corresponding to 0.562 kg metabolite/ha

**B.9.5.8. Additional information:**Methods

*The effect of flufenacet (purity 97%) on soil micro-organism respiration was studied using two different soils Table 1.*

*Flufenacet was applied to the soils at two rates, and 0.83 mg a.s./kg soil and 4.31 mg a.s./kg dws corresponding to field application rates of 0.62 and 3.10 kg a.s./ha, respectively.*

*To determine the influence of the product on glucose stimulated soil respiration, triplicate, moist samples (equivalent to 25 g dws) were taken from each treatment on day 0 (within 3 hours after treatment), and after 14 and 28 days of incubation. The samples were mixed with enough glucose to induce maximum respiration rates and poured into plastic cylinders. The cylinders were connected to a gas analyzer and the quantities of carbon dioxide released per hour per kg dry wt soil were measured for at least 12 hours.*

**Table B.9.5.8-1: Soil characteristic**

Soil	Corg	pH	Sand (%)	Silt (%)	Clay (%)	WHC %
silty sand	0.7	6.4-6.8	56.2%	19.2	3.6	40
Loamy silt	2.2	7.2-7.3	5.2	83.7	11.1	41.3

Results:

No statistically significant treatment related effects were found at two tested rates of 0.83 and 4.31 mg a.s./kg soil dw.

**B.9.6. EFFECTS ON TERRESTRIAL NON-TARGET HIGHER PLANTS****B.9.6.1. Summary of screening data**

Not submitted .

**B.9.6.2. Testing on non-target plants**

For study summaries of flufenacet to non-target plants, see Vol. 3 CP B.9 for the formulated product

**B.9.7. EFFECTS ON OTHER TERRESTRIAL ORGANISM (FLORA AND FAUNA)**

---

<b>Reference</b>	Investigation of effects of trifluoroacetate on vernal pool ecosystems
Author(s):	<b>Benesch, J.A., Gustin, M.S., Cramer, G.R., Cahill, T.M. (2002)</b>
Source:	Environmental Toxicology and Chemistry, Vol. 21, No. 3, pp. 640 - 647, 2002
Report No:	M-455780-01-1
Guidelines:	Not stated
GLP:	Not stated

---

This study focused on assessing the impact of TFA on vernal pool soil microbial communities as well as vernal pool and wetland plant species. Microbial respiration for three vernal pool soils and an agricultural soil was not affected by TFA exposures (0, 10, 100, 1000, and 10000 µg/L), and degradation of TFA by microbial communities was not observed in soils incubated for three months. TFA accumulated in foliar tissue of wetland plant species as a function of root exposure concentration (100 and 1000 µg/L TFA), and accumulation was found to stabilize or decrease after the second or third month of exposure. Seeds accumulated TFA as a function of root exposure concentration; however, germination success was not affected. No adverse physiological responses, including general plant health and photosynthetic and conductance rates, were observed for root exposures at the TFA concentrations used in this study.

Based on the soils and plant species used in this study, predicted TFA concentrations will not adversely affect the development of soil microbial communities and vernal pool plant species.

**MATERIAL AND METHODS****A. Material**1. Test material

Test item:	NaTFA (CF <sub>3</sub> COO-Na)
Active substance(s):	See above
Adjuvant / Surfactant:	Not stated
Source of test item:	Sigma Chemical, St. Louis, MO, USA (chemicals supplier)
Lot/Batch number:	Lot 106H3462
Purity:	Not stated
Storage conditions:	

2. Test solutions

Vehicle/solvent:	Not stated
Source of vehicle/solvent:	Not stated

Concentration of vehicle/solvent: Not stated

### 3. Test organism(s)

Species:	<p><u>Microbial soil communities</u>: MOs from three natural vernal pool soil and one agricultural soil;</p> <p><u>Plants</u>: (1) TFA uptake via roots: <i>Polypogon monspeliensis</i> (annual beardgrass), <i>Deschampsia elongata</i> (vernal pool hairgrass), <i>Lasthenia californica</i> (small sunflower), <i>Oryza sativa</i> (rice, M-201); (2) Biomass experiment: <i>D. elongata</i>, <i>O. Sativa</i> and <i>P. monspeliensis</i>; (3) Germination experiments: <i>Eryngium vaseyi</i> (Coyote thistle), <i>Epilobium densiflora</i> (Fleshy owl's clover), <i>L. californica</i> and <i>D. elongata</i>.</p>
Cultivar:	Not stated except for rice (M-201)
Source of test species:	<p><u>Microbial soil communities</u>: natural soil collected from vernal pools on the properties of the Rancho Seco Power Plant and Beale Air Force Base near Sacramento (CA, USA); Red Rock Playa, Stead (NV, USA; and agricultural soil from the University of Nevada Agriculture Experiment Station, Reno (NV, USA)</p> <p><u>Plants</u>: S&amp;S Seed, Capenteria, CA, USA; Pacific Coast Seed, Livermore, CA, USA; University of California, Davis, CA, USA)</p>
Age of test organisms at study initiation / Crop growth stage at treatment:	<p><u>Microbial soil communities</u>: (1) Exposure experiments: TFA was added to MOs at the beginning of the experiment rather than after microbial respiration had established.</p> <p><u>Plants</u>: (1) TFA uptake via roots: plants were <math>1.25 \pm 0.25</math> cm in height; (2) Biomass experiment: plants were <math>1.5 \pm 0.5</math> cm in height; (3) Germination experiments: Seeds of several wetland plant species</p>
Holding conditions prior to test / Preparation before experiments:	<p><u>Microbial soil communities</u>: (1) Exposure experiments: Soils air-dried, homogenized, and sieved to 0.2 mm before test start; (2) Microbial degradation of TFA: no further preparation.</p> <p><u>Plants</u>: (1) TFA uptake via roots: 4 species germinated and grown in 0.25 Hoagland's solution (<math>\text{pH } 6.0 \pm 0.5</math>). Silicon was added (<math>10 \mu\text{mol/L Na}_2\text{SiO}_3</math>) to the solution; (2) Biomass experiment: (a) <i>Deschampsia</i> seeds germinated in rock wool immersed in aerated hydroponic solutions until plants were <math>1.5 \pm 0.5</math> cm in height; (b) <i>Oryza</i> and <i>Polypogon</i> seeds germinated in vermiculite until plants were <math>1.5 \pm 0.5</math> cm in height; (3) Germination experiments: (a) first-generation seeds (seeds obtained from 4 plant species that had not been grown in TFA-containing solution) no preparation needed before test start, (b) second-generation seeds of had developed from <i>Lasthenia</i> and <i>Oryza</i> plants growing in solutions of 0, 100 and 1000 <math>\mu\text{g/L}</math> TFA and accumulated TFA.</p>
Acclimatisation:	Not stated

## B. Study design and methods

### 1. Test procedure

- Test system (study type): Laboratory studies assessing effects of TFA on vernal pool soils microbial communities and vernal pool and wetland plant species
- Guideline deviation: Not stated
- Duration of study: See below (treatment)
- Treatments: Microbial soil communities:
- (1) Exposure experiments: Glass microcosms (250-ml volume), fitted with gastight lids containing a septa port for sampling headspace gas using a gastight syringe, were filled with 50 g of dry soil. Solutions with different TFA concentrations (see below) were added to achieve an 80% saturation level (by weight). Microcosm headspace analysis of carbon dioxide was conducted by collection of triplicate samples of 500 ml of microcosm air that was immediately injected into a CO<sub>2</sub> analyzer. Methods and procedures for this study are similar to those described by Walton et al. (1989) and Taylor et al. (1996). After headspace sampling, the microcosms were opened for 30 min and allowed to degas. This procedure was maintained for 25 d for the first experiment that utilized all soils. The procedure was repeated for the second experiment for 14 d but utilized only the agricultural, Beale, and Red Rock soils.
- (2) Microbial degradation of TFA: One-gram samples of each soil type were placed into glass vials ( $n = 72$  per soil type) and spiked in the same manner as the two studies described above with solutions containing different TFA concentrations (see below). Twenty-four vials were used for each exposure concentration for each soil type. Test conditions are described below. Vials were incubated in 10-gal aquaria with 3 L of distilled water to maintain a relative humidity of  $85 \pm 5\%$  and temperatures of  $23.5^{\circ}\text{C}$  for the first month and  $20 \pm 2.58^{\circ}\text{C}$  for the remaining two months. Six vials from each concentration and soil type were collected at zero, one, two, and three months and placed into a  $-20^{\circ}\text{C}$  freezer. Three vials containing MilliQ-filtered ultra-high-purity water, which were incubated in the aquaria, were collected at each sample time from each aquaria to verify that TFA contamination had not occurred. Ten vials of each soil exposure were frozen at the initiation of the experiment, and 10 vials containing MilliQ ultra-high-purity water were also collected at the beginning of the experiment.

Plants:

(1) TFA uptake via roots: Two hundred plants of *Deschampsia*, *Lasthenia*, and *Oryza* were germinated under  $60 \mu\text{mol}/\text{m}^2/\text{s}$  fluorescent lighting rockwool immersed in aerated hydroponic solutions of different TFA concentrations (see below). After seedlings were  $1.25 \pm 0.25$  cm tall (14 d), they were randomly placed into triplicate Rubber-maid plastic tubs (23 L) containing the same respective concentration of TFA so that each tub contained 25 plants of the three species. Hydroponic solutions were replaced weekly. Plants were then grown in a greenhouse ( $25 \pm 15^\circ\text{C}$ ) under  $175 \mu\text{mol}/\text{m}^2/\text{s}$  cool-white florescent lighting supplementing natural greenhouse lighting for a 14-h/d light cycle. Individual plants of *Oryza* and *Deschampsia* were sampled from each tub 21, 42, and 84 d after germination. After 150 d, dry seeds from *Oryza* were collected. *Lasthenia* plants and flowers were collected at 21 and 42 d after germination, and at 72 d seeds were collected. After 84 d, the photosynthetic and conductance rates for six plants of *Oryza* and *Deschampsia* in each tub were measured using a LI-COR 6400 Photosynthetic System.

(2) Biomass experiment: Plants with 1.5 to 0.5 cm height were randomly placed in tubs with aerated hydroponic solutions containing different TFA concentrations. After 57 d, height and total foliar biomass was determined for each plant. Biomass and leaf length were also monitored for *Oryza* and *Polypogon* exposed to different TFA concentrations (see below). Plants were germinated in vermiculite, and after 7 d, five 1.5 to 0.5-cm seedlings of each species were transferred into hydroponic systems containing Hoagland's solution amended with TFA. After two months, leaf length was measured, as was root and leaf biomass. For both experiments, solutions were replaced weekly, solution pH was maintained at  $5.55 \pm 0.20$ , and plants were grown under a 14-h/d light cycle ( $70 \mu\text{mole}/\text{m}^2/\text{s}$ ) as described previously.

(3) Germination experiments: One first-generation germination experiment. Fifty seeds of each species were placed atop pieces of rockwool in tubs (3.5 L) containing Hoagland's solution spiked with different TFA concentrations (see below). The number of germinated seeds was counted daily until .50% had germinated. Seeds were germinated under the same lighting conditions as the biomass experiments. These germination experiments were performed twice. The temperatures for the first and second germination experiments were  $25 \pm 4^\circ\text{C}$  and  $24.5 \pm 2.5^\circ\text{C}$ , respectively. An additional first-generation germination experiment was conducted using *Oryza*, *Lasthenia*, and *Deschampsia* seeds. This germination experiment followed the same protocol as the one described previously, except 200 seeds of each species were used. Second-generation germination

experiments utilized *Lasthenia* and *Oryza* seeds that had developed from plants growing in solutions of different TFA contractions (see below). Seeds were collected after they reached full development and foliar tissue had dried. Two hundred *Lasthenia* seeds from each exposure concentration were germinated in triplicate in solutions at the same concentration as the parent plants had been grown. Fifty *Oryza* seeds were germinated similarly. In addition, 50 *Oryza* and 200 *Lasthenia* second-generation seeds were germinated in Hoagland's solution containing no TFA. These experiments were replicated twice.

Test concentrations	<u>Microbial soil communities:</u> (1) Exposure experiments: 0, 10, 100, 1000 and 10000 µg/L TFA; (2) Microbial degradation of TFA: 0, 0.3 and 1.5 µg/L TFA. <u>Plants:</u> (1) TFA uptake via roots: 0, 100 and 1000 µg/L TFA; (2) Biomass experiment: (a) <i>Deschampsia</i> seedlings: 0 and 100 µg/L TFA; (b) <i>Oryza</i> and <i>Polypogon</i> seedlings: 0, 10, 100 and 1000 µg/L TFA; (3) Germination experiments: (a) first generation experiment: 0, 10, 100, 1000 and 10000 µg/L TFA; (b) second generation experiment: 0, 100 and 1000 µg/L TFA
Number of replicates:	See above (treatments)
Individuals per replicate:	See above (treatments)
Test conditions:	See above (treatments)
Test units (type and size):	See above (treatments)
Application / device / nozzles:	See above (treatments)
Water volume:	See above (treatments)
Calibration of sprayer:	Not stated

## 2. Environmental conditions

Test medium:	See above (treatments)
Temperature / relative humidity:	See above (treatments)
Photoperiod:	See above (treatments)
Lighting	See above (treatments)
pH:	See above (treatments)
Organic matter (C <sub>org</sub> ):	Not stated
CaCO <sub>3</sub>	Not stated
Cation exchange capacity:	Not stated
Soil textural fractions / extractable micronutrient concentrations [mg per kg soil]:	Not stated
Fertilization:	Not stated

## 3. Observations and measurements:

Analytical parameters measured:	Analysis of TFA in solutions, soil and plant tissues was done using the method by Cahill et al. (1999)
Biological parameters measured:	<u>Microbial soil communities:</u> Soil respiration; microbial degradation of TFA. <u>Plants:</u> Uptake of TFA via root; morphology and biomass development; photosynthetic and conductance rates; germination

	success.
Measurement frequency:	See above (treatments)
Statistical analyses:	Data were evaluated using analysis of variance techniques (one-way, two-way). For biomass experiments, one-way ANOVA and two-tailed <i>t</i> tests, assuming equal variance, were used to compare leaf length, leaf weight, and root weight of exposed plants in comparison to control plants. A one-way ANOVA was used to compare soil TFA concentrations as a function of time. Germination and microbial results were compared using two-way ANOVA.

## RESULTS

### 1. Validity criteria:

No test guideline and no validity criteria were stated in this study.

### 2. Other measurements:

Please refer to point 3 'Biological findings'. Measurement of other parameters was not reported.

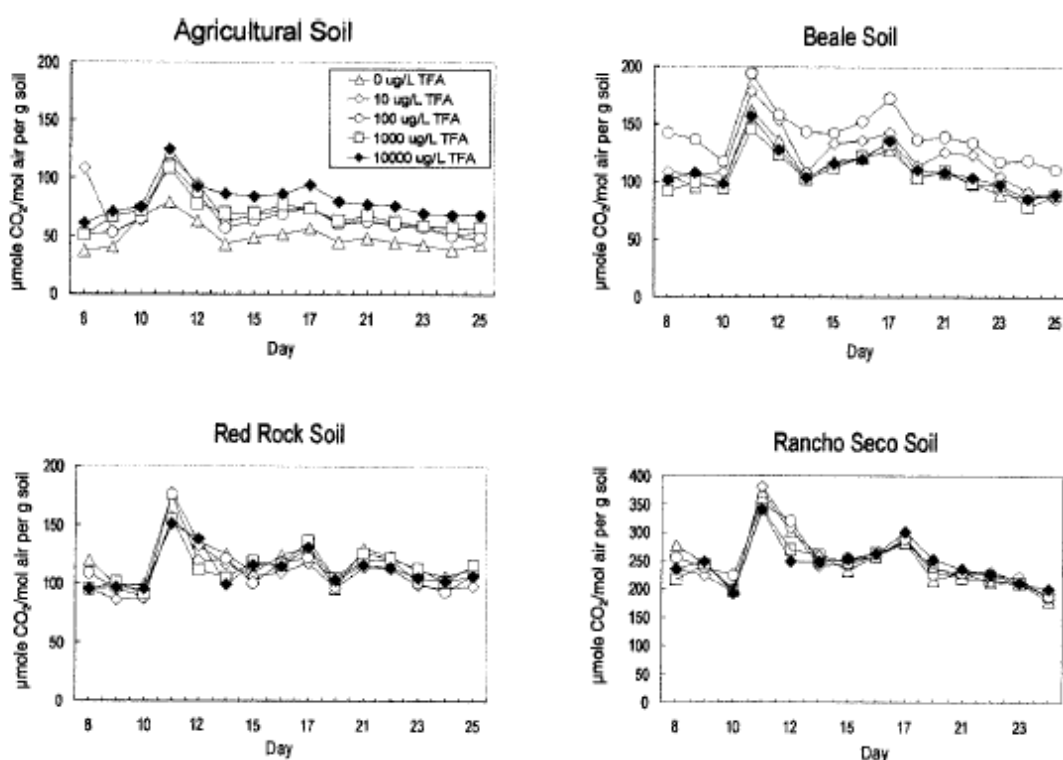
### 3. Biological findings:

#### **Microbial experiments:**

Respiration in microcosms containing vernal pool soils treated with TFA was not affected over time. Microbial respiration stabilized on approximately day 8, and respiration ranged between 75 and 300  $\mu\text{mole CO}_2/\text{mol air/g soil/d}$ . No significant difference was observed in the decline in respiration rates to day 8 as a function of TFA exposure concentration with time.

Vernal pool soils exhibited higher respiration rates ( $> 100 \mu\text{mole CO}_2/\text{mol air/g soil/d}$ ) than the agricultural soils ( $< 100 \mu\text{mole CO}_2/\text{mol air/g soil/d}$ ). No significant difference was observed in measured respiration as a function of TFA concentration for any of the soils exposed, except for the control agricultural soils that exhibited lower respiration than agricultural soil exposed to TFA. This experiment was replicated using agricultural, Beale, and Rancho Seco soils, and again no significant trends in respiration were observed as a function of exposure concentration.

In a further experiment, microbial degradation of TFA over a three-month time period was investigated. As a result, no significant difference was observed in the soil TFA concentrations at 0, 1, 2 and 3 months.



**Figure 1 (taken from Benesch et al., 2002):** Microbial respiration ( $\mu\text{mol CO}_2/\text{mol air/g soil}$ ) in microcosms amended with TFA as a function of time, starting with day 8, when respiration had stabilized. Each point represents a mean of three  $\text{CO}_2$  measurements for three replicate microcosms.

#### TFA uptake via roots

At TFA concentrations of 100 and 1,000  $\mu\text{g/L}$ , TFA taken up by plant roots was found to accumulate in foliar tissue as a function of concentration and time in the leaves of plants grown in aqueous medium. However, TFA concentrations in foliar tissue leveled off and/or declined with time.

Species	100- $\mu\text{g/L}$ TFA root exposure					1,000- $\mu\text{g/L}$ TFA root exposure				
	42 d	63 d	72 d	105 d	150 d	42 d	63 d	72 d	105 d	150 d
<i>Oryza</i> leaves	$26 \pm 5$ $n = 9$	$53 \pm 10$ $n = 9^*$		$56 \pm 9$ $n = 9$		$118 \pm 27$ $n = 9$	$289 \pm 92$ $n = 9^*$		$234 \pm 75$ $n = 9$	
<i>Oryza</i> seeds					$18 \pm 5$ $n = 9$					$17 \pm 3$ $n = 9$
<i>Lasthenia</i> leaves	$35 \pm 14$ $n = 18$	$75 \pm 19$ $n = 17^*$				$159 \pm 33$ $n = 9$	$295 \pm 50$ $n = 9^*$			
<i>Lasthenia</i> flowers	$12 \pm 2$ $n = 3$	$20 \pm 7$ $n = 3$				$81 \pm 27$ $n = 3$	$108 \pm 32$ $n = 3$			
<i>Lasthenia</i> seeds				$22 \pm 3$ $n = 9$				$17 \pm 2$ $n = 9$		
<i>Deschampsia</i> leaves	$27 \pm 7$ $n = 9$	$39 \pm 5$ $n = 9^*$		$30 \pm 7$ $n = 18^*$		$210 \pm 80$ $n = 9$	$171 \pm 52$ $n = 9$		$248 \pm 50$ $n = 9^*$	

**Table 1 (taken from Benesch et al., 2002):** Mean bioaccumulation factor ( $[\text{BCF}] = \mu\text{g trifluoroacetate [TFA]}/\text{g dry plant weight divided by } \mu\text{g TFA/g solution}$ ) values of *Oryza* leaves and seeds; *Lasthenia* leaves, flowers, and seeds; and *Deschampsia* leaves for the 100- and 1000- $\mu\text{g/L}$  exposures as a function of time. Data presented are mean  $\pm$  standard deviation of BCF value calculated for  $n$  plants. To convert BCF values to  $\mu\text{g TFA/g dry weight}$  for the 100- $\mu\text{g/L}$  exposure concentration, divide by a factor of 10. The BCF values listed for the 1000- $\mu\text{g/L}$  exposure are equivalent to  $\mu\text{g TFA/g dry-weight concentrations}$ . Asterisks indicate that data are statistically different ( $p < 0.05$ ) than prior sampling.

After 105 d, *Oryza* grown in 100  $\mu\text{g/L}$  TFA had accumulated  $5.6 \pm 0.9 \mu\text{g/g}$  TFA ( $n = 9$ ) in leaf tissue, whereas controls had  $< 0.05 \mu\text{g/g}$  TFA ( $n = 9$ ). After 63 d, leaf tissue of *Oryza* grown in 1000  $\mu\text{g/L}$  TFA exposure had

accumulated  $289 \pm 92$  µg/g TFA ( $n = 9$ ), and at 105 d concentration had declined by 19 % ( $p < 0.05$ ;  $234 \pm 75$  µg/g,  $n = 9$ ). *Deschampsia* also accumulated TFA as a function of exposure concentration; however, at 42, 63 and 105 d, foliar concentrations were roughly the same as reflected in the bioconcentration factors. The mean foliar concentration was  $3.0 \pm 0.7$  µg/g ( $n = 18$ ) for the 100-µg/L exposure and  $248 \pm 50$  µg/g ( $n = 9$ ) for the 1000-µg/L exposure at 105 d (controls contained  $< 0.02$  µg/g). *Lasthenia* plants did not live as long *Oryza* and *Deschampsia* and by day 63 had developed seeds and were beginning to die. After 42 d, their mean foliar concentration was  $7.5 \pm 1.9$  µg/g ( $n = 17$ ) for the 100-µg/L exposure and  $295 \pm 50$  µg/g ( $n = 9$ ) for the 1000-µg/L exposure (controls contained  $< 0.04$  µg/g). *Lasthenia* flowers also bioaccumulated TFA but to a lesser amount than the foliar tissue.

*Oryza* seeds accumulated  $1.8 \pm 0.5$  µg/g for the 100-µg/L exposure and  $17.6 \pm 3$  µg/g for the 1000-µg/L (controls contained  $< 0.07$  µg/g). *Lasthenia* seeds had TFA concentrations of  $2.2 \pm 0.3$  µg/g for the 100-µg/L exposure and  $17 \pm 2$  µg/g for the 1000-µg/L exposure (controls contained  $< 0.01$  µg/g). It is noteworthy that *Oryza* and *Lasthenia* seeds had similar TFA concentrations and bioconcentration factors despite the fact that they required different amounts of time to fully develop. No adverse physiological effects were observed for plants exposed to TFA concentrations as high as 1000 µg/L. Photosynthetic and conductance rates for exposed plants did not differ significantly ( $p < 0.05$ ) from the controls. Mean photosynthetic rates were  $19.0 \pm 3.6$  and  $11.1 \pm 4.2$  mmol CO<sub>2</sub>/mol air for *Oryza* and *Deschampsia*, respectively.

Mean conductance rates were  $0.4 \pm 0.2$  and  $0.20 \pm 0.09$  mol H<sub>2</sub>O/mol air for *Oryza* and *Deschampsia*, respectively. Photosynthetic rates reflect the plant's ability to fix CO<sub>2</sub>, and conductance rates reflect the plant's ability to transpire water.

### Biomass

After 57 d, *Deschampsia* exhibited no significant ( $p < 0.05$ ) difference in the plant height and biomass for the control versus the treatment plants (100 µg/L TFA). Leaf and root biomass and leaf length of *Polypogon* and *Oryza* harvested after two months of growth in 10-, 100-, and 1000-µg/L exposure concentrations were not significantly different from those plants grown in solutions containing no TFA with one exception. *Polypogon* exhibited a slight decline in leaf length with long-term exposure of 1000 µg/L TFA; however, no significant reduction was observed in development of biomass.

### Germination experiments

The first-generation germination experiments showed no significant effect at any TFA exposure concentration (100, 1000, and 10000 µg/L TFA) for *Eryngium* and *Epilobium*. In fact, *Eryngium* and *Epilobium* seeds exposed to solutions without TFA exhibited less germination success than those seeds exposed to TFA. In replication of this experiment, *Eryngium* and *Epilobium* seeds in control solutions exhibited better germination success for the first 9 d than seeds germinating in the 10000-µg/L TFA solution. *Lasthenia* seeds in control solutions exhibited significantly better germination success (~5-10 %) than seeds exposed to TFA in both replicate experiments. *Deschampsia* germination success was significantly better (~10-30 %) for the first 9 d for seeds in the 0- and 100-µg/L exposures than higher exposures in the first experiment.

However, in the duplicate experiment, *Deschampsia* seeds germinated in the 1000- and 10000-µg/L solutions exhibited greater success than the 0- and 100-µg/L exposures.

In the third first-generation germination experiment, which utilized 200 seeds of *Oryza sativa*, *Lasthenia californica* and *Deschampsia elongata*, both *Lasthenia* and *Deschampsia* seeds in 0-µg/L TFA solutions

exhibited significantly higher germination success than respective seeds in solutions containing TFA. For *Oryza*, no statistically significant difference was observed between success of seeds grown in the presence or absence of TFA.

Experiments in which *second-generation seeds* were germinated in solutions of the same concentration as the parent plants exhibited inconsistent results. In the first experiment, control seeds of *Lasthenia* exhibited significantly better germination success than seeds in the TFA-containing solutions. These results were not observed in the duplicate experiment, where the mean success for the three control exposures was not significantly different from germination success of seeds in the 1000-µg/L exposures.

The germination success of second-generation *Oryza* seeds in solutions of 0 µg/L TFA was less than for seeds exposed to TFA. In addition, *Lasthenia* and *Oryza* second-generation seeds germinated in solutions without TFA showed no significant difference in germination as a function of seed TFA concentration.

## RESULTS SUMMARY

Based on the results of this study investigating (a) vernal pool soil microbial communities with respect to soil respiration and (b) vernal pool and wetland plant species with respect to morphology and biomass development, photosynthetic and conductance rates, and germination success, no adverse effects as a consequence of environmentally relevant TFA exposures or even concentrations one order of magnitude higher need to be expected. In conclusion, it is unlikely that vernal pool microbial community and plant growth, development, and health will be impacted by the predicted TFA concentrations.

### Comments by the Notifier:

This study confirms the results from an existing study on effects of TFA on microbial nitrogen transformation. The microbial degradation is not affected due to the presence of TFA in soil. Thus, this study will not be further considered in the risk assessment.

### **RMS comments:**

The results of the study show that microbial respiration for three vernal pool soils and an agricultural soil was not affected by TFA exposures (0, 10, 100, 1000, and 10000 µg/L), and degradation of TFA by microbial communities was not observed in soils incubated for three months. However, the study is not standard regulatory study and the results can not be used in the regulatory assessment.

---

<b>Reference</b>	Effect of trifluoroacetate, a persistent degradation product of fluorinated hydrocarbons, on <i>Phaseolus vulgaris</i> and <i>Zea mays</i>
Author(s):	<b>Smit, M.F., van Heerden, P.D.R., Pienaar, J.J., Weissflog, L., Strasser, R.J., Krüger, G.H.J. (2009)</b>
Source:	Plant Physiology and Biochemistry 47 (2009) 623–634
Report No:	<u>M-455801-01-1</u>
Guidelines:	Not stated
GLP:	Not stated

---

## EXECUTIVE SUMMARY

The aim of this study was to quantify the effect of the pollutant, trifluoroacetate (TFA), on growth and photosynthesis of *Phaseolus vulgaris* (C3) and *Zea mays* (C4) in order to elucidate the physiological and biochemical basis of its inhibitory action. In whole plant studies, photosynthetic gas exchange, fast phase fluorescence kinetics and Rubisco activity were measured in parallel over a 14-day period in plants cultivated in a water culture system with NaTFA added at concentrations ranging from 0.625 to 160 mg L<sup>-1</sup>. Although initial stimulation of some photosynthetic parameters was observed at low TFA concentrations early on in the experiment, marked inhibition occurred at higher concentrations. In general *Z. mays* was affected more severely than *P. vulgaris* showing a large TFA-induced decrease in both apparent carboxylation efficiency and in vitro Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) activity. Analysis of photosynthetic gas exchange revealed that besides constraints on mesophyll processes such as Rubisco activity, stomatal limitation also increased with increasing TFA concentration, especially in *P. vulgaris*. In depth analysis of the fast phase fluorescence transients pointed at TFA-induced uncoupling of the oxygen evolving complex and inhibition of electron transport beyond Q<sub>a</sub> including possible constraints on the reduction of end electron acceptors of photosystem I.

## MATERIAL AND METHODS

### A. Material

#### 1. Test material

Test item:	Trifluoroacetate
Active substance(s):	See above
Chemical state and description:	Liquid
Source of test item:	Not stated
Batch number:	Not stated
Purity:	Not stated
Storage conditions:	Not stated
Water solubility:	Not stated

#### 2. Test solutions

Vehicle/solvent:	Not stated / not used
Source of vehicle/solvent:	See above
Concentration of vehicle/solvent:	See above

Method of preparation: See above  
 Evidence of unsolved material: See above

### 3. Test organism(s)

Species: *P. vulgaris* (genotype: Panthera); *Z. mays* (genotype: Jenny)  
 Common name: Not stated  
 Source of test species: Not stated

### 4. Culture conditions of test organism(s)

Culture medium: Hoagland's nutrient solution (pH 6.8)  
 Temperature: Unclear if culture conditions differ from test conditions (see below). Plants were cultured according to the method described in Hoagland & Arnon (1950)  
 Photoperiod: See above  
 Light intensity: See above  
 pH: See above  
 Oxygen saturation: See above

Food and feeding regime: Hoagland's nutrient solution was changed every 3 days  
 Acclimatisation prior to testing: A few days after germination in vermiculite plants were transferred to the water culture system, consisting of aerated opaque glass bottles filled with nutrient solution also used in the test  
 Observations during acclimatisation: Not stated

## **B. Study design and methods**

### 1. Test procedure

Test system: Laboratory test, water culture system  
 Test concentration(s): 0.625, 2.5, 10, 40 and 160 mg TFA L<sup>-1</sup>  
 Control(s): Water culture solution without test item  
 Number of replicates: 4 replicates per treatment group and control  
 Treatments / Test conditions: Experiments were carried out over a 14-day treatment period on plants grown in growth chambers under vigorously controlled conditions, i.e.: 15-h photoperiod and 26°C / 20°C day/night temperatures. The irradiance intensity of 1000 µmol m<sup>-2</sup> s<sup>-1</sup> at the level of the plant canopy in the chambers was provided by a combination of fluorescent (Sylvania Cool White VHO, 215 W) and incandescent (Sylvania, 100 W) lamps. The CO<sub>2</sub> concentration inside the chambers was controlled at 350 µmol mol<sup>-1</sup> by a built-in infrared gas analyser connected to CO<sub>2</sub> gas cylinders.  
 When the third leaves of *P. vulgaris* and *Z. mays* reached maturity chlorophyll a fluorescence, photosynthetic gas exchange and the chlorophyll content index were measured in these leaves. In addition, the plastochron index (in the case of *P. vulgaris*) of each plant was determined. Thereafter, NaTFA was applied to the water culture solution at different concentrations (see above). Measurements were taken 4, 8 and 12 days after application. Measurements throughout the experiment were done on the same mature leaves.  
 Feeding: Fresh nutrient solutions were applied on days 5 and 9

---

Medium renewal:	See above
Frequency of test item application:	NaTFA was applied at test start and on days 5 and 9 (together with the exchange of the nutrient solution)
Test duration:	14 day treatment period
Endpoints:	Measurement of plant development (plastochron index), biomass; CO <sub>2</sub> assimilation; determination of chlorophyll content index; measurements of oxygen evolution/consumption on isolated thylakoids, chlorophyll a fluorescence and rubisco activity [for details on methods, please refer to the study]
Statistics:	In data sets with parametric distribution, significant differences between treatment means were determined using Student's t-test.

## 2. Measurements during the test

Water/medium parameters:	Not stated. However, nutrient solution was exchanged on days 5 and 9.
--------------------------	---

## 3. Sampling

Sampling frequency:	Measurements were taken 4, 8 and 12 days after application.
Transport/storage of samples:	Not stated

## 4. Chemical analysis

Guideline/protocol:	Concentrations of the test item were not confirmed by appropriate analytical verification.
Method:	See above
Pre-treatment of samples:	See above
Conduction:	See above
Reference item:	See above
Recovery:	See above
Limit of detection:	See above
Limit of quantification:	See above

# RESULTS

## 1. Validity criteria:

Study was not conducted according to an official test guideline, e.g. OECD or EU guideline. No validity criteria were determined.

## 2. Analytical findings:

Concentrations of the test item were not confirmed by appropriate analytical verification. Nutrient solutions together with the test item were exchanged on days 5 and 9.

## 3. Other measurements:

Please refer to point 3 'Biological findings'. Measurement of other parameters was not reported.

## 4. Biological findings:

**Effects on plant growth:** From day 7 to 14 growth rates of *P. vulgaris* (measured by the plastochron index, in ΔPI units per day) declined with increasing concentration of TFA ranging from 0.625 to 160 mg NaTFA L<sup>-1</sup>. The respective growth rate reductions were 13 %, 12 %, 48 %, 48 % and 76 %. The reductions in growth at the 0.625 and the 2.5 mg L<sup>-1</sup> concentrations were not statistically significant. At the end of the treatment period significant

differences occurred in the final PI values corresponding to decreases of 11 %, 30 %, 27 % and 38 % for the NaTFA concentration of 2.5, 10, 40 and 160 mg L<sup>-1</sup>, respectively. *Z. mays* plants also displayed a reduction in plant height and growth rate with increasing TFA concentration. Due to their monocotyledonous growth form, however, no PI values could be measured.

TFA treated *Z. mays* plants displayed signs of increasing chlorosis and reduction in plant height with increasing TFA concentration. The visible chlorotic symptoms in *Z. mays* corresponded to the actual decreases in measured chlorophyll content index values ranging from 17 % to 70 % for the 0.625 – 160 mg L<sup>-1</sup> treatments, respectively. In contrast to *Z. mays*, in *P. vulgaris* no significant chlorosis occurred at any TFA level applied. Severe epinasty, wrinkling and necrosis of young *Z. mays* and *P. vulgaris* leaves were observed in the 40 and 160 mg L<sup>-1</sup> treatments. No visual symptoms were, however, observed on the mature leaves which were used for physiological measurements.

Observations of reduction in plant growth and development also correlated with the shoot and root biomass data: Shoot growth was stimulated (although not statistically significantly;  $p > 0.05$ ) at 0.625 and 2.5 mg L<sup>-1</sup> in *P. vulgaris*, but was significantly inhibited at all higher concentrations in both species. Since root growth was inhibited much more than shoot growth in both species, increased shoot:root ratios occurred. *Z. mays* however displayed a larger inhibition of root growth than *P. vulgaris*.

### **Inhibition of photosynthetic CO<sub>2</sub> assimilation by TFA**

*Inhibition of photosynthetic CO<sub>2</sub> assimilation:* The constraints imposed by TFA on photosynthetic gas exchange of the test plants were evaluated by analysis of CO<sub>2</sub> response curves, i.e. CO<sub>2</sub> assimilation rate plotted vs. intercellular CO<sub>2</sub> concentration response curves. The data of the study revealed that *P. vulgaris* and *Z. mays* responded differently to TFA treatment. The initial slope of the demand function, which is a measure of the apparent carboxylation efficiency, was much more effected in *Z. mays* (69 % decrease at the 160 mg L<sup>-1</sup> concentration) than in *P. vulgaris*. On the other hand the supply function, which is related to the stomatal conductance, was inhibited more in *P. vulgaris* (58 % decrease) at the 160 mg L<sup>-1</sup> concentration than in *Z. mays* (43 % decrease). Early on, after 4 days of treatment at 0.625 mg L<sup>-1</sup>, an increase of 55 % in stomatal conductance was apparent in *P. vulgaris*.

This initial increase in stomatal conductance however soon gave way to a decrease in stomatal conductance at all TFA concentrations. In *Z. mays*, a C<sub>4</sub> plant, J<sub>max</sub> which is determined by either Rubisco activity, PEP regeneration capacity or photosynthetic electron transport rate, was already reached at a C<sub>i</sub> value of below 500 mmol mol<sup>-1</sup>, a phenomenon typical of C<sub>4</sub> plants. A very pronounced decrease in J<sub>max</sub> of up to 33 % at the highest TFA concentration occurred. In *P. vulgaris*, the corresponding TFA induced changes in J<sub>max</sub>, which is an indicator of RuBP regeneration capacity, were much less pronounced, showing only a 19 % decrease at the highest concentration. From the calculated intercellular CO<sub>2</sub> concentration (C<sub>i</sub>) values, corresponding to the respective actual CO<sub>2</sub> assimilation rate, it was evident that in the case of *Z. mays*, C<sub>i</sub> almost remained constant, while in the case of *P. vulgaris*, C<sub>i</sub> decreased with increasing TFA concentration.

*Inhibition of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity:* In *P. vulgaris* statistically non-significant decreases in total Rubisco activity, calculated on a leaf area basis, namely 8 %, 14 %, 29 %, 27 % and 15 % occurred at the 0.625, 2.5, 10, 40 and 160 mg L<sup>-1</sup> NaTFA treatments respectively. In *Z. mays* on the other hand decreases of 20 %, 8 %, 32 %, 52 % and 46 % were observed at the corresponding concentrations. Since the initial Rubisco activity changed in parallel with total Rubisco activity in both *P. vulgaris* and *Z. mays*, no significant change in Rubisco activation state occurred.

**Inhibition of photosynthetic electron transport in thylakoids of *P. vulgaris***

TFA had marked concentration dependent effects on the electron transport of isolated thylakoid membranes in the system,  $\text{H}_2\text{O} - \text{PSII} - \text{FeCy}$ . In this case, the oxygen evolution rate was used as measure of electron transport rate. At the lowest TFA treatment of  $0.00005 \text{ mmol L}^{-1}$ , a significant stimulation of 9 % occurred in the oxygen evolution rate, while a significant decrease ranging from 10 % to 52 % occurred at increasing concentrations ranging from 0.005 to  $100 \text{ mmol L}^{-1}$  respectively. TFA also had marked concentration dependent effects on electron transport of isolated thylakoid membranes in the system,  $\text{DCPIP/Asc} - \text{PSI} - \text{MV/Na}_3\text{N}$ . In this case the oxygen consumption rate was used as measure of electron transport rate. At the lowest TFA treatment of  $0.0001 \text{ mmol L}^{-1}$  no significant inhibition occurred in oxygen consumption rate, while a significant decrease ranging from 1 % to 33 % occurred at concentrations ranging from 0.001 to  $100 \text{ mmol L}^{-1}$  respectively.

**Inhibition of PSII function and photosynthetic electron transport in vivo**

Analysis of the recorded chlorophyll a fluorescence transients showed that TFA-concentration dependant changes occurred in both the specific (per reaction centre) and the phenomenological (per cross-section) energy fluxes through PSII in both *P. vulgaris* and *Z. mays*. In *P. vulgaris* significant decreases occurred in the electron transport per cross-section of 2 %, 3 % and 12 % as well as concurrent decreases in density of reaction centres of 3 %, 6 % and 7 % at the 10, 40 and  $160 \text{ mg L}^{-1}$  concentrations, respectively. Concomitantly significant increases occurred in “antenna size” of 5 %, 9 %, 16 % and 13 % as well as decreases of 4 %, 3 %, 14 % and 10 % in the specific trapping flux from the 2.5 to the  $160 \text{ mg L}^{-1}$  concentration, respectively.

Also *Z. mays* displayed significant decreases in the electron transport per cross-section namely 8 %, 11 % and 15 % with concomitant decreases of 11 %, 12 % and 8 % in density of reaction centres at the 10, 40 and  $160 \text{ mg L}^{-1}$  concentrations, respectively. Concurrently a significant increase in “antenna size” of 10 %, 15 % and 17 % and an increase of 8 %, 12 % and 14 % in the specific trapping flux occurred for the 10, 40 and  $160 \text{ mg L}^{-1}$  concentrations.

The performance index calculated on an absorption basis ( $\text{PI}_{\text{total}}$ ) was found to be a very sensitive parameter for quantification of TFA-effects in both *P. vulgaris* and *Z. mays*. For *P. vulgaris*, changes in  $\text{PI}_{\text{total}}$  after 12 days of treatment corresponded well to the corresponding gas exchange data. The  $\text{PI}_{\text{total}}$  of treated *P. vulgaris* plants decreased significantly between 10 % and 35 % for concentrations ranging from 0.625 to  $160 \text{ mg L}^{-1}$  respectively.

The individual effect on the component parameters of  $\text{PI}_{\text{total}}$  was as follows: the efficiency of absorption of light decreased significantly by 7 %, 15 % and 11 % in the range 10– $160 \text{ mg L}^{-1}$  respectively; the performance due to the quantum efficiency of primary photochemistry decreased significantly by 2 %, 7 %, 8 % and 13 % from the 2.5 to the  $160 \text{ mg L}^{-1}$  concentrations; the performance due to the quantum efficiency of the conversion of excitation energy to electron transport decrease by 7 %, 6 %, 13 %, 22 % and 30 % from the 0.625 to the  $160 \text{ mg L}^{-1}$  treatment; the performance due to the quantum efficiency of the reduction of end acceptors decreased by 11 % at the  $10 \text{ mg L}^{-1}$  concentration. At the 40 –  $160 \text{ mg L}^{-1}$  concentrations, it showed a significant increase of 10 % and 19 % respectively.

In *Z. mays* the  $\text{PI}_{\text{total}}$  decreased significantly by between 6% and 48 % from the 0.625 to the  $160 \text{ mg L}^{-1}$  concentrations respectively. The effect on the component parameters of  $\text{PI}_{\text{total}}$  was as follows: the efficiency of light absorption decreased significantly by 9 %, 13 % and 14 % from the 10 to the  $160 \text{ mg L}^{-1}$  concentrations respectively; the performance due to the quantum efficiency of primary photochemistry displayed a significant decrease of 9 %, 13 % and 10 % from the 10 to the  $160 \text{ mg L}^{-1}$  concentrations; the performance due to the quantum efficiency of the conversion of excitation energy to electron transport displayed a decrease of 10 %, 20

% and 30 % from the 10 to the 160 mg L<sup>-1</sup> concentrations; the performance due to the quantum efficiency of the reduction of end acceptors decreased by 8 % at the 0.625 mg L<sup>-1</sup> concentration and showed a maximum decrease of 18 % at the 40 mg L<sup>-1</sup> concentration.

## RESULTS SUMMARY

This study reported on adverse effects on growth as well as the physiological and biochemical basis of the inhibition of photosynthesis in *P. vulgaris* and *Z. mays* plants which were induced by NaTFA applied to growth medium (water culture system instead of soil culture system). However, TFA levels tested in this study are much greater (by orders of magnitude) than the levels currently found in the environment.

### Comments by the Notifier:

This study reports physiological effects of TFA in two plant species. These endpoints are not comparable to endpoints obtained from tests with non-target plants (i.e. emergence, survival, biomass). Thus, this study will not be further considered in the risk assessment.

### **RMS comments:**

The treatments are given in mg NaTFA/L in hydroculture systems, such endpoint cannot be used in the regulatory assessment.

\*\*\*\*\*

<b>Report:</b>	<b>KCA 8.7/01; Oehrle, N.WGreen, L.S., Karr, D.B., Emerich, D.W. (2004)</b>
Title:	The HFC/HCFC breakdown product trifluoroacetic acid (TFA) and its effects on the symbiosis between <i>Bradyrhizobium japonicum</i> and soybean ( <i>Glycine max</i> )
Source:	Soil Biology & Biochemistry 36 (2004) 333–342
DOI No:	doi:10.1016/j.soilbio.2003.10.00
Document No:	M-455785-01-1
Guidelines:	Not stated
GLP:	Not stated

## EXECUTIVE SUMMARY

The study was performed in accordance with the Alternative Fluorocarbon Environmental Acceptability Study (AFEAS). Those results are presented in addition to the findings of further experimentation on the initial interaction of *B. japonicum* with soybean. Three levels of TFA (0.67, 6.74 and 67.40 µL TFA kg<sup>-1</sup> soil; 0.003, 0.031 and 0.314 µL TFA L<sup>-1</sup>) were used for soil and hydroponics conditions and three levels (10, 100 µM and 1 mM) in bacterial culture. The results demonstrate that TFA affects growth of *B. japonicum* significantly, but does not affect PHB accumulation. Also no F<sup>-</sup> was found in cultures grown on TFA. Attachment of *B. japonicum* to soybean roots was enhanced with the lowest level of acetate or TFA and was significantly reduced with 1 mM acetate or TFA. Cultures grown on acetate or acetate with TFA do not attach well, with those grown with 1 mM TFA the least. Both effects may be attributed to pH. Soybean seedlings had significantly retarded development with levels of TFA at or above 6.74 µL TFA kg<sup>-1</sup> soil and 0.031 µL TFA L<sup>-1</sup> nutrient solution. No nodules formed on those plants treated with these levels of TFA except in the hydroponics trials. Nodule location was not

affected regardless of the TFA level. At the lowest level used we found no effects on soybean or symbiotic nitrogen fixation. In some cases, nodulation was enhanced, but nodule weight reduced. Anaerobically isolated bacteroids had normal levels of acetylene reduction activity regardless of the level of TFA used.

In summary, soybean is much more sensitive to low levels of TFA than its symbiotic counterpart *B. japonicum*. No detrimental effects on symbiotic nitrogen fixation in soybean should be expected unless large bioaccumulation of TFA occurs in agricultural areas.

## MATERIAL AND METHODS

### A. Material

#### 1. Test material

Test item:	Trifluoroacetic acid (TFA)
Active substance(s):	See above (MW 114.03)
Adjuvant / Surfactant:	Gluconate or acetate as carbon source
Source of test item:	E.I. DuPont de Nemours & Co., Inc. in conjunction with the AFEAS. Additional TFA from Fisher Scientific
Lot/Batch number:	Not stated
Purity:	Not stated
Storage conditions:	Not stated

#### 2. Test solutions

Vehicle/solvent:	Not stated
Source of vehicle/solvent:	Not stated
Concentration of vehicle/solvent:	Not stated

#### 3. Test organism(s)

Species:	<i>Bradyrhizobium japonicum</i> strains USDA 110, 2143 and 184; <i>G. max</i> seedlings (cv Williams 82)
Source of test species:	Not stated
Age of test organisms at study initiation /	Not relevant / not stated
Crop growth stage at treatment:	
Holding conditions prior to test	See below (treatments)
Acclimatisation:	See below (treatments)

### B. Study design and methods

#### 1. Test procedure

Test system (study type):	Laboratory study investigating effects of TFA on symbiotic nitrogen fixation in soybeans
Guideline deviation:	No official test guideline available
Duration of study:	Unclear. Approximately 40 days
Treatments:	<u>TFA in culture (free living state)</u> : Strains grown in Tully's (T.) defined liquid media without vitamins (exact composition is given in the study) with acetate, pH 6.8 (T. acetate). Liquid cultures were grown at 28 °C, monitored over time and sampled for optical density (O.D.) readings at A <sub>630</sub> using a Cary 1Bio UV-Visible. Growth curves (three trials) were performed on <i>B. japonicum</i> 2143 using three different starting O.D. ( $5 \times 10^6$ , $1 \times 10^7$ and $5 \times 10^7$ cells mL <sup>-1</sup> ) and were monitored periodically at A <sub>630</sub> until stationary growth phase. To test the effects of TFA on growth of this strain, three different concentrations (10, 100 µM and 1 mM) of TFA

were added to T. acetate. Inorganic acids, organic acids and free fluoride content of *B. japonicum* 2143 grown on gluconate, acetate and acetate þ TFA, were analyzed in duplicate cultures which had reached late-log phase (method is described in the study). The extent of PHB accumulation within *B. japonicum* strain 2143 grown on gluconate, acetate and acetate + TFA, was performed with UV detection of crotonic acid (from PHB acid-catalyzed chemical depolymerization) at 210 nm (method is described in the study).

Attachment of *B. japonicum* to soybean roots: *B. japonicum* strain 2143, either grown on T. gluconate, T. acetate or T. acetate plus TFA were diluted to a standard  $1 \times 10^7$  cells ml<sup>-1</sup> with a buffered solution, then incubated with the roots of whole soybean seedlings (cv Williams 82) and the cells were allowed to attach to the roots for 3 or 6 min. Cells were removed with low intensity sonication, aliquots plated in replicate and colonies counted to quantify the number of cells attached to the roots. In a separate experiment, strain 2143 grown in T. gluconate was compared for attachment in the presence of three levels of TFA in the attachment buffer with the controls having equal amounts of acetate. For this experiment the attachment buffer (which has a low buffering capacity) was allowed to undergo a pH change from the addition of the three levels of acetate or TFA. The pH of each solution of acetate or TFA dissolved in the attachment media was 10 mM acetate (pH 6.8), 100 mM acetate (pH 6.7), 1 mM acetate (pH 4.6), 10 µM TFA (pH 6.8), 100 µM TFA (pH 6.5) and 1mM TFA (pH 3.4). Results are presented as the number of cells attached per root from three separately inoculated seedlings, done in replicate.

Symbiosis: The effect of TFA on symbiosis was tested in two different growth regimes. First regime utilized a sterile Missouri silt loam soil (< 10 % organic matter) with TFA incorporated at levels of 0.674, 6.74 and 67.4 µL TFA kg<sup>-1</sup> of dry soil. Second regime utilized a hydroponics system where the root system was bathed in a nitrogen free plant nutrient solution contained within sterile clear plastic growth pouches with TFA incorporated at levels of 0.003, 0.031 and 0.314 µL TFA L<sup>-1</sup>. Both experiments done with soybean cv Williams 82. Environmental conditions: experiments utilized in a growth chamber with 50 % relative humidity and a 16 h light/8 h dark cycle. Plants were inoculated with *B. japonicum* strain 110, 2143 or 184 depending on the experimental parameter.

Test concentrations	See above (Treatments)
Number of replicates:	See above (Treatments)
Individuals per replicate:	See above (Treatments)
Test conditions:	See above (Treatments)
Test units (type and size):	See above (Treatments)
Application / device / nozzles:	See above
Water volume:	See above
Calibration of sprayer:	Not relevant / not stated

<u>2. Environmental conditions</u>	See above (Treatments)
Test medium:	See above
Temperature / relative humidity:	See above
Photoperiod:	See above

---

Lighting	See above
pH:	See above
Organic matter (C <sub>org</sub> ):	See above
CaCO <sub>3</sub>	See above
Cation exchange capacity:	See above
Soil textural fractions / extractable micronutrient concentrations [mg per kg soil]:	See above
Fertilization:	See above

### 3. Observations and measurements:

Analytical parameters measured:	Concentrations of the test item were not confirmed by appropriate analytical verification.
Biological parameters measured:	See above (Treatments)
Measurement frequency:	See above (Treatments)
Statistical analyses:	Statistical significance for the majority of experiments was determined using the t-test for significance as control and experimental standard deviation values were re-evaluated for each experimental trial. Chi <sup>2</sup> analysis was performed on those experiments where the control was replicated enough to serve as an expected value for each data point.

## RESULTS

### 1. Validity criteria:

No official test guideline available and thus no validity criteria.

### 2. Other measurements:

Please refer to point 3 'Biological findings'. Measurement of other parameters was not reported.

### 3. Biological findings:

**TFA in culture (free living state):** *B. japonicum* 2143 grown in liquid culture with 10 mM gluconate as the carbon source had a doubling time of 10 h. The same strain grown both on 10 mM acetate or 10 mM acetate with TFA added revealed that growth was slower with increasing amounts of TFA. The doubling times were acetate (14 h), acetate with 10 µM TFA (15 h), acetate with 100 µM TFA (20 h) and acetate with 1 mM TFA (28 h). Analysis of PHB content of *B. japonicum* 2143 grown in the presence of TFA revealed that accumulation of PHB is three times higher in those cells grown on acetate compared to those grown on gluconate. In addition, the presence of TFA had no effect on PHB accumulation in those cells grown only on acetate, regardless of the level of TFA in addition to the acetate. Fluoride was not detected in any cultures grown in the presence of TFA, however, small levels of fluoride were detected in those grown on acetate and gluconate.

**Attachment of *B. japonicum* to soybean roots:** The effect of TFA on attachment of *B. japonicum* to soybean roots was determined under two conditions. The first condition was the attachment of *B. japonicum* grown on gluconate and then assayed for attachment in the presence of acetate or TFA (see Fig. 1). When cells were grown on gluconate and then exposed to low levels of TFA or acetate during the attachment assay, the number of cells adhering to the root increased. The number of cfu significantly increased after a 6 min incubation; the trend was obvious at 3 min but not significant. *B. japonicum* incubated with the highest level of acetate or TFA demonstrated reduced attachment. This reduction could have been the result of a drop of pH in the attachment medium, since authors of this study have found correlation between low rhizosphere pH and reduced attachment

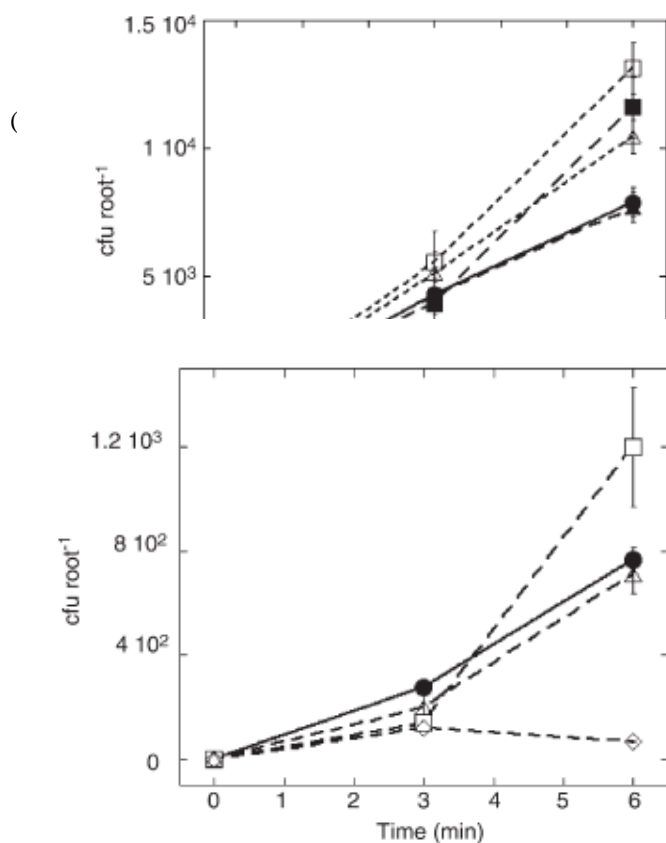


Fig. 2. Attachment (in cfu root<sup>-1</sup>) of *B. japonicum* strain 2143 grown on T. acetate (●), and T. acetate plus 10 μM TFA (□), 100 μM TFA (Δ) or 1 mM TFA (◇). Data point error is reported as SEM.

The second condition was attachment of *B. japonicum* grown on acetate in the absence or presence of TFA (see Fig. 2). TFA was removed immediately prior to the assay. Growth on acetate markedly reduced the number of cells capable of attachment. This result is in contrast to the effect of acetate in the attachment medium of cells grown on gluconate. This demonstrates that acetate affects the attachment process differentially depending on whether acetate is the primary carbon source for growth or is an exogenous effector. Growth on acetate in the presence of low levels of TFA enhanced attachment at 6 min. In the first attachment experiment, acetate and TFA yielded similar effects, but in this experiment different results were obtained suggesting that acetate and TFA may have different mechanisms of action. The highest level of TFA reduced attachment significantly. The effects of TFA observed here were not due to pH as the growth medium was highly buffered, but TFA did significantly increase culture doubling time.

**Symbiosis:** Effects of TFA on early plant growth (post-germination) were examined when both the soybean seedling and *B. japonicum* were pre-incubated for 1 h with the three levels of TFA. There was no statistically

significant difference in the fresh weights of plants between controls not treated with TFA and those treated with  $0.674 \mu\text{L TFA kg}^{-1}$  soil (see Figure 3A). However, those plants treated with the two highest levels of TFA were developmentally stunted and had shoot weights that were significantly reduced. Those plants treated with  $0.674 \mu\text{L TFA kg}^{-1}$  soil developed root systems similar to those of the control plants and they developed very normal nodules capable of nitrogen fixation. The nodule weight (see Fig. 3B) of these plants was not significantly different from the control plants nor was the acetylene reduction activity (see Fig. 3C) of these plants different from control plants: However, higher levels of TFA significantly affected plant development and shoot fresh weight when pre-incubated with TFA. Those plants treated with  $6.74 \mu\text{L kg}^{-1}$  soil developed secondary shoots with small leaf-like structures at the internodes, but these structures remained small and never developed into mature leaves. Internodal expansion was much less than those of the untreated control, resulting in stunted growth. The growth of most of these plants halted between eight and ten days. The root systems of these plants were considerably shorter and less developed compared to untreated plants. These plants occasionally developed root nodules, but they were small and ineffective. Those plants treated with  $67.4 \mu\text{L TFA kg}^{-1}$  soil never progressed beyond the cotyledon stage of plant development. That is, these plants germinated and the seeds opened to expose the cotyledons as they normally do, but the secondary shoot never emerged. All growth ceased at three days but the cotyledons remained green and succulent throughout the experiment. Root development was also severely reduced. None of these plants developed root nodules.

Additional experiments were performed in which *B. japonicum* and soybean seedlings were allowed to begin the infection process before being planted into soil containing TFA. The plants grown in soil with the two highest levels of TFA ( $6.74$  and  $67.4 \mu\text{L TFA kg}^{-1}$  soil) showed very similar developmental effects to those plants described in the previous experiment, so little or no nodule data could be collected. The  $0.674 \mu\text{L TFA kg}^{-1}$  treatment was reduced in terms of acetylene reduction activity relative to the control at 32 dpi. This difference was not significantly less in conjunction with all other time points, which were very similar. The decrease in acetylene reduction activity at the peak time was unexpected since the number of nodules formed per plant was significantly higher. Consequently, the average nodule weight of those grown with TFA was much less of those harvested at 32 and 35 dpi, respectively.

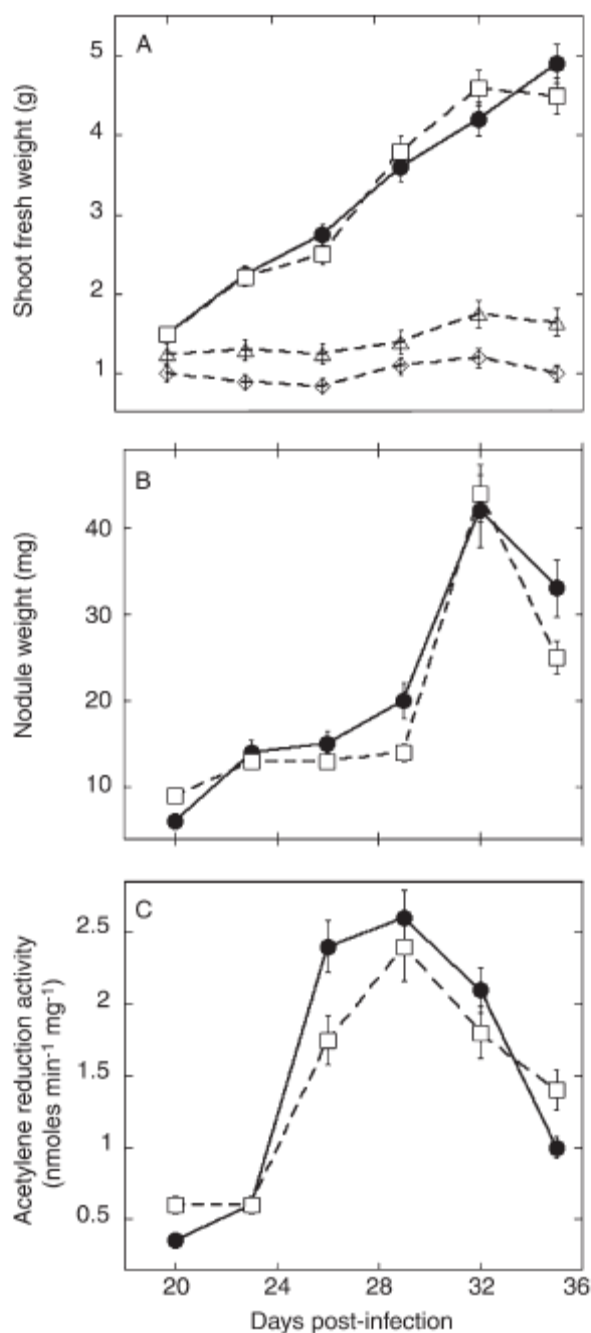


Fig. 3. (A) Shoot weights of soybean when both plant and bacteria were pre-incubated with TFA for 1 h before inoculating and planting in soil containing either no TFA (●), 0.674  $\mu\text{l TFA kg}^{-1}$  soil (□), 6.74  $\mu\text{l TFA kg}^{-1}$  (Δ) and 67.4  $\mu\text{l TFA kg}^{-1}$  (◇). (B) The nodule weight of those plants in (A) not treated (●) or treated with the lowest level of TFA (□). (C) Acetylene reduction activity of those nodules collected in (B) not treated with TFA (●) or treated with the lowest level of TFA (□). Data point errors are reported as SEM.

To determine whether greater concentrations of TFA could affect the reduction of atmospheric dinitrogen, bacteroids, the symbiotic form of *B. japonicum*, were isolated from 4-week old nodules on plants grown in soil without TFA. The bacteroids were measured ex planta in the presence of TFA by the acetylene reduction technique, which serves as an index of nitrogen fixation activity. The bacteroid acetylene reduction activity for

each level of TFA tested were statistically no different from control plants suggesting that TFA has no direct effect on the nitrogenase enzyme complex.

The results from using TFA with strain 110 and soybean under hydroponics conditions were different from those obtained from both soil experiments. As with the soil experiments,  $0.003 \mu\text{L TFA L}^{-1}$  of solution had no measurable effect on plant growth whereas  $0.031$  and  $0.314 \mu\text{L TFA L}^{-1}$  of solution retarded plant development. However, these conditions were sufficiently different from the soil trials in that nodules were able to form on the roots of all plants regardless of treatment. The pronounced effects of the two highest TFA levels on plant development in soil conditions precluded obtaining nodule number, mass or development. With these plants however, nodulation onset was normal regardless of treatment. These plants were not pre-incubated with TFA and were only subjected to it under growth conditions. The average nodule weight from these same plants (see Fig. 4) indicates that TFA at all three levels had some effect on nodule mass, especially in the latter portion of the nitrogen fixation time course. At 24 and 27 dpi there was a statistically significant reduction in nodule mass with the lowest level of TFA. At the two higher levels of TFA, even though the plants had the same number of nodules, they were significantly reduced in mass.

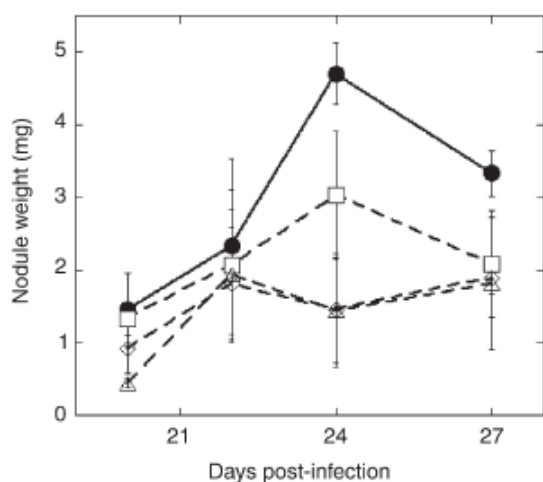


Fig. 4. Nodule weight of soybean seedlings grown hydroponically after inoculation with *B. japonicum* strain 110. Seedling were grown with no TFA (●),  $0.003 \mu\text{L TFA L}^{-1}$  nutrient solution (□),  $0.031 \mu\text{L TFA L}^{-1}$  (△) and  $0.314 \mu\text{L TFA L}^{-1}$  (◇). Data point errors are reported as SEM.

By measuring nodule appearance, a judgment can be made as to the whether TFA affects infection. These studies in conjunction with analysis of attachment provide information as to when an exogenous substance has an effect on symbiosis. In a separate experiment, strains 110, 2143 and 184 were each used as inoculum and the plants monitored within clear plastic growth pouches. The presence of  $0.003 \mu\text{L TFA L}^{-1}$  did not affect the rate of appearance of nodules by inoculation with strain 110, but did cause a slight delay in the appearance of nodules with strain 2143.

The delay observed with strain 2143 in the presence of TFA was similar to that with strain 184, a mutant that consistently demonstrates a delay in nodulation. Though nodule appearance was slightly delayed with strain 2143, the presence of TFA did increase the number of nodules per plant to a significant degree. This was also true with strain 110.

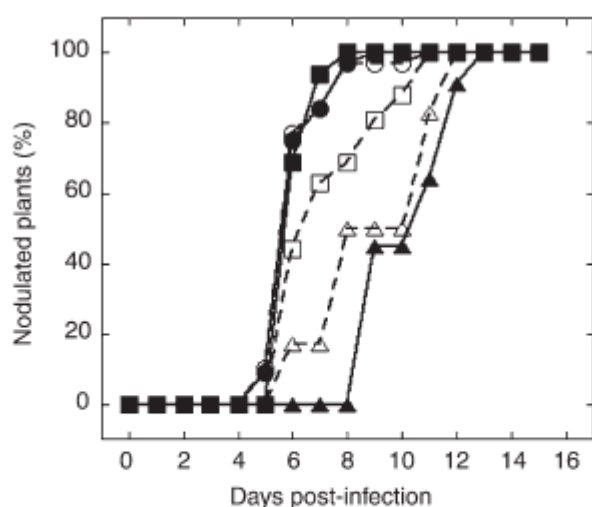


Fig. 5. Nodulation kinetics of *B. japonicum* strain 110 (●), 2143 (■) and 184 (▲) in the absence of TFA on hydroponically grown soybean seedlings. In the presence of  $0.003 \mu\text{l TFA l}^{-1}$  nutrient solution, strain 110 (○) was unaffected, strain 2143 (□) had reduced nodulation and mutant strain 184 (Δ) nodulated earlier.

In the presence of the lowest level of TFA, the mutant that normally displays a delay in nodule appearance had a slightly earlier appearance. The location of the first nodule relative to the root tip at the time of inoculation (nodule geometry) was also examined during all the hydroponics experiments. The results demonstrated a slight downward trend in nodule location in the presence of all three levels of TFA and with all strains (data not shown), however none of these differences were significant.

## RESULTS SUMMARY

In summary, at very low levels TFA has little or no effect on either symbiosis or the two partners involved. As the level of TFA increase, the effects become detrimental, with the plant being more affected at lower levels. However, the lowest concentration of TFA used in the study ( $0.674 \mu\text{L kg}^{-1}$ ) is at least 25 times greater than the levels currently found in some contaminated surface waters and is 1000 to 10000 times greater than the projected for the near future. Thus, TFA at the levels currently found in the environment will not have an adverse effect on symbiotic nitrogen fixation in soybeans.

### Comment of the notifier:

The treatment level in the study mentioned above are by far higher than the maximum PECsoil-figures for TFA, which occur after the application of flufenacet. Thus, the study is not relevant for the risk assessment.

### **RMS comments:**

Due to that fact the lowest treatment level of TFA ( $0.674 \mu\text{L kg}^{-1}$ ) tested in the present study is far higher than the maximum PECsoil for this metabolite which occur after application of flufenacet, the study can not be used in the standard risk assessment.

**B.9.8. EFFECTS ON BIOLOGICAL METHODS FOR SEWAGE TREATMENT**

For the first EU approval of the active substance flufenacet a study on effects on biological methods for sewage treatment (Kanne R., 1989) was submitted. The study was performed according to ISO 8192-1986/B guideline and resulted in  $EC_{50} > 10000$  mg a.s./L. However, this endpoint was not mentioned in the Review Report for flufenacet.

For the renewal of the EU approval a new activated sludge, respiration inhibition test done according to OECD 209 test guideline, was submitted. The endpoint from it was considered valid and was used in the risk assessment.

The study summaries are provided in the Table B.9.8-1 below.

**Table B.9.8-1: Effects data of flufenacet to activated sludge.**

Organism	Test design	Test substance	Endpoint	Reference
Activated sludge	Respiration inhibition, 3 h, static	Flufenacet	Activated sludge, inhibition of respiratory activity $EC_{50} > 10000$ mg a.s./L	Kanne R. (1989) M-004740-02-1
		Flufenacet	Activated sludge, inhibition of respiratory activity $EC_{50} > 10000$ mg a.s./L	Weyers A (2007) M-283846-01-1

**B.9.8.1. Flufenacet TC - Toxicity to bacteria**

<b>Reference:</b>	Flufenacet TC - Toxicity to bacteria
<b>Author(s), year:</b>	Weyers, A (2007)
<b>Report/Doc. number:</b>	Study No: 2006/0171/01, Reference BCS No: M-283846-01-1
<b>Guideline(s):</b>	OECD Guideline 209 (1984)
<b>GLP:</b>	Yes

Material and methods:

Test substance: Flufenacet tech CAS:142459-58-3, batch No: EDHB001715, purity: 97%.

Test species: Activated sludge (mixed population of aquatic microorganisms)

Source: aeration tank of a domestic sewage treatment plant  
(Cologne-Stammheim)

Type of test, duration: Laboratory aerobic activated sludge inhibition test, 3 hours

Applied concentration:

Nominal: 10, 100, 10000 mg a.s./L

Toxic reference: 3,5-dichlorophenol (purity 97%) tested at concentrations of 5, 10 and 20 mg/L,  $EC_{50} = 11.2$  mg item/l

Substrate:	Activated sludge and synthetic sewage feed (according OECD guideline) treated with the test item, reference substance and control. Additional vessels to determine the physico-chemical oxygen consumption were prepared containing the test item (10 000 mg a.s/L) without activated sludge.
Incubation time:	Aerated for 3 hours at $20 \pm 2^{\circ}\text{C}$
Test parameters:	Inhibition of respiration rate (rate of oxygen uptake) pH-value, temperature and dissolved oxygen concentration were determined at the start and at the end of the test. The respiration rate of the activated sludge was measured after a contact time of 3 hours. The respiration rate of the same activated sludge in the presence of various concentrations of the test item, under otherwise identical conditions, is also measured. The inhibitory effect of the test item at a particular concentration is expressed as a percentage of the mean of the respiration rates of two controls.
Statistic:	Test item: $\text{EC}_{50}$ was determined directly from the raw data. Toxic reference: $\text{EC}_{50}$ was calculated based on probit analyses.
Findings:	Oxygen consumption: Flufenacet showed 6.3% respiration inhibition of activated sludge at a test item concentration of 10000 mg/L. The effect value relates to nominal concentration.

Table B.9.8-1: Effect of flufenacet on the respiration rate of acitved sludge.

Test substance	Nominal concentration mg/L	Temp. [°C]	pH	Respiratory rate [mg O <sub>2</sub> /L/h]	Oxygen concentration [mg O <sub>2</sub> /L]		Inhibition [%]
					Start	end	
Control 1	-	18.8	7.9	30.0	4.8	3.3	-
Control 2	-	19.5	7.9	34.0	5.5	3.8	-
Control 3 <sup>a</sup>	10000	19.2	7.2	0.0	7.6	7.6	-
Flufenacet	10	19.2	7.9	34	4.4	2.7	-
	100	19.2	7.9	36	4.1	2.9	-
	10000	19.3	7.9	30	4.8	3.3	6.3
Toxic reference	5	18.8	7.9	24	5.3	4.1	25
	10	18.9	7.9	20	5.8	4.8	37.5
	20	19	7.9	7.2	7.1	6.5	77.5

<sup>a</sup> The physico-chemical oxygen consumption has been determined at 10000 mg/L test item concentration.

As no physico-chemical oxygen consumption was observed at that test item concentration this observation also holds true for the lower test item concentrations.

Conclusion:

EC<sub>50</sub> > 10 000 mg a.s./L, based on nominal concentrations.

**Comments RMS:**

The study was conducted according to the OECD test guideline 209 (1984).

Taking into account the validity criteria given in the test guideline 209 (1984) the study is considered acceptable. The respiratory rates of the two control groups differ less than 15% from each other. The EC<sub>50</sub> of the reference compound 3,5-Dichlorophenol is in the range of 5 and 30 mg a.s./L (being 11.2 mg a.s./L).

Taking into account the current valid OECD test guideline 209 (July 2010) the following validity criteria are given:

-The blank controls (without the test substance or reference substance) oxygen uptake rate should not be less than 20 mg oxygen per one gram of activated sludge (dry weight of suspended solids) in an hour. The inoculum used in the experiment was 0.96 g activated sludge/L of the test solution. Taking into account the fact that the respiration activity of the control samples in 1 hour was at least 30 mg O<sub>2</sub>/L solution, that validity criterion was fulfilled.

-The coefficient of variation of oxygen uptake rate in control replicates should not be more than 30% at the end of definitive test ( being 8.8%).

-The EC<sub>50</sub> of 3,5-DCP was found in the range 2 mg/L to 25 mg/L for total respiration ( being 11.2 mg a.s./l)

The test also meets the given validity criteria according to the OECD test guideline 209 (2010).

**Agreed endpoint:**

EC<sub>50</sub> > 10000 mg a.s./L

**9.8.2. Additional information**

Oxygen consumption test with activated sludge submitted and evaluated in the first EU approval of the active substance and was summarised below:

Test substance:	FOE 5043 98.1 % Lot NLL 3643-5
Authors	Kanne. R
Report No.	Report No: BCS No: M-004740-02-1
Guideline:	ISO 8192-1986 /BETAD 103/OECD 209)
Inoculum:	3L Laboratory Facility (OECD)
Exposure time:	3 hours
Reference substance:	3,5-dichlorophenol
Date of testing:	Dec. 7, 1989

*Summary:*

*The effects of flufenacet on activated sludge were determined in a 3-hour exposure laboratory study. Activated sludge was exposed to controls (water) and of the test item at nominal concentration of 100, 1000 and 10000 mg a.s./L. A toxic reference, 3,5-dichlorophenol, was tested at three concentrations of 1 and 20 mg/L to demonstrate the sensitivity of the test system.*

<b>Nominal concentration mg a.s./L</b>	<b>Mean respiration rate (mg O<sub>2</sub>/L/h)</b>	<b>% inhibition</b>
<i>C (control)</i>	<i>30</i>	<i>-</i>
<i>100 flufenacet/L</i>	<i>30</i>	<i>0</i>
<i>1000 flufenacet/L</i>	<i>30</i>	<i>0</i>
<i>10000 flufenacet/L</i>	<i>27</i>	<i>10</i>
<i>3,5-dichlorophenol (1 mg L)</i>	<i>30</i>	<i>0</i>
<i>3,5-dichlorophenol (20 mg /L)</i>	<i>6</i>	<i>80</i>

**Results:**

*A risk to biological sewage treatment process has not be anticipated*

*EC<sub>50</sub> > 10000 mg a.s./L.*

*Due to lack of bacteriacidal activity of flufenacet, a risk to biological sewage treatment processes has not be anticipated.*

*The study is considered acceptable.*

**B.9.9. MONITORING DATA**

Monitoring data concerning adverse effects of the active substance to non target organism are not available.

**B.9.10. BIOLOGICAL ACTIVITY OF METABOLITES POTENTIALLY OCCURRING IN GROUNDWATER****B.9.10.1. Evaluation of the pre-emergence biological activity of FOE 5043-Oxalate (code: BCS-AB16305) a metabolite of Flufenacet.**

<b>Reference:</b>	Evaluation of the pre-emergence biological activity of FOE 5043-Oxalate (code: BCS-AB16305) a metabolite of Flufenacet.
<b>Author(s), year:</b>	Hills, M., 2009
<b>Report/Doc. number:</b>	Study No. PP09022, Reference BCS No:M-353844-01-1
<b>Guideline(s):</b>	Not applicable
<b>GLP:</b>	No

Test substance:	Test item 1 FOE 5043 (AE F133402), FOE 5043-Oxalate, M01, metabolite of flufenacet. Batch Number: BCS-AB16305 (batch SES 10564-3-1) formulated as WP 05 Test item 2 Flufenacet coded: FOE 5043 (AE F133402), Batch ID: 488, formulated as a WP 05
-----------------	---

Methods and test condition:

The test was designed to determine the biological activity of FOE 5043-Oxalate a metabolite of Flufenacet.

The study was conducted under standardized glasshouse conditions using a WP 05 formulation of the metabolite in comparison with a WP 05 formulation of the parent Flufenacet. Jiffy pots (8 cm diameter) were filled to within 2 cm of the top with a silt-loam soil (20% sand, 57% silt, 23% clay, pH 6,8 and 1,4 % organic matter). Seeds of the weed species listed in Table B. 9.10.1-1 were sown into these pots and covered with 0,5 to 1 cm of the same soil mixed 1 to 1 with sharp sand.

After sowing the pots were placed into a glasshouse set 20°C+/-2°C day and 12°C+/-2°C night and watered from the top according to need. High pressure sodium lamps (400W) were used to augment daylight during cloudy conditions and to extend the day length to 14 hours.

The parent Flufenacet and metabolite formulations were diluted in de-ionized water to obtain the required dose rates. Flufenacet was applied at 600 / 500 / 250 / 125 / 60g a.s./ha. BCS-AB16305 the Oxalate metabolite was applied with the equivalent dose rate of 372 / 310 / 155 / 77 / 37g a.s./ha, adjusted by the molar mass of the metabolite in comparison with the active substance (molar mass for Flufenacet 363,3g/mol and for FOE 5043-Oxalate (BCS-AB16305) 225g/mol.). The pre-emergence applications were made using the Herbicide Research track-sprayer with a spray volume of 300 l/ha via a flat fan nozzle (Tee Jet 80015 SS).

Four weeks after application the treated plants were visually assessed for injury compared with the untreated control plants. The assessments were on a percentage basis (0 = no effects, 100 = complete kill).

**Table B.9.10.1-1: Plant species used in pre-emergence screening test.**

Plant species	Assigned number
Triticum aestivum (TRZAW)	1
Zea mays var vulgaris (ZEAMA)	2
Glycine max (GLXMA)	3
Alopecurus myosuroides (ALOMY)	4
Apera spica-venti (APESV)	5
Digitaria sanguinalis (DIGSA)	6
Echinochloa crus-galli (ECHCG)	7
Panicum miliaceum (PANMI)	8
Setaria viridis (SETVI)	9
Sorghum halepense (SORHA)	10
Amaranthus retroflexus (AMARE)	11
Ambrosia artemisiifolia (AMBEL)	12
Chenopodium album (CHEAL)	13
Galium aparine (GALAP)	14

**Findings:**

Flufenacet demonstrated control of all of the 11 weed species tested and some severe damage to wheat and corn crops with some degree of selectivity on soybeans.

The metabolite FOE-oxalate (BCS-AB16305) showed no biological activity on any of the 14 tested plant species.

**Table B.9.10.1-2: The observed injury (%) of plants, 28 days after application.**

Species name	kg a.s/ha	1	2	3	4	5	6	7	8	9	10	11	12	13	14	MEAN*
Observed injury (%) of plants, 28 days after application																
Flufenacet	0.600	96	63	20	100	100	100	100	100	100	100	100	96	96	98	99
	0.500	95	40	20	99	100	100	100	100	100	100	100	93	95	98	99
	0.250	93	20	20	98	100	100	100	99	100	100	98	75	95	96	96
	0.125	73	15	25	97	99	99	100	97	99	97	75	50	85	92	90
	0.060	65	0	0	97	99	99	99	97	90	97	55	18	75	85	83
FOE oxalate	0.372	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.310	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Species name	kg a.s./ha	1	2	3	4	5	6	7	8	9	10	11	12	13	14	MEAN*
	0.155	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.077	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.037	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

\* The results of the visual assessments are presented as means from two replicates.

#### Conclusions:

In a direct comparison study, it could be shown that FOE oxalate (BCS-AB16305), a metabolite of flufenacet, had no pre-emergence biological activity when tested on a range of weeds and crops under highly sensitive screening conditions. No injuries caused by flufenacet-oxalate metabolite of all tested plants were recorded at all tested application rates.

#### RMS comments:

Summarized above study was performed in order to demonstrate pre - emergence biological activity of metabolite of flufenacet (FOE 5043-oxalate, M01, (AE F133402) ) in comparison with the parent substance. Used method was sufficient to demonstrate that metabolite is significantly less biologically active compared to a.s.- flufenacet. The study is considered acceptable and provides supplemental information .

#### B.9.10.1.2. Screening and Efficacy Data for WAK6222 (metabolite of FOE 5043)

<b>Reference:</b>	Screening and Efficacy Data for WAK6222 (metabolite of FOE 5043)
<b>Author(s), year:</b>	Dahmen P., 2004
<b>Report/Doc. number:</b>	Report No: PF-F-HB-WAK6222-01, Reference BCS No: M- 089475-01-1
<b>Guideline(s):</b>	Not applicable
<b>GLP:</b>	No

Test substance:	WAK6222, Sulphonic acid, M02, metabolite of flufenacet ), Batch No: not given
Application solvent:	The test material was applied as active ingredient with a solvent (H <sub>2</sub> O) and application volume 30 ml water.
Application method:	30 ml substance solution to pour the surface of test pots.
Test location:	Greenhouses of the Business Group Crop Protection, - Biological Research, Institute for Herbicides, Agricultural Centre Monheim, D-51368 Leverkusen, Germany.
Test species:	Nine monocotyledonous and five dicotyledoneous plant species listed in the Table B.9.10.1.2-1.
Horticultural Conditions:	Pre-emergence

Seeds of all mentioned plant species were planted into soil (sandy loam, organic matter of 2.5 - 3 %) in greenhouse pots (420 cm<sup>2</sup> surface, pot T59). Seeds were sown 24 hours prior to application of the test substance.

The soil used allowed good germination.

Growing condition: The plants were kept at a day/night cycle of 22°C/15 °C, respectively.

The relative humidity in the test chamber was set at 50% and the illumination at 8000 lux under a 14-hour day cycle.

Test methods:

Plants used for pre-emergence treatments were sown, sprayed with the test material and then directly placed under the specified growing conditions.

The final evaluation was performed 21 days after treatment initiation.

For the metabolite the same application rates as for the parent compound were used, corrected for the molecular mass of FOE-sulfonic acid.

Evaluation of phytotoxicity was done by visual observations using a rating scale of 0 to 100%, where 100% represented complete destruction of above ground parts and 0% represented no visual damage (normal growth) as compared to untreated plants.

**Table B.9.10.1.2-1. Plants species used in the pre-emergency test.**

Plant species	
Monocotyledonous	Dicotyledonous
Triticum aestivum (TRZAW)	Glycine max (GLXMA)
Zea mays (ZEAMX)	Amaranthus retroflexus (AMARE)
Alopecurus myosuroides (ALOMY)	Ambrosia elatior (AMBEL)
Apera spica-venti (APESV)	Chenopodium album (CHEAL)
Digitaria sanguinalis (DIGSA)	Gallium aparine (GALAP)
Echinochloa crus-galli (ECHCG)	
Panicum miliaceum (PANMI)	
Setaria viridis (SETVI)	
Sorghum halepense (SORHA)	

**Table B.9.10.1.2-2: Results of % injuries caused by flufenacet at different application rates .**

FOE5043 (flufenacet)	Code	Results (% injury) at different application rates				
Pre-emergence (g a.s./ha)						
Test species		600	500	250	125	60
Monocotyledonae						
Triticum aestivum	TRZAW	5	0	0	0	0
Zea mays	ZEAMX	5	0	0	0	0
Alopecurus myosuroides	ALOMY	100	100	100	100	100
Alpera spica-venti	APESV	100	100	100	100	100
Digitaria sanguinalis	DIGSA	100	100	100	100	100
Echinochloa crus-galli	ECHCG	100	100	100	100	0
Panicum miliaceum L.	PANMI	100	100	99	99	99
Setaria viridis	SETVI	100	100	100	100	100
Sorghum halepense	SORHA	100	100	100	100	100
Dicotyledonae						
Glycine max	GLXMA	0	0	0	0	0
Amaranthus retroflexus	AMARE	100	100	100	90	20
Ambrosia elatior	AMBEL	95	90	70	40	40
Chenopodium album	CHEAL	99	99	95	60	40
Gallium aparine	GALAP	99	95	95	95	20

**Table B.9.10.1.2-3: Results of % injuries caused by WAK6222 (FOE sulfonic acid) at different application rates.**

WAK6222 (FOE sulfonic acid)	Code	Results (% injury) at different application rates				
Pre-emergence (g a.s./ha)						
Test species	Code	455	379	189	95	45
Monocotyledonae						
Triticum aestivum	TRZAW	0	0	0	0	0
Zea mays	ZEAMX	0	0	0	0	0
Alopecurus myosuroides	ALOMY	0	0	0	0	0
Alpera spica-venti	APESV	0	0	0	0	0
Digitaria sanguinalis	DIGSA	0	0	0	0	0
/Echinochloa crus-galli	ECHCG	0	0	0	0	0
Panicum miliaceum L.	PANMI	0	0	0	0	0
Setaria viridis	SETVI	0	0	0	0	0
Sorghum halepense	SORHA	0	0	0	0	0
Dicotyledonae						

WAK6222 (FOE sulfonic acid)	Code	Results (% injury) at different application rates				
Pre-emergence (g a.s./ha)						
Glycine max	GLXMA	0	0	0	0	0
Amaranthus retroflexus	AMARE	0	0	0	0	0
Ambrosia elatior	AMBEL	0	0	0	0	0
Chenopodium album	CHEAL	0	0	0	0	0
Gallium aparine	GALAP	0	0	0	0	0

### Conclusion

At none of the tested rates any phytotoxic effects were observed. Based on these results it is concluded that FOE sulfonic acid (M02, WAK6222) has no herbicidal activity.

### **RMS comments:**

Summarized above study was performed in order to demonstrate pre - emergence biological activity of metabolite of flufenacet -FOE sulfonic acid (M02, WAK6222) in comparison with the parent substance. Used method was sufficient to demonstrate that metabolite FOE sulfonic acid is significantly less biologically active compared to a.s.- flufenacet. The study is considered acceptable and provides supplemental information .

### **B.9.10.1.2.4. Evaluation of the pre-emergence biological activity of flufenacet and its metabolite BCS-CU 62474.**

<b>Reference:</b>	Evaluation of the pre-emergence biological activity of flufenacet and its metabolite BCS-CU 62474.
<b>Author(s), year:</b>	Noeding S., 2012 c
<b>Report/Doc. number:</b>	Study No: FFS125049, Reference BCS No: M-460336-01-1
<b>Guideline(s):</b>	Not applicable
<b>GLP:</b>	No

Test substance:	BCS-CO 62475 WP20 =SP102000027668, FOE 5043-trifluoroethanesulfonic acid (M44) AE F133402 WP20 = Flufenacet, SP102000027667 The WP 20 formulations of both flufenacet and its metabolite BCS-CO 62475were used.
Nominal application:	180, 90, 45 and 23 g metabolite/ha 240, 120,60 and 30 g flufenacet/ha
Test location:	The study was conducted under standardized glasshouse conditions.

The testing was carried out in the Bayer CropScience Building H872 (BCS-R&D-CPR-Research Weed Control) on the “Industrial Park Höchst” /Frankfurt, Germany.

#### Methods and test condition:

Jiffy pots (7 cm diameter) were filled to within 2 cm of the top with a silt-loam soil (20% sand, 57% silt, 23% clay, pH 6.8 and 1.4% organic matter). Seeds of the weed species listed in Table 9.10.1.2.4-1 were sown into these pots and covered with 0.5 to 1 cm of the same soil mixed 1 to 1 with sharp sand. The sowing density was selected based on prior experience to provide approximately 60-70% soil cover by the plants at application timing.

After sowing the pots were watered slightly.

All compounds used in the test were dissolved in deionized water and diluted to obtain the required dose rates. The pre-emergence application (25 OCT 2012) was made using the track-sprayer of Research Weed Control with a spray volume of 300 L/ha via a flat fan nozzle (TeeJet 8001).

After application, the pots were placed into a glasshouse set 21°C+/-2°C day and 12°C+/-2°C night and watered according to need. High pressure sodium lamps (400W) were used to augment daylight during cloudy conditions and to extend the day length to 14 hours.

Two weeks and four weeks after application, the treated plants were visually assessed for injury compared with the untreated control plants. The assessments were on a percentage basis (0 = no effects, 100 = complete kill).

**Table 9.10.1.2.4-1: The plants used in the study.**

Plant species	EPPO Code
<i>Zea mays</i>	ZEAMA
<i>Triticum aestivum</i>	TRZAS
<i>Alopecurus myosuroides</i>	ALOMY
<i>Apera spica-venti</i>	APESV
<i>Digitaria sanguinalis</i>	DIGSA
<i>Echinochloa crus-galli</i>	ECHCG
<i>Setaria viridis</i>	SETVI
<i>Sorghum halepense</i>	SORHA
<i>Lolium perenne</i>	LOLPE

**Table 9.10.1.2.4-2: Results of phytotoxicity of flufenacet and it metabolite BCS-CU 62474 after post emergence application.**

Substance	g a.s/ha	ZEAMA	TRZAW	ALOMY	APESV	DIGSA	ECHCG	SETVI	SORHA	LOLPE
Observed injury (%) of plants, 14 days after application										
flufenacet	240	0	40	97	99	99	95	95	99	99
	120	0	20	90	98	99	95	95	99	99
	60	0	10	93	97	99	98	93	96	75
	30	0	0	65	95	99	90	93	96	40
BCS-CU62474 (FOE 5043-	123	0	0	15	0	0	0	30	30	0
	62	0	0	15	0	0	0	30	20	0

Substance	g a.s/ha	ZEAMA	TRZAW	ALOMY	APESV	DIGSA	ECHCG	SETVI	SORHA	LOLPE
trifluoroethanesulfonic acid	31	0	0	0	0	0	0	30	10	0
	15	0	0	0	0	0	0	30	n.a	0
Blank formulation WP 20	960*	0	0	0	0	0	0	0	0	0
	480*	0	0	0	0	0	0	0	0	0
	240*	0	0	0	0	0	0	0	0	0
	120*	0	0	0	0	0	0	0	0	0
Observed injury (%) of plants, 28 days after application										
flufenacet	240	0	50	97	100	100	99	97	99	100
	120	0	20	88	100	100	98	94	97	99
	60	0	0	98	97	100	92	88	85	68
	30	0	0	80	92	100	96	40	63	35
BCS-CU62474 (FOE 5043-trifluoroethane sulfonic acid)	123	0	0	0	0	0	0	0	0	0
	62	0	0	0	0	0	0	0	0	0
	31	0	0	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0	0	0
Blank formulation WP20	960*	0	0	0	0	0	0	0	0	0
	480*	0	0	0	0	0	0	0	0	0
	240*	0	0	0	0	0	0	0	0	0
	120*	0	0	0	0	0	0	0	0	0

amount of blank formulation applied

#### Conclusion:

In a direct comparison study under highly sensitive glasshouse screening conditions, the metabolite BCS-CO 62475 showed no biological activity 28 days after treatment against the range of weeds tested. After 14 days the phytotoxic effects were observed in some plants (*Alopecurus myosuroides*, *Setaria viridis*, *Sorghum halepense*) ranged between 10-30 %. In that same time the % of injuries caused by flufenacet in these plants were most pronounced and was closed to 90% of injuries.

#### **RMS comments:**

Summarized above study was performed in order to demonstrate pre-emergence biological activity of metabolite of flufenacet - BCS-CO 62475, in comparison with the parent substance.

Used method was sufficient to demonstrate that metabolite BCS-CO 62475 is significantly less biologically active compared to a.s.- flufenacet.

The study is considered acceptable and provides supplemental information .

#### **B.9.10.1.2.5. Evaluation of the post emergence herbicidal activity of trifluoroethanesulfonic acid sodium-salt (metabolite of flufenacet) in comparison with flufenacet.**

**Reference:** Evaluation of the post emergence herbicidal activity of trifluoroethanesulfonic acid sodium-salt (metabolite of flufenacet) in comparison with flufenacet.

**Author(s), year:** Jans, D.; 2013

---

<b>Report/Doc. number:</b>	Report No: RF13/035, Reference BCS No: M-460341-01-1
<b>Guideline(s):</b>	EPPO standards for the efficacy evaluation of plant protection products. PP 1/135 (3), 2006.
<b>GLP:</b>	No

---

Test substance:	<u>Test item 1</u> Flufenacet WP 20, Batch No: 2012-00524, content of a.s. 20.1% w/w. <u>Test item 2</u> Trifluoroethansulfonicacid sodium-salt WP 20 (BCS-CU62474), Batch No: 2012-005249, content of a.s.19.4%
Test soil	Standard soil “ Riedberg: soil from Bayer Crop Science AG Horticulture. The composition of soil according to DIN : clay 21.4%, 64.5% silt and 14.1 % sand, Cation exchange: 14.5 meq/100 g Organic carbon content : 0.86 %C pH=6.91
Test condition:	Greenhouse condition  Temperature:23°C at day and 18°C at night Humidity: 70% Light regime: 16light :8 dark hours, with natural daylight supplemented by artificial lighting. Number of replicates: 4 (one replicate per one pot, each pot contained 4 plants. Total 16 plants per test groups were tested

#### Methods

To reach the 2-4 leaf stage at the start of testing, sowing was started prior to testing. Seeds were introduced manually in the soil. The seeds were pressed into the soil surface and completely invisible covered with a layer of the sand-soil mixture.

The flower pots used were commercial plastic flower pots (13 cm diameter). The suspensions of the two test items were prepared on the day of application with the following rates:

Control : 200 l /ha deionised water.

Test item 1: (Flufenacet): 140, 180 and 240 g a.s./ha in 200 l deionised water

Test item: 2 (BCF-CU62474): 72,92 and 123 g a.s./ha ( rates are adjusted according to the molecular weight of test item 2 in comparison with item 1

The spray solution was applied once at test initiation onto the leaves and above-ground portions of the plants using a linear cabinet track sprayer [specially constructed, permitting area application].

After application the pots of each plant species were transferred to the greenhouse and placed on the tables within one species. One to four days prior to the final assessment, the pots of each plant species were arranged according to their treatment level as a benefit for the final assessment.

After application by Track sprayer (spray Lab SLGH 2500), bottom watering was performed with saucers standing below each pot. Water was given and retained within the saucer according to the need of the plants in order to have an optimal water supply for plant growth.

Assessment of phytotoxicity effects:

Phytotoxicity records: Visual phytotoxicity ratings (chlorosis, necrosis, bleaching, leaf deformation, stunting) were recorded for the replicates at each application rate on day 7 (7 DAA), 14 (14 DAA) and 21 (21 DAA) days after application.

**Table 9.10.1.2.5-1: The plants used in the study.**

Species name	Common name	Family	EPPO code
<i>Alopecurus</i>	Meadow foxtail	Poaceae	ALOMY
<i>Apera spica-venti</i>	Silky wind grass	Poaceae	APESV
<i>Digitaria sanguinalis</i>	Hairy fingergrass	Poaceae	DIGSA
<i>Echinochloa crus-galli</i>	Cockspurgrass	Poaceae	ECHCG
<i>Lolium perenne</i>	Ryegrass	Poaceae	LOLPE
<i>Setaria viridis</i>	green foxtail	Poaceae	SETVI
<i>Sorghum halepense</i>	Johnson grass	Poaceae	SORHA
<i>Triticum aestivum</i>	Spring wheat	Poaceae	TRZAS
<i>Zea mays</i>	Corn	Poaceae	ZEAMA

**Table 9.10.1.2.5-2: Results of % injuries caused by flufenacet and its metabolite BCS-CU62474 at different application rates.**

Species name	g a.s/ha	ZEAMA	TRZAS	ALOMY	APESV	DIGSA	ECHCG	SETVI	SORHA	LOLPE
Observed injury (%) of plants, 7 days after application										
Control	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
flufenacet	240	0.0	5.0	20.0	52.5	40.0	42.5	42.5	40.0	37.5
	180	0.0	5.0	27.5	52.5	32.5	42.5	40.0	32.5	27.5
	140	0.0	2.5	30.0	52.5	30.0	35.0	27.5	35.0	22.5
BCS-CU62474 (FOE 5043-trifluoroethane - sulfonic acid)	123	0.0	7.5	0.0	12.5	0.0	0.0	0.0	0.0	0.0
	92	0.0	5.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0
	72	0.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Observed injury (%) of plants, 14 days after application										
Control	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Species name	g a.s/ha	ZEAMA	TRZAS	ALOMY	APESV	DIGSA	ECHCG	SETVI	SORHA	LOLPE
flufenacet	240	0.0	0.0	40.0	100.0	75.0	82.5	80.0	90.0	62.5
	180	0.0	0.0	55.0	97.5	70.0	80.0	82.5	80.0	47.5
	140	0.0	0.0	50.0	95.0	70.0	75.0	65.0	72.5	42.5
BCS-CU62474 (FOE 5043-trifluoroethane - sulfonic acid)	123	0.0	0.0	0.0	20.0	0.0	0.0	0.0	0.0	0.0
	92	0.0	0.0	0.0	12.5	0.0	0.0	0.0	0.0	0.0
	72	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Observed injury (%) of plants, 21 days after application										
Control	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
flufenacet	240	0.0	5.0	77.5	100.0	90.0	92.5	92.5	97.5	90.0
	180	0.0	0.0	67.5	100.0	87.5	87.5	92.5	90.0	72.5
	140	0.0	0.0	32.5	95.0	85.0	85.0	80.0	85.0	65.0
BCS-CU62474 (FOE 5043-trifluoroethane - sulfonic acid)	123	0.0	0.0	0.0	20.0	7.5	0.0	0.0	0.0	0.0
	92	0.0	0.0	0.0	10.0	2.5	0.0	0.0	0.0	0.0
	72	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	0.0

Description of symptoms assessed:

*b* = necrosis (brown shoot tissue)  
*c* = bleaching (shoot tissue without pigmentation)  
*d* = leaf deformation (leaf curl, abnormal leaf shape)  
*e* = stunting (plant height reduced with shorter internode length)  
*f* = reddening (reddening of green shoot tissue)  
50, 60% severe symptom (s)  
70, 80% total plant symptom (s)  
90% moribund  
100% complete kill

Rating system for recording the severity of phytotoxic symptoms:  
Phytotoxicity was recorded at each assessment date following a 0-100% rating system. The phytotoxicity is a subjective assessment and was rated in 10% increments as follows:  
0% no injury or effect  
10, 20% slight symptom (s)  
30, 40% moderate symptom (s)

Conclusion:

Compared to Flufenacet the herbicidal activity of the sodium salt of Trifluoroethansulfonicacid is clearly reduced. 21 days after application. Trifluoroethansulfonicacid did not cause any phytotoxic effects in six of the nine plant species tested. The 5%-effect at 72 g a.s./ha on *Setaria viridis* is clearly not dose-related. The observed effects of Trifluoroethansulfonicacid on *Apera spica venti* and *Digitaria sanguinalis* do not exceed 20% and are clearly reduced compared to Flufenacet.

Overall, it can be concluded that Trifluoroethansulfonic acid has no herbicidal activity.

#### RMS comments:

Summarized above study was performed in order to demonstrate pre - emergence biological activity of metabolite of flufenacet - BCS-CO 62475, in comparison with the parent substance.

Used method was sufficient to demonstrate that metabolite BCS-CO 62475 is significantly less biologically active compared to a.s.- flufenacet.

The study is considered acceptable and provides supplemental information .

**B.9.10.1.2.6. Evaluation of the pre-emergence herbicidal activity of flufenacet and its metabolite BCS-AZ 56567.**

<b>Reference:</b>	Evaluation of the pre-emergence herbicidal activity of flufenacet and its metabolite BCS-AZ 56567.
<b>Author(s), year:</b>	S. Nöding, 2013
<b>Report/Doc. number:</b>	Study No: FFS135016, Reference BCS No: M-461398-01-1)
<b>Guideline(s):</b>	None
<b>GLP:</b>	No

Test substance :

Test substance:

Test item 1

BCS-AZ 56567 WP20,=trifluoroacetic acid (Na-Salt) = TFA).  
metabolite of flufenacet. Batch Number: 2013 - 002927

Test item 2

AE F133402 WP20 = Flufenacet, SP102000027667

Application rate

The study was conducted under standardized glasshouse conditions using WP 20 formulations of both flufenacet and its metabolite BCS-AZ 56567 (trifluoroacetic acid (Na-Salt) = TFA).

The application rate of the metabolite was adjusted taking into account the differences in molecular weight.

BCS-AZ 56567 WP20 was applied at rate of 22.5 g a.s./ha

AE F133402 WP20 was applied as 60 g a.s./ha

Methods:

Jiffy pots (7 cm diameter) were filled to within 2 cm of the top with a silt-loam soil (20% sand, 57% silt, 23% clay, pH 6.8 and 1.4% organic matter). Seeds of the weed species listed in Table 9.10 .1.2.6-1 were sown into these pots and covered with 0.5 to 1 cm of the same soil mixed 1 to 1 with sharp sand. The sowing density was selected based on prior experience to provide approximately 60-70% soil cover by the plants at application timing.

After sowing the pots were watered slightly.

All compounds used in the test were dissolved in deionized water and diluted to obtain the required dose rates.

The pre-emergence application was made using the track-sprayer of Research Weed Control with a spray volume of 300 L/ha via a flat fan nozzle (TeeJet 8001).

After application, the pots were placed into a glasshouse set 21°C+/-2°C day and 12°C+/-2°C night and watered according to need. High pressure sodium lamps (400W) were used to augment daylight during cloudy conditions and to extend the day length to 14 hours.

Two weeks and four weeks after application, the treated plants were visually assessed for injury compared with the untreated control plants. The assessments were on a percentage basis (0 = no effects, 100 = complete kill).

**Table 9.10.1.2.6-1: Plants used in the study.**

Plant species	EPPO Code
<i>Alopecurus myosuroides</i>	ALOMY
<i>Apera spica-venti</i>	APESV
<i>Digitaria sanguinalis</i>	DIGSA
<i>Echinochloa crus-galli</i>	ECHCG
<i>Setaria viridis</i>	SETVI
<i>Sorghum halepense</i>	SORHA
<i>Lolium perenne</i>	LOLPE

**Table 9.10.1.2.6-2: The visual pytoxiicty effects 14 and 28 days after application <sup>1</sup>**

	g a.s./ha	ALOMY	APESV	DIGSA	ECHCG	SETVI	SORHA	LOLPE
Observed injury (%) of plants, 14 days after application								
AE F133402 WP 20	60	60	99	99	97	94	99	95
BCS-AZ 56567	22.5	0	0	0	0	0	0	0
Blank Formulation WP20	240*	0	0	0	0	0	0	0
Observed injury (%) of plants, 28 days after application								
AE F133402 WP 20	60	75	100	100	98	89	89	92
BCS-AZ 56567	22.5	0	0	0	0	0	0	0
Blank Formulation WP20	240*	0	0	0	0	0	0	0

\* amount of blank formulation applied

<sup>1</sup> Two replicates for each tested plants**Conclusion:**

In a direct comparison study under highly sensitive glasshouse screening conditions, the metabolite BCS-AZ 56567 (trifluoroacetic acid (Na-Salt) = TFA) showed no biological activity 14 and 28 days after treatment against the range of weeds tested.

**RMS comments:**

Summarized above study was performed in order to demonstrate pre - emergence biological activity of metabolite of flufenacet-BCS-AZ 56567 (trifluoroacetic acid (Na-Salt) in comparison with the parent substance.

The metabolite was tested at lower application rate than the parent. However, by taking into consideration the above studies with ( trifluoroacetic acid (Na –Salt) the metabolite may be considered as less biologically active than parent

The study is considered acceptable and provides supplemental information.

**Overall summary on biological activity testing:**

The four metabolites FOE oxalate (M01), FOE sulfonic acid (M02), FOE 5043- trifluoroethanesulfonic acid (M44) and trifluoroacetic acid (TFA) (M45) were tested for their biological activity in direct comparison studies under highly sensitive screening conditions. In these tests, none of the metabolites showed any significant herbicidal activity, i.e. the observed activity was always clearly lower than 50% compared to the parent active substance flufenacet. Since the activity threshold of 50% as given in Guidance Document Sanco/221/2000 rev. 10 (Anonymous, 2003) was never exceeded, all metabolites are considered to be non-relevant. Therefore it is concluded that all metabolites tested were considered to be not herbicidal active.

**B.9.11. LITERATURE DATA****Open literature review**

Complying with the data requirement set in the Article 8 point 5 of the Regulation (EC) 1107/2009, the Applicant submitted the following report presenting the results of the search of the scientific peer-reviewed open literature, according to EFSA Guidance Document “Submission of scientific peer-reviewed open literature for approval of pesticide active substances under Regulation (EC) No 1107/2009 [*EFSA Journal*; **2001**; 9(2); 2092]:

*Study 1:*

**Report:** Derpmann J., Teubner. L.; **2014**: “*Summary of the literature data for Flufenacet*”; Bayer CropScience; Document MCA: Section 9 Literature Data Flufenacet (unpublished document No. M-482180-01-1); issuing date: 2014. 03. 18;

**Guidelines:** EFSA Guidance Document “Submission of scientific peer-reviewed open literature for approval of pesticide active substances under Regulation (EC) No 1107/2009 [*EFSA Journal*; **2001**; 9(2); 2092]

**GLP:** no, not required

**RMS comments:** The literature search was performed in line with the recommendations of the EFSA Guideline, therefore, it can be considered acceptable.

Summary

The aim was to carry out the literature search in a comprehensive and transparent way, in order to fulfil the data requirement set, as mandatory for the AIR 3 compounds (such as flufenacet), by, at the time of submission, Regulation (EU) No 844/2012, subsequently repealed by the Regulation (EC) 283/2013, but in which this requirement was maintained. It was performed in line with the EFSA Guidance document on the “Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009” published in *EFSA Journal*; **2001**; 9(2); 2092 (further referred to as *EFSA Guidance document on literature search*). Presenting the adopted search strategy the Applicant stated that the evaluation was based on the examination of the abstracts of all references identified as potentially relevant. Patents were not considered in the literature search, because they are not included into the definition of “scientific peer-reviewed open literature”. The literature search covered the period of thirteen years predating the submission - from the 1<sup>st</sup> January 2000 to the 13<sup>th</sup> November 2013. Several data bases were used by the Applicant to perform the search. Their list is provided below, on figure B.9.11\_CA-1 (table reproduced from the report submitted by the Applicant).

Table 1: List of data bases for the literature search flufenacet and date of last database update

Database Name	Trifluoroacetic acid (C10)	Flufenacet and metabolites (except C10)
Agricola	2013-10-22	2013-11-05
Biosis	2013-10-30	2013-11-06
CABA	2013-10-30	2013-11-13
Chemical Abstracts	2013-11-01	2013-11-12
Derwent Drug File (DRUG)	2013-10-31	2013-11-07
EMBASE	2013-11-01	2013-11-12
Esbiobase	2013-10-28	2013-11-11
IPA	2013-10-16	2013-11-04
Medline	2013-11-02	2013-11-12
Pascal	2013-10-28	2013-11-11
PQSciTech	2013-10-18	2013-11-12
Registry	2013-10-31	2013-11-11
Scisearch	2013-10-28	2013-11-11
Toxcenter	2013-10-29	2013-11-12
Ulidat	2013-08-14	2013-08-14
FSTA	2013-10-28	2013-11-11

Figure B.9.11\_CA-1. List of data bases used By Applicant in literature search for Flufenacet (reoroduced from Applicact report)

It was indicated that as a preferred provider was selected STN – a scientific information platform hosted by CAS, division of the American Chemical Society.

The search was performed using the following key pieces of information: IUPAC name, CAS name/number, common names, codes and abbreviations, molecular structure, molecular formula, molar mass and other available names and/or codes.

The example of search input parameters is given below (for flufenacet) on figure B.9.11\_CA-2.

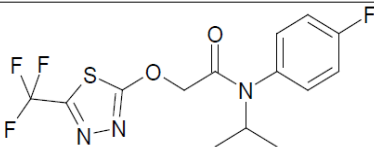
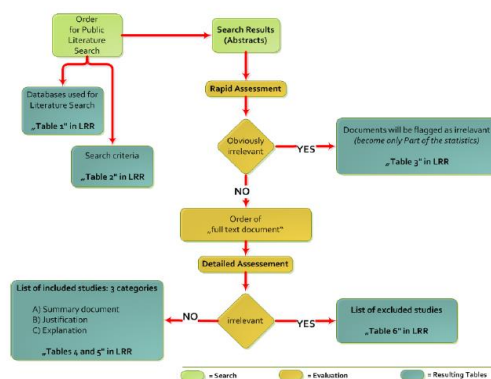
i) Flufenacet	
IUPAC name:	N-(4-fluorophenyl)-N-isopropyl-2-[[5-(trifluoromethyl)-1,3,4-thiadiazol-2-yl]oxy]acetamide
CAS number:	142459-58-3
STN Query	(142459-58-3 OR FLUFENACET OR FLUTHIAMID OR FLUTHIAMIDE OR THIAFLUAMIDE OR FOE 5043 OR (DRAGO OR DRADO OR CADOU OR TIARA OR FIREBRID OR FOSBURI OR HEROLD OR ANTILOPE)(W)(RTM OR R OR TM)) AND PY>1999 NOT P/DT
Molecular structure:	

Figure B.9.11\_CA-2: The example of search input parameters used by the Applicant in the literature search for Flufenacet (reproduced from the Applicant's report).

The search was performed for Flufenacet and its following degradation products: **FOE oxalate (M1)** – code C01, **FOE sulfonic acid (M2)** – code C02, **FOE-Thaidone (M9)** – code C03, **FOE Methylsulfone (M7)** – code C04, **FOE Thioglycolate sulfoxide** – code C05, **3-((2-((4-fluorophenyl)(isopropyl)amino)-2-oxoethyl)sulfinyl)-2-hydroxypropanoic acid** – code C06, **FOE-conjugate with cysteine** – code C07, **FOE trifluoroethane sulfonic acid** – code C08, **FOE Methylsulfide (M5)** – code C09 and **Trifluoroacetic acid (TFA, M45)** – code C10.

In case of Flufenacet and its degradation products bearing the codes C01 – C09 no keyword filter was used. Such filter was however used in case of TFA, for which 53 chains of refining key words (L2 – L54), encompassing all areas of evaluation, were used. They were all listed by the Applicant in the Appendix II to the study report. The whole search and evaluation procedure followed the conceptual scheme presented below on the figure B.9.11\_CA-3.



**Figure B.9.11\_CA-3:** The conceptual scheme of search and evaluation procedure used in the literature search for Flufenacet (reproduced from the Applicant’s report).

Initially 3489 publications were found, of which 369 were those for flufenacet and 3120 for its degradation products. Of that number 3089 were for TFA (including salts). No publications were found for either FOE Methylsulfide (M5) or FOE Trifluroethane sulfonic acid. Of the remaining 31, 11 publications were found for FOE oxalate (M1) and the same number for FOE sulfonic acid (M2), 4 for FOE-Thaidone (M9) and 2 for FOE Methylsulfone (M7). In case of the remaining four degradation products, for each of them 1 potentially relevant publication was found.

Evaluation of the search results was performed according to the rules set by the *EFSA Guidance document on literature search*.

At the “Rapid assessment” stage the following topics were used in assessment of the relevance of publications (considered as the exclusion criteria):

- Efficacy;
- Analytical method development;
- New ways of synthesis;
- Studies on a molecular level, which cannot be related to risk assessment (RA);
- Non-EU monitoring studies;
- Publications in non-EU language without English abstract;
- Abstract refers to a conference contribution and does not contain data, full text not available;
- Not relevant due to missing information: Studies with target organisms.

Using these criteria the Applicant excluded from the further assessment as not relevant 278 of 369 publications found for Flufenacet, 3036 of 3089 of those found for TFA, 8 for 11 found for FOE Oxalate, 7 of 11 found for

FOE sulfonic acid, 2 of 4 found for FOE-Thaidone and all found for four remaining degradation products. The remaining 153 publications – 91 for Flufenacet and 62 for its degradation products (including 53 for TFA) underwent the detailed assessment based on the examination of their full text versions.

In total 9 publications were found relevant or of unclear relevance and included into the dossier, as inform the results of the publication selection process presented in the Table 3 of the Applicant's report. The results of the detailed assessment were presented in three separate tables, in line with the recommendations given in the *EFSA Guidance document on literature search*. Tables 4 and 5 contain the relevant publications included into the dossier after the detailed assessment of full-text documents for relevance, ordered by data requirement (Table 4) and by authors (Table 5). These Tables contain 7 publications each. In Table 6 were listed all remaining publications evaluated at the "Detailed assessment" step, but found not relevant. The table contains 137 positions arranged in alphabetical order with regard to their first authors. For each of the listed publication their full citation data were provided together with a short rationale for their non-relevance. It was also stated that the Table 6 lists only 137 publications, while their number should be higher – 144. This difference may however be explained by the fact that, as declared the Applicant, double entries, resulting from the separated search performed for the compounds, were removed from the Table 6.

Below are presented the results of the detailed assessment of the publications found during the literature search (original Table 6).

They are given in tabularised form. First table – B.9.11\_CA-1, provides the list of publications considered not relevant by the Applicant. RMS decided to maintain the format of the table proposed by the Applicant.

**Table B.9.11\_CA-1: Results of the "Detailed assessment" stage of the literature search – publications not included into the dossier on the basis of their non-relevance (table reproduced from the Applicant's report, modified by the RMS).**

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Allesoe, Morten; Van Den Berg, Frans; Cornett, Claus; Joergensen, Flemming Steen; Halling-Soerensen, Bent; De Diego, Heidi Lopez; Hovgaard, Lars; Aaltonen, Jaakko; Rantanen, Jukka</b>	2008	Solvent diversity in polymorph screening	Journal of Pharmaceutical Sciences ( 2008 ), 97(6), 2145-2159	The investigation does not report results in values reflecting agreed determinants for the hazard characterization or risk assessment under Reg. EC No 1107/2009 and information is insufficient to transfer values into such determinants
<b>Amvrazi, Elpiniki G.; Papadi-Psyllou, Asimina T.; Tsiropoulos, Nikolaos G.</b>	2010	Pesticide enrichment factors and matrix effects on the determination of multiclass pesticides in tomato samples by single-drop microextraction (SDME) coupled with gas chromatography and comparison study between SDME and acetone-partition extraction procedure.	Int. J. Environ. Anal. Chem., Volume 90, Issue 3-6, Page 245-259, Publication Year 2010	No endpoint can be derived

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Anagnostopoulos, Chris; Bourmpopoulou, Aikaterini; Miliadis, George</b>	2013	Development and validation of a dispersive solid phase extraction liquid chromatography mass spectrometry method with electrospray ionization for the determination of multiclass pesticides and metabolites in meat and milk	Analytical Letters ( 2013 ), 46(16), 2526-2541	Findings not related to a certain test system
<b>Arcangeli, G.; Pasotti, A.</b>	2000	Flufenacet , a new selective herbicide on maize, soyabean, tomato and potato. Flufenacet , nuovo diserbante selettivo per mais, soia, pomodoro e patata.	Atti, Giornate fitopatologiche, Perugia, 16-20 aprile, 2000, Volume 2 (2000) , pp. 417-424.	The article reports on positive effects on crop yield
<b>Banerjee, Kaushik; Utture, Sagar; Dasgupta, Soma; Kandaswamy, Chandrasekar; Pradhan, Saswati; Kulkarni, Sunil; Adsule, Pandurang</b>	2012	Multiresidue determination of 375 organic contaminants including pesticides, polychlorinated biphenyls and polyaromatic hydrocarbons in fruits and vegetables by gas chromatography-triple quadrupole mass spectrometry with introduction of semi-quantification approach	Journal of Chromatography, A (2012), 1270, 283-295	No endpoint can be derived
<b>Barron, Leon; Paull, Brett [Reprint Author]</b>	2004	Determination of haloacetic acids in drinking water using suppressed micro-bore ion chromatography with solid phase extraction.	Analytica Chimica Acta, ( September 27 2004 ) Vol. 522, No. 2, pp. 153-161. print. ISSN: 0003-2670 (ISSN print).	Findings not related to a certain test system
<b>Basa Cesnik, Helena; Velikonja Bolta, Spela; Gregorcic, Ana.</b>	2012	Pesticide residues in samples of apples, lettuce and potatoes from integrated pest management in Slovenia from 2005-2009.	Acta Agric. Slov., Volume 99, Issue 1, Page 49-56, Publication Year 2012	Findings not related to a certain test system; monitoring data, not related to GAP
<b>Bazoobandi, M.; Yaduraju, N. T.; Kulshrestha, G.</b>	2000	Analysis of flufenacet in soil, wheat grain and straw by gas chromatography	Journal of Chromatography, A ( 2000 ), 886(1+2), 319-322	The article does not contain information related to the substance of concern, as flufenacet was not detected in any of the investigated water samples and is therefore assessed as irrelevant.
<b>Benesch, J. A.; Gustin, M. S.</b>	2002	Uptake of trifluoroacetate by Pinus ponderosa via atmospheric pathway	Atmospheric Environment ( 2002 ), 36(7), 1233-1235	An unphysiological exposure pathway is presented in this publication. TFA from PPP use is not airborne.
<b>Bhatarai, Barun; Gramatica, Paola</b>	2010	Per-and Polyfluoro Toxicity ( LC50 Inhalation ) Study in Rat and Mouse Using QSAR Modeling	Chemical Research in Toxicology ( 2010 ), 23(3), 528-539	Test method does not cover the right targets
<b>Brimblecombe P (Reprint) Lifongo L L; Bowden D J</b>	2004	Photodegradation of haloacetic acids in water	CHEMOSPHERE, ( APR 2004 ) Vol. 55, No. 3, pp. 467-476. ISSN: 0045-6535.	The article does not contain information related to the substance of concern
<b>Campagna, G.; Paci, F.; Fabbi, A.; Rapparini, G. Editor(S): Brunelli, A.; Canova, A.; Collina, M.</b>	2006	Percolation of acetochlor, dimethenamid, flufenacet and s-metolachlor applied in columns. Studio in colonna della percolazione di alcuni diserbanti residuali del mais.	Giornate Fitopatologiche 2006, Riccione (RN), 27-29 marzo 2006. Atti, volume primo (2006) , pp. 591-598.	The test design used does not fulfill the requirements of current test guidelines. Furthermore, the test was performed with a formulation and hence, the results are not useable for RA of ai

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Cesnik, Helena Basa; Bolta, Spela Velikonja; Gregorcic, Ana.</b>	2009	Pesticide residues in agricultural products of the slovene origin found in 2007.	Acta Chim. Slov., Volume 56, Issue 2, Page 484-493, Publication Year 2009	Findings not related to a certain test system; monitoring data not related to GAP
<b>Chen, Baiyang; Lee, Wontae; Westerhoff, Paul K.; Krasner, Stuart W.; Herckes, Pierre</b>	2010	Solar photolysis kinetics of disinfection byproducts	Water Research ( 2010 ), 44(11), 3401-3409	The article does not contain information related to the substance of concern
<b>Chiaia-Hernandez, Aurea C.; Krauss, Martin; Hollender, Juliane</b>	2013	Screening of Lake Sediments for Emerging Contaminants by Liquid Chromatography Atmospheric Pressure Photoionization and Electrospray Ionization Coupled to High Resolution Mass Spectrometry	Environmental Science and Technology ( 2013 ), 47(2), 976-986; Environmental Science and Technology (2013), 47(2), 976-986	The article does not contain information related to the substance of concern, as flufenacet was not detected in any of the investigated sediment samples.
<b>Claudio L; Kwa W C; Russell A L; Wallinga D</b>	2000	Testing methods for developmental neurotoxicity of environmental chemicals.	Toxicol.Appl.Pharmacol. (164, No. 1, 1-14, 2000) 3 Tab. 94 Ref. CODEN: TXAPA9	The article reports about resistance of organisms considered detrimental to human welfare and its fundaments (eg. nutrition, disease control) and deals as such on target organisms
<b>Conte, E.; Rossi, E.; Spera, G.; Pompei, V.; Carfi, F.; Spadoni, A. R.; Rosati, M.; Montereali, M. R.; Donnarumma, L.; Perconti, W.</b>	2003	Presence of plant protection products in three agricultural areas of Regione Lazio	Communications in Agricultural and Applied Biological Sciences ( 2003 ), 68(4b), 865-874	The observations made are based upon unknown exposure. Flufenacet was only found in soil of agricultural fields and not in water or air. However, it is not known at all if FFA was used since the agricultural uses were not reported.
<b>Cunha, S. C.; Fernandes, J. O.</b>	2011	Multipesticide residue analysis in maize combining acetonitrile-based extraction with dispersive liquid-liquid microextraction followed by gas chromatography-mass spectrometry.	J. Chromatogr., A, Volume 1218, Issue 43, Page 7748-7757, Publication Year 2011	Findings not related to a certain test system
<b>Cus, Franc; Cesnik, Helena Basa; Bolta, Spela Velikonja; Gregorcic, Ana.</b>	2009	Pesticide residues and microbiological quality of bottled wines.	Food Control, Volume 21, Issue 2, Page 150-154, Publication Year 2009	Findings not related to a certain test system
<b>Cus, Franc; Cesnik, Helena Basa; Bolta, Spela Velikonja; Gregorcic, Ana.</b>	2010	Pesticide residues in grapes and during vinification process.	Food Control, Volume 21, Issue 11, Page 1512-1518, Publication Year 2010	Target substance not a test item
<b>Deon, Jessica C.; Hurley, Michael D.; Wallington, Timothy J.; Mabury, Scott A.</b>	2006	Atmospheric Chemistry of N-methyl Perfluorobutane Sulfonamidoethanol, C <sub>4</sub> F <sub>9</sub> SO <sub>2</sub> N(CH <sub>3</sub> )CH <sub>2</sub> CH <sub>2</sub> OH: Kinetics and Mechanism of Reaction with OH	Environmental Science and Technology ( 2006 ), 40(6), 1862-1868	The article does not contain information related to the substance of concern
<b>Donovan S F; Pescatore M C</b>	2002	Method for measuring the logarithm of the octanol-water partition coefficient by using short octadecyl-poly(vinyl alcohol) high-performance liquid chromatography columns.	J.Chromatogr. (952, No. 1-2, 47-61, 2002) CODEN: JOCRAM	No endpoint can be derived

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Fantke, Peter; Juraske, Ronnie; Anton, Assumpcio; Friedrich, Rainer; Jolliet, Olivier.</b>	2011	Dynamic Multicrop Model to Characterize Impacts of Pesticides in Food.	Environ. Sci. Technol., Volume 45, Issue 20, Page 8842-8849, Publication Year 2011	No endpoint can be derived
<b>Fatemi, Mohammad Hossein; Heidari, Afsane; Ghorbanzade, Mehdi.</b>	2010	Prediction of aqueous solubility of drug-like compounds by using an artificial neural network and least-squares support vector machine.	Bull. Chem. Soc. Jpn., Volume 83, Issue 11, Page 1338-1345, Publication Year 2010	The article reports on chemical synthesis or development of methods for measurements of the chemical without its application to natural samples
<b>Fennimore, Steven A.; Lanini, W. Thomas; McGiffen, Milton E.; Bell, Carl E.</b>	2000	Evaluation of low-rate herbicides for potential use in vegetable crops	Proceedings of the Western Society of Weed Science ( 2000 ), 53, 53-57	Rates tested were 0.525 to 0.675 lb/acre which refers to 588 to 756 g/ha. Such high application rates are not relevant for EU risk assessments.
<b>Ferrer, Imma; Thurman, E. Michael.</b>	2007	Multi-residue method for the analysis of 101 pesticides and their degradates in food and water samples by liquid chromatography/time-of-flight mass spectrometry.	J. Chromatogr., A, Volume 1175, Issue 1, Page 24-37, Publication Year 2007	Findings not related to a certain test system; The article does not contain information related to the substance of concern
<b>Ferrer, Imma; Thurman, E. Michael; Zweigenbaum, Jerry A.</b>	2007	Screening and confirmation of 100 pesticides in food samples by liquid chromatography/tandem mass spectrometry.	Rapid Commun. Mass Spectrom., Volume 21, Issue 23, Page 3869-3882, Publication Year 2007	Findings not related to a certain test system
<b>Finizio, A.; Villa, S.; Vighi, M.</b>	2005	Predicting pesticide mixtures load in surface waters from a given crop.	Agric., Ecosyst. Environ., Volume 111, Issue 1-4, Page 111-118, Publication Year 2005	The article does not contain information related to the substance of concern
<b>Gajbhiye, V. T.; Suman Gupta; Agnihotri, N. P.; Gupta, S.</b>	2000	Gas liquid chromatographic method of analysis for a herbicide flufenacet (FOE 5043).	Pesticide Research Journal (2000) Volume 12, Number 1, pp. 41-47.	The article reports on chemical synthesis or development of methods for measurements of the chemical without its application to natural samples
<b>Godejohann, Markus; Berset, Jean-Daniel; Muff, Daniel.</b>	2011	Non-targeted analysis of wastewater treatment plant effluents by high performance liquid chromatography-time slice-solid phase extraction-nuclear magnetic resonance/time-of-flight-mass spectrometry.	J. Chromatogr., A, Volume 1218, Issue 51, Page 9202-9209, Publication Year 2011	The investigation does not report results in values reflecting agreed determinants for the hazard or exposure characterization or risk assessment under Reg. EC No 1107/2009. Flufenacet not in the supplementary list of analysed compounds
<b>Gravell, A.; Mills, G. A.; Civil, W.</b>	2012	Screening of pollutants in water samples and extracts from passive samplers using LC-MS and GC-MS.	LC-GC Eur., Volume 25, Issue 8, Page 404-406, 408, 410, 412, 414, 416, Publication Year 2012	The article does not contain information related to the substance of concern
<b>Grayson, Richard; Kay, Paul; Foulger, Miles</b>	2008	The use of GIS and multi-criteria evaluation (MCE) to identify agricultural land management practices which cause surface water pollution in drinking water supply catchments	Water Science and Technology ( 2008 ), 58(9), 1797-1802	The article does not contain information related to the substance of concern

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Gru, Yvan; Colin, Ronan; Le Cloirec, Pierre.</b>	2009	Organic molecule identification and confirmation by on-line SPE coupled to hybrid triple quadripole-linear ion trap mass spectrometry. Use of MRM-full scan MS/MS in environmental analysis.	Spectra Anal., Volume 38, Issue 270, Page 22-31, Publication Year 2009	No endpoint can be derived
<b>Gugaa, M.; Zarzecka, K.; Mystkowska, I.</b>	2009	The influence of herbicides on selected qualitative traits in potato. Wpływ herbicydów na wybrane cechy jakości bulw ziemniaka.	Progress in Plant Protection (2009) Volume 49, Number 1, pp. 436-439.	The investigation does not allow attributing the observations made to the substance of concern (e.g. mixture of substances, origin of exposure unclear)
<b>Gupta, Suman; Gajbhiye, Vijay T.</b>	2002	Effect of concentration, moisture and soil type on the dissipation of flufenacet from soil	Chemosphere ( 2002 ), 47(9), 901-906	The article descr. 4 tests in total. Only 1 was conducted in style of current guidelines. Its results are in line with known results, not influencing the RA. The other tests were performed at extreme conditions & are therefore not reliable & not usable for RA
<b>Hamilton, M. C.; Woudneh, M.; Grace, R.</b>	2007	Analysis of current use pesticides in environmental and wastewater samples by high resolution GC with high resolution mass spectrometric detection.	Organohalogen Compd., Volume 69, Page 600/1-600/4, Publication Year 2007	The article does not contain information related to the substance of concern, as flufenacet was not detected in any of the investigated water samples and is therefore assessed as irrelevant.
<b>Hanson M L (Reprint) Sibley P K; Ellis D A; Fineberg N A; Mabury S A; Solomon K R; Muir D C</b>	2002	Trichloroacetic acid fate and toxicity to the macrophytes <i>Myriophyllum spicatum</i> and <i>Myriophyllum sibiricum</i> under field conditions	AQUATIC TOXICOLOGY, ( MAR 2002 ) Vol. 56, No. 4, pp. 241-255. ISSN: 0166-445X.	The article does not contain information related to the substance of concern
<b>Hanson M L (Reprint) Sibley P K; Ellis D A; Mabury S A; Muir D C G; Solomon K R</b>	2002	Evaluation of monochloroacetic acid (MCA) degradation and toxicity to <i>Lemna gibba</i> , <i>Myriophyllum spicatum</i> , and <i>Myriophyllum sibiricum</i> in aquatic microcosms	AQUATIC TOXICOLOGY, ( 3 DEC 2002 ) Vol. 61, No. 3-4, pp. 251-273. ISSN: 0166-445X.	The article does not contain information related to the substance of concern
<b>Hanson M L (Reprint) Solomon K R</b>	2002	New technique for estimating thresholds of toxicity in ecological risk assessment	ENVIRONMENTAL SCIENCE and TECHNOLOGY, ( 1 AUG 2002 ) Vol. 36, No. 15, pp. 3257-3264. ISSN: 0013-936X.	The article does not contain information related to the substance of concern
<b>Harris, Kate A.; Dangerfield, Neil; Woudneh, Million; Brown, Tom; Verrin, Stacey; Ross, Peter S.</b>	2008	Partitioning of current-use and legacy pesticides in salmon habitat in British Columbia, Canada.	Environ. Toxicol. Chem., Volume 27, Issue 11, Page 2253-2262, Publication Year 2008	The article does not contain information related to the substance of concern
<b>Heidari, Mahmoud; Bahrami, Abdolrahman; Ghiasvand, Ali Reza; Shahna, Farshid Ghorbani; Soltanian, Ali Reza</b>	2013	A needle trap device packed with a sol-gel derived, multi-walled carbon nanotubes/silica composite for sampling and analysis of volatile organohalogen compounds in air	Analytica Chimica Acta (2013), 785, 67-74	The article does not contain information related to the substance of concern

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Hemmerling, Christlieb; Augustyniak, Bettina; Maye, Astrid; Seidl, Gilda; Warschewske, Guido.</b>	2009	Multi residual methods for pesticide analysis in fruit and vegetable sampling. Potentials and limits - Part 2: method comparison in samples with grown residuals.	Dtsch. Lebensm.-Rundsch., Volume 105, Issue 7, Page 435-443, Publication Year 2009	Findings not related to a certain test system or GAP
<b>Henne, S.; Shallcross, D. E.; Reimann, S.; Xiao, P.; Boulos, S.; Gerecke, A. C.; Brunner, D.</b>	2012	Environmental impacts of HFO-1234yf and other HFOs	Moving Towards Sustainability, ASHRAE/NIST Refrigerants Conference, Gaithersburg, MD, United States, Oct. 29-30, 2012 (2012), 13/1-13/13.	The observation made is based upon a route of exposure that cannot be considered representative for the intended use of the substance of concern
<b>Henne, Stephan [Reprint Author]; Shallcross, Dudley E.; Reimann, Stefan; Xiao, Ping; Brunner, Dominik; Odoherly, Simon; Buchmann, Brigitte</b>	2012	Future Emissions and Atmospheric Fate of HFC-1234yf from Mobile Air Conditioners in Europe.	Environmental Science and Technology, (FEB 7 2012) Vol. 46, No. 3, pp. 1650-1658.	The observation made is based upon a route of exposure that cannot be considered representative for the intended use of the substance of concern
<b>Hetherton, Christel L.; Sykes, Mark D.; Fussell, Richard J.; Goodall, David M.</b>	2004	A multi-residue screening method for the determination of 73 pesticides and metabolites in fruit and vegetables using high-performance liquid chromatography/tandem mass spectrometry.	Rapid Commun. Mass Spectrom., Volume 18, Issue 20, Page 2443-2450, Publication Year 2004	Findings not related to a certain test system
<b>Hiemstra, Maurice; De Kok, Andre.</b>	2007	Comprehensive multi-residue method for the target analysis of pesticides in crops using liquid chromatography-tandem mass spectrometry.	J. Chromatogr., A, Volume 1154, Issue 1-2, Page 3-25, Publication Year 2007	Findings not related to a certain test system
<b>Howard, Philip H.; Muir, Derek C. G.</b>	2010	Identifying New Persistent and Bioaccumulative Organics Among Chemicals in Commerce	Environmental Science and Technology ( 2010 ), 44(7), 2277-2285	The article does not contain information related to the substance of concern
<b>Hukushima, Kouhei; Masunaga, Miki; Miyahara, Yosiko; Tobino, Toshiaki.</b>	2009	Pesticide residue monitoring method in foods with tandem mass spectrometry. (2).	Kumamoto-ken Hoken Kankyo Kagaku Kenkyushoho, Volume 37, Page 36-47, Publication Year 2009	Findings not related to a certain test system
<b>Hurley, M. D.; Sulbaek Andersen, M. P.; Wallington, T. J.; Ellis, D. A.; Martin, J. W.; Mabury, S. A.</b>	2004	Atmospheric Chemistry of Perfluorinated Carboxylic Acids: Reaction with OH Radicals and Atmospheric Lifetimes	Journal of Physical Chemistry A ( 2004 ), 108(4), 615-620	The article addresses the kinetics of the atmospheric reaction of OH radicals with trifluoroacetic acid, not the substance of environmental relevance trifluoroacetate . In addition trifluoroacetate coming from herbicide use will not reach the atmosphere
<b>Hurley, M. D.; Wallington, T. J.; Andersen, M. P. Sulbaek; Ellis, D. A.; Martin, J. W.; Mabury, S. A.</b>	2004	Atmospheric Chemistry of Fluorinated Alcohols: Reaction with Cl Atoms and OH Radicals and Atmospheric Lifetimes	Journal of Physical Chemistry A ( 2004 ), 108(11), 1973-1979	The article does not contain information related to the substance of concern

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Inoue, Tomonori; Nagatomi, Yasushi; Kinami, Tomohisa; Uyama, Atsuo; Mochizuki, Naoki.</b>	2010	Fate of pesticides in a distilled spirit of barley shochu during the distillation process.	Biosci., Biotechnol., Biochem., Volume 74, Issue 12, Page 2518-2522, Publication Year 2010	Study design / test system not adequate
<b>Inoue, Tomonori; Nagatomi, Yasushi; Suga, Keiko; Uyama, Atsuo; Mochizuki, Naoki.</b>	2011	Fate of Pesticides during Beer Brewing.	J. Agric. Food Chem., Volume 59, Issue 8, Page 3857-3868, Publication Year 2011	Test method does not cover the right targets
<b>Ishii, Rie; Takahashi, Kunihiko; Horie, Masakazu.</b>	2006	Simultaneous determination of pesticide residues in crops by liquid chromatography with tandem mass spectrometry.	Shokuhin Eiseigaku Zasshi, Volume 47, Issue 5, Page 201-212, Publication Year 2006	No endpoint can be derived
<b>James Grichar, W.; Dotray, Peter A.; Ray Langham, D.</b>	2009	Sesame ( <i>Sesamum indicum</i> L.) response to preemergence herbicides.	Crop Prot., Volume 28, Issue 11, Page 928-933, Publication Year 2009	The article does not contain information related to the substance of concern
<b>Jones, N. E.; Smith, B. M. Editor(S): Boatman, N.; Bradbury, R.; Critchley, N.; Holland, J.; Marshall, J.; Ogilvy, S.</b>	2007	Effects of selective herbicide treatment, row width and spring cultivation on weed and arthropod communities in winter wheat.	Aspects of Applied Biology (2007) , Number 81, pp. 39-46.	The investigation does not allow attributing the observations made to the substance of concern (e.g. mixture of substances, origin of exposure unclear)
<b>Jursik, Miroslav; Soukup, Josef; Holec, Josef; Andr, Jiri.</b>	2011	Mechanisms of action of herbicides and manifestations of their effects on plants. Inhibitors of the biosynthesis of long chains of fatty acids.	Listy Cukrov. Reparske, Volume 127, Issue 1, Page 15-19, Publication Year 2011	The article reports on positive effects on crop yield
<b>Kern, Susanne; Singer, Heinz; Hollender, Juliane; Schwarzenbach, Rene P.; Fenner, Kathrin.</b>	2011	Assessing Exposure to Transformation Products of Soil-Applied Organic Contaminants in Surface Water: Comparison of Model Predictions and Field Data.	Environ. Sci. Technol., Volume 45, Issue 7, Page 2833-2841, Publication Year 2011	This article assesses the quality of a new modeling approach. However, the modeled is not validated and the measured surface water concentrations of FFA, FOE sulfonic acid and FOE oxalate are in line with known results not influencing RA
<b>Konieczka, Christopher M.; Colquhoun, Jed B.; Rittmeyer, Richard A.</b>	2009	Swamp dodder ( <i>Cuscuta gronovii</i> ) management in carrot production.	Weed Technol., Volume 23, Issue 3, Page 408-411, Publication Year 2009	The article reports on positive effects on crop yield
<b>Kowal, Sebastian; Balsaa, Peter; Werres, Friedrich; Schmidt, Torsten C.</b>	2013	Fully automated standard addition method for the quantification of 29 polar pesticide metabolites in different water bodies using LC-MS/MS	Analytical and Bioanalytical Chemistry ( 2013 ), 405(19), 6337-6351	Catchment areas for water body samples were not further specified (e.g. urban uses or agricultural use areas). Therefore, the relevance of the results can not be assessed adequately.
<b>Kutsuna, Shuzo [Reprint Author]; Hori, Hisao</b>	2008	Experimental determination of Henry's law constants of trifluoroacetic acid at 278-298 K.	Atmospheric Environment, ( MAR 2008 ) Vol. 42, No. 7, pp. 1399-1412. ISSN: 1352-2310.	The article addresses the Henry's law constant of trifluoroacetic acid. The substance of environmental relevance is the acetate for which measured data are available.

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Lacina, Ondrej; Zachariasova, Milena; Urbanova, Jana; Vaclavikova, Marta; Cajka, Tomas; Hajslova, Jana.</b>	2012	Critical assessment of extraction methods for the simultaneous determination of pesticide residues and mycotoxins in fruits, cereals, spices and oil seeds employing ultra-high performance liquid chromatography-tandem mass spectrometry.	J. Chromatogr. A, Volume 1262, Page 8-18, Publication Year 2012; J. Chromatogr., A, Volume 1262, Page 8-18, Publication Year 2012	No endpoint can be derived
<b>Lam, Christopher K.; McKinney, Mary K.; Clay, Val E.</b>	2002	Evaluation of laboratory and field extraction methods: extraction of [phenyl-U-14C] flufenacet from aged soils	ACS Symposium Series ( 2002 ), 813(Pesticide Environmental Fate), 153-166	Only two sampling intervals (DAT-0 and DAT-32) were analyzed and no half-lives could be calculated. The reported max. occurrence of the DPs is in line with known results and thus, not influencing RA
<b>Lechelt-Kunze, Christa; Meissner, Ruth C.; Drewes, Mark; Tietjen, Klaus</b>	2003	Flufenacet herbicide treatment phenocopies the fiddlehead mutant in Arabidopsis thaliana	Pest Management Science ( 2003 ), 59(8), 847-856.	Experiment was conducted with a mutant of Arabidopsis. Not relevant for natural occurring non-target plants
<b>Lee, Sung Joong; Park, Semin; Choi, Jin Young; Shim, Jae-Han; Shin, Eun-Ho; Choi, Jeong-Heui; Kim, Soo Taek; Abd El-Aty, A. M.; Jin, Jong Sung; Bae, Dong Won; Shin, Sung Chul.</b>	2009	Multiresidue analysis of pesticides with hydrolyzable functionality in cooked vegetables by liquid chromatography tandem mass spectrometry.	Biomed. Chromatogr., Volume 23, Issue 7, Page 719-731, Publication Year 2009	No endpoint can be derived
<b>Lee, Sung Jung; Park, Hyeong Jin; Kim, Wooseong; Jin, Jong Sung; Abd El-Aty, A. M.; Shim, Jae-Han; Shin, Sung Chul.</b>	2009	Multiresidue analysis of 47 pesticides in cooked wheat flour and polished rice by liquid chromatography with tandem mass spectrometry.	Biomed. Chromatogr., Volume 23, Issue 4, Page 434-442, Publication Year 2009	No endpoint can be derived
<b>Lehotay, Steven J.; De Kok, Andre; Hiemstra, Maurice; Van Bodegraven, Peter.</b>	2005	Validation of a fast and easy method for the determination of residues from 229 pesticides in fruits and vegetables using gas and liquid chromatography and mass spectrometric detection.	J. AOAC Int., Volume 88, Issue 2, Page 595-614, Publication Year 2005	Findings not related to a certain test system
<b>Li, Fei; Zhang, Chaojie; Qu, Yan; Chen, Jing; Hu, Xiang; Zhou, Qi (Reprint)</b>	2011	Method development for analysis of short-and long-chain perfluorinated acids in solid matrices	INTERNATIONAL JOURNAL OF ENVIRONMENTAL ANALYTICAL CHEMISTRY, ( 2011 ) Vol. 91, No. 12, pp. 1117-1134. ISSN: 0306-7319.	Study design / test system not relevant to EU data requirements
<b>Li, Yongjun; Wang, Meiling; Yan, Hongfei; Fu, Shanliang; Dai, Hua</b>	2013	Simultaneous determination of multiresidual phenyl acetanilide pesticides in different food commodities by solid-phase cleanup and gas chromatography-mass spectrometry	Journal of Separation Science ( 2013 ), 36(6), 1061-1069	Study design / test system not relevant to EU data requirements

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Lifongo, Lydia L.; Bowden, Derek J.; Brimblecombe, Peter</b>	2010	Thermal degradation of haloacetic acids in water	International Journal of Physical Sciences ( 2010 ), 5(6), 738-747	The article reports on chemical synthesis or development of methods for measurements of the chemical without its application to natural samples
<b>Loos, Martin; Krauss, Martin; Fenner, Kathrin.</b>	2012	Pesticide Nonextractable Residue Formation in Soil: Insights from Inverse Modeling of Degradation Time Series.	Environ. Sci. Technol., Volume 46, Issue 18, Page 9830-9837, Publication Year 2012	The article does not contain information related to the substance of concern
<b>Mabury, Scott A.</b>	2002	Redefining persistence .apprx. fluorinated pollutants in the environment	Proceedings of the Biennial International Conference on Monitoring and Measurement of the Environment, 4th, Toronto, ON, Canada, May 27-30, 2002 ( 2002 ), 35-40.	The observation made is based upon a route of exposure that cannot be considered representative for the intended use of the substance of concern. TFA as metabolite from thermolysis of perfluorinated polymers.
<b>Machefer, G. [Reprint Author]</b>	2000	Alternatives to isoproturon based products for the control of grasses and broad-leaved weeds in cereals. Original Title: Alternative losungen zu isoproturon-haltigen produkten bei der ungras-und unkrautbekämpfung in getreide.	Zeitschrift fuer Pflanzenkrankheiten und Pflanzenschutz, ( 2000 ) No. Sp. Iss. 17, pp. 501-508.	The article reports on desired effects on organisms considered as such target organisms
<b>Mezcua, Milagros; Malato, Octavio; Garcia-Reyes, Juan F.; Molina-Diaz, Antonio; Fernandez-Alba, Amadeo R.</b>	2009	Accurate-Mass Databases for Comprehensive Screening of Pesticide Residues in Food by Fast Liquid Chromatography Time-of-Flight Mass Spectrometry.	Anal. Chem. (Washington, DC, U. S.), Volume 81, Issue 3, Page 913-929, Publication Year 2009	No endpoint can be derived
<b>Milan, Marco; Ferrero, Aldo; Letey, Marilisa; De Palo, Fernando; Vidotto, Francesco</b>	2013	Effect of buffer strips and soil texture on runoff losses of flufenacet and isoxaflutole from maize fields	Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes ( 2013 ), 48(12), 1021-1033	Experimental design not well reported (e.g. no application rates and used formulation reported - NO monitoring study)
<b>Nguyen, Thanh Dong; Han, Eun Mi; Seo, Mi Suk; Kim, SA Ra; Yun, Mi Young; Lee, Dae Myung; Lee, Gae-Ho.</b>	2008	A multi-residue method for the determination of 203 pesticides in rice paddies using gas chromatography/mass spectrometry.	Anal. Chim. Acta, Volume 619, Issue 1, Page 67-74, Publication Year 2008	Study design / test system not relevant to EU data requirements
<b>Nguyen, Thanh Dong; Lee, Myoung Hee; Lee, Gae Ho.</b>	2008	Multiresidue determination of 156 pesticides in watermelon by dispersive solid phase extraction and gas chromatography/mass spectrometry.	Bull. Korean Chem. Soc., Volume 29, Issue 12, Page 2482-2486, Publication Year 2008	Test system not relevant to representative uses/GAPs
<b>Nilsson, E. J. K.; Nielsen, O. J.; Johnson, M. S.; Hurley, M. D.; Wallington, T. J.</b>	2009	Atmospheric chemistry of cis-CF <sub>3</sub> CH equals CHF: Kinetics of reactions with OH radicals and O <sub>3</sub> and products of OH radical initiated oxidation	Chemical Physics Letters ( 2009 ), 473(4-6), 233-237	The article does not contain information related to the substance of concern
<b>Ninomiya, Katsuyuki.</b>	2009	Study on simultaneous analysis of pesticides by LC/MS/MS.	Yokohama-shi Kankyo Kagaku Kenkyushoho, Volume 33, Page 86-90, Publication Year 2009	The article reports on chemical synthesis or development of methods for measurements of the chemical without its application to natural samples

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Nunez, Oscar; Gallart-Ayala, Hector; Ferrer, Imma; Moyano, Encarnacion; Galceran, Maria Teresa.</b>	2012	Strategies for the multi-residue analysis of 100 pesticides by liquid chromatography-triple quadrupole mass spectrometry.	J. Chromatogr., A, Volume 1249, Page 164-180, Publication Year 2012	Findings not related to a certain test system
<b>Nurmi, Joonas; Pellinen, Jukka.</b>	2011	Multiresidue method for the analysis of emerging contaminants in wastewater by ultra performance liquid chromatography-time-of-flight mass spectrometry.	J. Chromatogr., A, Volume 1218, Issue 38, Page 6712-6719, Publication Year 2011	No endpoint can be derived; The article does not contain information related to the substance of concern
<b>Oeberg, Tomas.</b>	2005	A QSAR for the hydroxyl radical reaction rate constant: validation, domain of application, and prediction.	Atmos. Environ., Volume 39, Issue 12, Page 2189-2200, Publication Year 2005	This article is not relevant as trifluoroacetate derived from the use of a herbicide will not reach the atmosphere.
<b>Pacanoski, Zvonko; Kostov, Tasko; Glatkova, Gordana; Knezevic, Branislav</b>	2007	Effect of soil applied herbicides and depth of sowing on common cocklebur ( <i>Xanthium strumarium</i> L.) and maize ( <i>Zea mays</i> L.) emergence and early growth	Plant Protection Science ( 2007 ), 43(3), 117-121	The investigation does not allow attributing the observations made to the substance of concern (e.g. mixture of substances, origin of exposure unclear)
<b>Padilla, S.; Corum, D.; Padnos, B.; Hunter, D. L.; Beam, A.; Houck, K. A.; Sipes, N.; Kleinstreuer, N.; Knudsen, T.; Dix, D. J.; Reif, D. M.</b>	2012	Zebrafish developmental screening of the ToxCast Phase I chemical library.	Reprod. Toxicol., Volume 33, Issue 2, Page 174-187, Publication Year 2012	The observations made are based upon an application or exposure that does not allow transferring the results into a value that can be used for the purpose of the hazard assessment or risk characterization.
<b>Pelletier, Jeffrey C.; Chengalvala, Murty; Cottom, Josh; Feingold, Irene; Garrick, Lloyd; Green, Daniel; Hauze, Diane; Huselton, Christine; Jetter, James; Kao, Wenling; Kopf, Gregory S.; Lundquist, Joseph T.; Mann, Charles; Mehlmann, John; Rogers, John; S</b>	2008	2-Phenyl-4-piperazinylbenzimidazoles: Orally active inhibitors of the gonadotropin releasing hormone (GnRH) receptor	Bioorganic and Medicinal Chemistry ( 2008 ), 16(13), 6617-6640	Target substance not a test item
<b>Perreau, Francois; Einhorn, Jacques</b>	2006	Determination of frequently detected herbicides in water by solid-phase microextraction and gas chromatography coupled to ion-trap tandem mass spectrometry	Analytical and Bioanalytical Chemistry ( 2006 ), 386(5), 1449-1456	No endpoint can be derived; The article reports on chemical synthesis or development of methods for measurements of the chemical without its application to natural samples
<b>Pesek, Joseph J.; Matyska, Maria T.; Fischer, Steven M.; Sana, Theodore R.</b>	2008	Analysis of hydrophilic metabolites by high-performance liquid chromatography-mass spectrometry using a silica hydride-based stationary phase	Journal of Chromatography, A ( 2008 ), 1204(1), 48-55	No endpoint can be derived

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Pizzutti, Ionara R.; De Kok, Andre; Hiemstra, Maurice; Wickert, Cristine; Prestes, Osmar D.</b>	2009	Method validation and comparison of acetonitrile and acetone extraction for the analysis of 169 pesticides in soya grain by liquid chromatography-tandem mass spectrometry.	J. Chromatogr., A, Volume 1216, Issue 21, Page 4539-4552, Publication Year 2009	Findings not related to a certain test system. The article reports about resistance of organisms considered detrimental to human welfare and its fundaments (eg. nutrition, disease control) and deals as such on target organisms
<b>Polgar, Laszlo; Garcia-Reyes, Juan F.; Fodor, Peter; Gyepes, Attila; Dernovics, Mihaly; Abranko, Laszlo; Gilbert-Lopez, Bienvenida; Molina-Diaz, Antonio.</b>	2012	Retrospective screening of relevant pesticide metabolites in food using liquid chromatography high resolution mass spectrometry and accurate-mass databases of parent molecules and diagnostic fragment ions.	J. Chromatogr., A, Volume 1249, Page 83-91, Publication Year 2012; J. Chromatogr. A, Volume 1249, Page 83-91, Publication Year 2012	No endpoint can be derived
<b>Ramsak, A.</b>	2001	Flufenacet (Terano, Axiom, Plateen): a new type of broadleaf and grass-weed control. Flufenacet (Terano, Axiom, Plateen): nov nacin zatiranja širokolistnih in ozkolistnih plevelov.	Proceedings of the 5th Slovenian Conference on Plant Protection, Catez ob Savi, Slovenia, 6-8 March 2001, pp. 404-408.	The article reports on positive effects on crop yield
<b>Rani, Sunita; Kumari, Beena; Kathpal, T. S.</b>	2006	Effect of pH on the dissipation behaviour of flufenacet (FOE-5043) in water	Pesticide Research Journal ( 2006 ), 18(2), 201-204	The test system is not clearly characterized, e.g. use of steile aqueous buffer or natural, microbial active water, the obtained results can not be ranged in the overall environmental fate of FFA
<b>Rapparini, G.; Campagna, G.; Campagna, S.</b>	2002	Sensitivity of replacement cultures to herbicides applied to maize and other plants. Sensibilita di colture di sostituzione a erbicidi impiegati su mais e altre sarchiate.	Informatore Agrario (2002) Volume 58, Number 14, pp. 83-87.	Data only for full application rates, thus not relevant for NTP risk assessment
<b>Rayne, Sierra; Forest, Kaya</b>	2009	Congener-specific organic carbon-normalized soil and sediment-water partitioning coefficients for the C1 through C8 perfluoroalkyl carboxylic and sulfonic acids	Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering ( 2009 ), 44(13), 1374-1387	The investigation does not report results in values reflecting agreed determinants for the hazard characterization or risk assessment under Reg. EC No 1107/2009 and information is insufficient to transfer values into such determinants
<b>Reagen, William K.; Lindstrom, Kent R.; Thompson, Kathy L.; Flaherty, John M.</b>	2004	Analytical Techniques and Method Validation for the Measurement of Selected Semivolatile and Nonvolatile Organofluorochemicals in Air	Journal of Occupational and Environmental Hygiene ( 2004 ), 1(9), 559-569	No endpoint can be derived
<b>Reboud, X.</b>	2002	Response of Chlamydomonas reinhardtii to herbicides: negative relationship between toxicity and water solubility across several herbicide families.	Bulletin of Environmental Contamination and Toxicology (2002) Volume 69, Number 4, pp. 554-561.	The investigation does not allow attributing the observations made to the substance of concern (e.g.mixture of substances, origin of exposure unclear)

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Reemtsma, Thorsten; Alder, Lutz; Banasiak, Ursula.</b>	2013	A multimethod for the determination of 150 pesticide metabolites in surface water and groundwater using direct injection liquid chromatography-mass spectrometry.	J. Chromatogr., A, Volume 1271, Issue 1, Page 95-104, Publication Year 2013	No endpoint can be derived; The article reports on chemical synthesis or development of methods for measurements of the chemical without its application to natural samples
<b>Renner, Rebecca</b>	2008	A tale of two fish.	Environmental Science and Technology, (15 Sep 2008) Vol. 42, No. 18, pp. 6784-6785. ISSN: 0013-936X CODEN: ESTHAG	The article does not contain information related to the substance of concern
<b>Roberti, R.; Badiali, F.; Pisi, A.; Veronesi, A.; Pancaldi, D.; Cesari, A.</b>	2006	Sensitivity of <i>Clonostachys rosea</i> and <i>Trichoderma</i> spp. as potential biocontrol agents to pesticides	Journal of Phytopathology ( 2006 ), 154(2), 100-109	The publication is regarded as an exploratory study as it provides information on effects on cellular or molecular level, investigates on biochemical or clinical responses or reports on metabolism or degradation from isolated micro-organisms
<b>Roberti, R.; Pisi, A.; Veronesi, A.; Cesari, A. Editor(S): Elad, Y.; Pertot, I.; Enkegaard, A.</b>	2004	Effect of fungicides and herbicides on in vitro sensitivity of <i>Clonostachys rosea</i> and different strains of <i>Trichoderma</i> .	Bulletin OILB/SROP (2004) Volume 27, Number 8, pp. 75-78.	The publication is regarded as an exploratory study as it provides information on effects on cellular or molecular level, investigates on biochemical or clinical responses or reports on metabolism or degradation from isolated micro-organisms
<b>Rouchaud, J.; Neus, O.; Eelen, H.; Bulcke, R.</b>	2001	Persistence, mobility, and adsorption of the herbicide flufenacet in the soil of winter wheat crops	Bulletin of Environmental Contamination and Toxicology ( 2001 ), 67(4), 609-616	The test design used does not fulfill the requirements of current test guidelines
<b>Sakkas, V. A.; Calza, P.; Vlachou, A. D.; Medana, C.; Minero, C.; Albanis, T.</b>	2011	Photocatalytic transformation of flufenacet over TiO <sub>2</sub> aqueous suspensions: Identification of intermediates and the mechanism involved	Applied Catalysis, B: Environmental ( 2011 ), 110, 238-250	The investigation does not report results in values reflecting agreed determinants for the hazard or exposure characterization or risk assessment under Reg. EC No 1107/2009. Phototransformation experiments all in combination with TiO <sub>2</sub>
<b>Saraji Mohammad; Bidgoli Ali Akbar Hajialiakbari</b>	2009	Single-drop microextraction with in-microvial derivatization for the determination of haloacetic acids in water sample by gas chromatography-mass spectrometry.	Journal of chromatography. A, (2009 Feb 13) Vol. 1216, No. 7, pp. 1059-66. Electronic Publication: 2008-12-27.	Study design / test system not adequate
<b>Schmatz, Ruediger; Ormerod, Corinna; Dick, Christian.</b>	2009	Herbicide tests in lemon balm ( <i>Melissa officinalis</i> L.) in Thuringia.	Gesunde Pflanz., Volume 61, Issue 3-4, Page 113-122, Publication Year 2009	The investigation does not allow attributing the observations made to the substance of concern (e.g. mixture of substances, origin of exposure unclear)

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Scott, B. F.; Spencer, C.; Martin, J. W.; Barra, R.; Bootsma, H. A.; Jones, K. C.; Johnston, A. E.; Muir, D. C. G.</b>	2005	Comparison of Haloacetic Acids in the Environment of the Northern and Southern Hemispheres	Environmental Science and Technology ( 2005 ), 39(22), 8664-8670	Provides monitoring information in air (not relevant for TFA from PPPs); in soil (no trend was observed from 1865 - 1956. Therefore, no link to use of PPPs) and in conifer needles (source of exposure unknown). Sources (anthropogenic or not) not clarified
<b>Shankle, Mark W. [Reprint Author]; Villordon, Arthur Q.; Cannon, James M.; Monks, David W.</b>	2002	Weed control and sweetpotato tolerance with flufenacet .	Hortscience, ( August, 2002 ) Vol. 37, No. 5, pp. 755.	The investigation does not allow attributing the observations made to the substance of concern (e.g.mixture of substances, origin of exposure unclear)
<b>Shoemaker, Jody A.</b>	2002	Method development for alachlor ESA and other acetanilide herbicide degradation products	Preprints of Extended Abstracts presented at the ACS National Meeting, American Chemical Society, Division of Environmental Chemistry ( 2002 ), 42(2), 311-316	The article does not contain information related to the substance of concern
<b>Sipes, Nisha S.; Martin, Matthew T.; Reif, David M.; Kleinstreuer, Nicole C.; Judson, Richard S.; Singh, Amar V.; Chandler, Kelly J.; Dix, David J.; Kavlock, Robert J.; Knudsen, Thomas B.</b>	2011	Predictive Models of Prenatal Developmental Toxicity from ToxCast High-Throughput Screening Data.	Toxicol. Sci., Volume 124, Issue 1, Page 109-127, Publication Year 2011	Does not report results in values reflecting agreed determinants for the risk assessment under 1107/2009. The publication is regarded as an exploratory study.
<b>Solomon K R (Reprint) Hanson M L; Sibley P K; Mabury S A; Muir D C G</b>	2003	Field level evaluation and risk assessment of the toxicity of dichloroacetic acid to the aquatic macrophytes Lemna gibba, Myriophyllum spicatum, and Myriophyllum sibiricum	ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY, ( MAY 2003 ) Vol. 55, No. 1, pp. 46-63. ISSN: 0147-6513.	The article does not contain information related to the substance of concern
<b>Soroka, S. V.; Soroka, L. I.; Guk, N. V.</b>	2002	Chemical control of weeds in winter wheats cultivated with alfalfa as a companion crop. Chemiczne zwalczanie chwastow w zbozach ozimych z wsiewka, koniczyny akowej.	Progress in Plant Protection (2002) Volume 42, Number 1, pp. 244-248	The article reports on desired effects on organisms considered as such target organisms
<b>Sulbaek Andersen, Mads P.; Axson, Jessica L.; Michelsen, Rebecca R. H.; Nielsen, Ole John; Iraci, Laura T.</b>	2011	Solubility of Acetic Acid and Trifluoroacetic Acid in Low-Temperature (207-245 K) Sulfuric Acid Solutions: Implications for the Upper Troposphere and Lower Stratosphere	Journal of Physical Chemistry A ( 2011 ), 115(17), 4388-4396	Solubility of trifluoroacetic acid (TFA) in aqueous Sulphuric acid solutions not relevant
<b>Tonnina, Daniele; Campanella, Luigi; Sammartino, Maria Pia; Visco, Giovanni</b>	2002	Integral toxicity test of sea waters by an algal biosensor	Annali di Chimica (Rome, Italy) ( 2002 ), 92(4), 477-484	The article reports on chemical synthesis or development of methods for measurements of the chemical without its application to natural samples

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Tsai, Wen-Tien</b>	2009	Environmental hazards and health risk of common liquid perfluoro-n-alkanes, potent greenhouse gases	Environment International ( 2009 ), 35(2), 418-424	The observation made is based upon a route of exposure that cannot be considered representative for the intended use of the substance of concern
<b>Tsai, Wen-Tien</b>	2011	Environmental property modeling of perfluorodecalin and its implications for environmental fate and hazards	Aerosol and Air Quality Research ( 2011 ), 11(7), 903-907	The observation made is based upon a route of exposure that cannot be considered representative for the intended use of the substance of concern
<b>Tsai, Wen-Tien</b>	2013	Environmental risks of new-generation fluorocarbons in replacement of potent greenhouse gases	International Journal of Global Warming [Int. J. Global Warming]. Vol. 5, no. 1, pp. 84-95. 2013.	The article does not contain information related to the substance of concern
<b>Ulrich, U.; Dietrich, A.; Fohrer, N.</b>	2013	Herbicide transport via surface runoff during intermittent artificial rainfall: A laboratory plot scale study	Catena ( 2013 ), 101, 38-49	Research paper employing an artificial study design at laboratory scale for basic research about runoff influencing factors. Results not transferable to real world situation, not suitable for regulatory risk assessments.
<b>Ulrich, Uta (Reprint) Ulrich, Uta (Reprint); Fohrer, Nicola Zeiger, Marcus</b>	2013	Soil structure and herbicide transport on soil surfaces during intermittent artificial rainfall	ZEITSCHRIFT FÜR GEOMORPHOLOGIE, ( MAR 2013 ) Vol. 57, Supp. [1], pp. 135-155. ISSN: 0372-8854.	Research paper employing an artificial study design at laboratory scale for basic research about runoff influencing factors. Results not transferable to real world situation, not suitable for regulatory risk assessments.
<b>Vargo, John D.; Lee, Edward A.; Fuhrman, John D.</b>	2003	Interlaboratory comparison and validation of methods for chloroacetanilide and chloroacetamide soil degradates in environmental waters	ACS Symposium Series ( 2003 ), 850(Liquid Chromatography/Mass Spectrometry, MS/MS and Time of Flight MS), 273-290	The article does not contain information related to the substance of concern
<b>Vasilakoglou, I. B.; Eleftherohorinos, I. G.; Dhima, K. B.</b>	2001	Activity, adsorption and mobility of three acetanilide and two new amide herbicides	Weed Research ( 2001 ), 41(6), 535-546	Test design used does not fulfill the requirements of current test guidelines. Test was performed with a formulation. Hence the results are not useable for RA of a.i.
<b>Vecitis, Chad D.; Cheng, Jie; Hoffmann, Michael R. (Reprint) Park, Hyunwoong Choi, Wonyong Mader, Brian T.</b>	2009	Reductive Defluorination of Aqueous Perfluorinated Alkyl Surfactants: Effects of Ionic Headgroup and Chain Length	JOURNAL OF PHYSICAL CHEMISTRY A, ( 29 JAN 2009 ) Vol. 113, No. 4, pp. 690-696. ISSN: 1089-5639.	The investigation does not report results in values reflecting agreed determinants for the hazard characterization or risk assessment under Reg. EC No 1107/2009 and information is insufficient to transfer values into such determinants
<b>Wagner, Andrea; Raue, Brigitte; Brauch, Heinz- Juergen; Worch, Eckhard; Lange, Frank T.</b>	2013	Determination of adsorbable organic fluorine from aqueous environmental samples by adsorption to polystyrene-divinylbenzene based activated carbon and combustion ion chromatography	Journal of Chromatography A (2013), 1295, 82-89	The article reports on chemical synthesis or development of methods for measurements of the chemical with application in natural and artificial samples. However, natural samples were not analyzed regarding TFA.

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Wakabayashi K; Boger P</b>	2002	Target sites for herbicides: entering the 21st century.	Pest Manage.Sci. (58, No. 11, 1149-54, 2002)	The publication is regarded as an exploratory study as it provides information on effects on cellular or molecular level, investigates on biochemical or clinical responses or reports on metabolism or degradation from isolated micro-organisms
<b>Walgren, Jennie L.; Jollow, David J.; Mcmillan, Joellyn M.</b>	2004	Induction of peroxisome proliferation in cultured hepatocytes by a series of halogenated acetates	Toxicology ( 2004 ), 197(3), 189-197	No endpoint can be derived
<b>Wallace, Russell W. [Reprint Author]</b>	2004	Pre and Early Postemergence Herbicide Phytotoxicity and Yield Effects in Transplanted Peppers.	Hortscience, ( JUL 2004 ) Vol. 39, No. 4, pp. 746.	The observations made are based upon an application or exposure that does not allow transferring the results into a value that can be used for the purpose of the hazard assessment or risk characterization
<b>Walorczyk, Stanisław (Correspondence); Gnusowski, Bogusław</b>	2009	Development and validation of a multi-residue method for the determination of pesticides in honeybees using acetonitrile-based extraction and gas chromatography-tandem quadrupole mass spectrometry.	Journal of Chromatography A, (11 Sep 2009) Vol. 1216, No. 37, pp. 6522-6531.	No endpoint can be derived; The investigation does not report results in values reflecting agreed determinants for the hazard characterization or risk assessment under Reg. EC No 1107/2009 and information is insufficient to transfer values into such determinants
<b>Walorczyk, Stanisław; Drozdzyński, Dariusz.</b>	2012	Improvement and extension to new analytes of a multi-residue method for the determination of pesticides in cereals and dry animal feed using gas chromatography-tandem quadrupole mass spectrometry revisited.	J. Chromatogr. A, Volume 1251, Page 219-231, Publication Year 2012; J. Chromatogr., A, Volume 1251, Page 219-231, Publication Year 2012	Findings not related to a certain test system
<b>Wang Leo; Schnute William C.</b>	2009	Determination of Trifluoroacetic Acid Using Ion Chromatography Mass Spectrometry	LC GC North America, (2009) (FEV, SUP), p. 18, 5 refs. ISSN: 1527-5949	No endpoint can be derived; The article reports on chemical synthesis or development of methods for measurements of the chemical without its application to natural samples
<b>Wang, Fang; Dicinoski, Greg W.; Haddad, Paul R. (Correspondence)</b>	2004	Simultaneous determination of monofluoroacetate, difluoroacetate and trifluoroacetate in environmental samples by ion chromatography.	Journal of Chromatography A, (2 Apr 2004) Vol. 1032, No. 1-2, pp. 31-35. Refs: 19 ISSN: 0021-9673 CODEN: JCRAEY	The article reports on chemical synthesis or development of methods for measurements of the chemical with application to natural samples outside Europe.
<b>Wang, Fang; Dicinoski, Greg W.; Zhu, Yan; Haddad, Paul R.</b>	2004	Simultaneous determination of fluoroacetates, chloroacetates, and bromoacetates in soil samples by ion chromatography	Australian Journal of Chemistry ( 2004 ), 57(10), 1005-1010	No endpoint can be derived; The article reports on chemical synthesis or development of methods for measurements of the chemical with application to natural samples outside Europe.

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
<b>Wang, Qiao-Yun; Ding, Xiang; Li, De-Jun; Wang, Xin-Ming</b>	2009	Determination of trifluoroacetic acid in environmental water samples by gas chromatography-mass spectrometry	Fenxi Shiyanshi ( 2009 ), 28(5), 68-71	The article reports on chemical synthesis or development of methods for measurements of the chemical. The publication is a matter of observations for environmental responses in a region outside Europe.
<b>Wilson, S. R.; Solomon, K. R.; Tang, X.</b>	2007	Changes in tropospheric composition and air quality due to stratospheric ozone depletion and climate change	Photochemical and Photobiological Sciences ( 2007 ), 6(3), 301-310	The article reports on chemical synthesis or development of methods for measurements of the chemical with application to natural samples outside Europe.
<b>Wlodarczyk, M.; Wybieralski, J.</b>	2006	Adsorption kinetics of atrazine and flufenacet and their water/soil partition coefficients Kd and KOC	Ekologia i Technika ( 2006 ), 14(1), 16-22	The test design used does not fulfill the requirements of current test guidelines. Furthermore, the test was performed with a mixed formulation (atrazine + flufenacet)
<b>Wlodarczyk, Malgorzata</b>	2009	Influence of adjuvant Adpros 850 SL on adsorption of flufenacet on soils with different organic carbon content	Progress in Plant Protection ( 2009 ), 49(3), 1456-1460	Test design used does not fulfill requirements of current test guidelines. Test was performed with a formulation. Hence, the results are not useable for RA of a.i.
<b>Wong, Jon; Hao, Chunyan; Zhang, Kai; Yang, Paul; Banerjee, Kaushik; Hayward, Douglas; Iftakhar, Imran; Schreiber, Andre; Tech, Katherine; Sack, Chris; Smoker, Michael; Chen, Xiangru; Utture, Sagar C.; Oulkar, Dasharath P.</b>	2010	Development and Interlaboratory Validation of a QuEChERS-Based Liquid Chromatography-Tandem Mass Spectrometry Method for Multiresidue Pesticide Analysis.	J. Agric. Food Chem., Volume 58, Issue 10, Page 5897-5903, Publication Year 2010	Findings not related to a certain test system
<b>Xiang W (Reprint) Xiang J; Zhang J G; Wu F; Tang J H</b>	2005	Geochemical transformation of trichloroacetic acid to chloroform in fresh waters - The results based upon laboratory experiments	WATER AIR AND SOIL POLLUTION, ( NOV 2005 ) Vol. 168, No. 1-4, pp. 289-312. ISSN: 0049-6979.	The article does not contain information related to the substance of concern
<b>You Qiang; Cheng Linling; Ju Cynthia</b>	2010	Generation of T cell responses targeting the reactive metabolite of halothane in mice .	Toxicology letters, (2010 May 4) Vol. 194, No. 3, pp. 79-85. Electronic Publication: 2010-02-13.	Observations can not be transferred into an endpoint
<b>Young, Cora J.; Hurley, Michael D.; Wallington, Timothy J.; Mabury, Scott A.</b>	2009	Atmospheric chemistry of CF <sub>3</sub> CF <sub>2</sub> H and CF <sub>3</sub> CF <sub>2</sub> CF <sub>2</sub> CF <sub>2</sub> H: Kinetics and products of gas-phase reactions with Cl atoms and OH radicals, infrared spectra, and formation of perfluorocarboxylic acids	Chemical Physics Letters ( 2009 ), 473(4-6), 251-256	The article reports on chemical synthesis or development of methods for measurements of the chemical without its application to natural samples

Author(s)	Year	Title	Source	Reason(s) for not including publication in dossier
Zarzecka, Krystyna (Correspondence); Gugaa, Marek; Mystkowska, Iwona	2010	Herbicide residues and nitrate concentration in tubers of table potatoes.	Journal of Toxicology and Environmental Health - Part A: Current Issues, (January 2010) Vol. 73, No. 17-18, pp. 1244-1249.	Test method does not cover the right targets
Zhang, Kai; Wong, Jon W.; Yang, Paul; Tech, Katherine; Dibenedetto, Alex L.; Lee, Nathaniel S.; Hayward, Douglas G.; Makovi, Carolyn M.; Krynitsky, Alexander J.; Banerjee, Kaushik; Jao, Lillian; Dasgupta, Soma; Smoker, Michael S.; Simonds, Roger; Schreibe	2011	Multiresidue pesticide analysis of agricultural commodities using acetonitrile salt-out extraction, dispersive solid-phase sample clean-up, and high-performance liquid chromatography-tandem mass spectrometry.	J. Agric. Food Chem., Volume 59, Issue 14, Page 7636-7646, Publication Year 2011	No endpoint can be derived

In the second table – B.9.11\_CA-2, are given the publications considered relevant by the Applicant after the detailed assessment and included into the documentation submitted for evaluation. As it was in case of the excluded publications, RMS presented only those publications that may be considered as addressing the issues related to the assessment of the ecotoxicology according to the data requirements set by the Regulation (EC) 283/2013.

**Table 9.11\_CA-2.: Report of all relevant publications that are included in the dossier after detailed assessment of full-text documents for relevance: ordered by data requirement.**

KCA - SANCO Data Point	KCP - SANCO Data Point	Author(s)	Year	Title	Source	Justification for classification of the publication
KCA 8.2.2.1. Fish early life stage toxicity test		Ulhaq, Mazhar; Carlsson, Gunnar (Correspondence); Orn, Stefan; Norrgren, Leif	2013	Comparison of developmental toxicity of seven perfluoroalkyl acids to zebrafish embryos	Environmental Toxicology and Pharmacology, (September 2013) Vol. 36, No. 2, pp. 423-426.	b) As no early life stage fish study is available for TFA, the NOEC of 300 mg a.s./L will be used in the risk assessment.
KCA 8.2.7. Effects on aquatic macrophytes		Hanson, Mark L.; Solomon, Keith R.	2004	Haloacetic acids in the aquatic environment. Part I: macrophyte toxicity	Environmental Pollution (Amsterdam, Netherlands) (2004), 130(3), 371-383.	b) As no endpoint for aquatic plant species is available for TFA, the lowest endpoint of this publication will be used for the risk assessment ( <i>Myriophyllum</i> sp.: 312.9 mg as/L).

KCA - SANCO Data Point	KCP - SANCO Data Point	Author(s)	Year	Title	Source	Justification for classification of the publication
KCA 8.7. Effects on other terrestrial organisms		Benesch, Jody A.; Gustin, Mae S.; Cramer, Grant R.; Cahill, Thomas M.	2002	Investigation of effects of trifluoroacetate on vernal pool ecosystems	Environmental Toxicology and Chemistry (2002), 21(3), 640-647.	b) This study confirms the results from an existing study of TFA on microbial nitrogen transformation. The microbial degradation is not affected due to the presence of TFA in soil.
KCA 8.7. Effects on other terrestrial organisms		Oehrle, Nathan W.; Green, Laura S.; Karr, Dale B.; Emerich, David W.	2004	The HFC/HCFC breakdown product trifluoroacetic acid (TFA) and its effects on the symbiosis between <i>Bradyrhizobium japonicum</i> and soybean ( <i>Glycine max</i> )	Soil Biology and Biochemistry (2004), 36(2), 333-342.	b) Soil concentrations at which effects were observed are far higher than PEC <sub>soil</sub> -figures.

\*double entries (due to the separated search for the compounds) were removed from this table.

In total, 4 relevant ecotoxicological publications were found by Applicant.

**RMS comments:**

The literature review is adequate.

For Flufenacet no information was identified which was considered to have impact on an EU - agreed endpoint or would require to adopt any of the risk assessment in the flufenacet supplementary ( renewal) dossier.

---

**Appendix II – Keywords used in the search for trifluoroacetic acid**

## Appendix B:

- L2 QUE SPE=ON ABB=ON PLU=ON ABNORMAL BEHAVIOUR OR ABORTION OR ACCEPTABLE DIETARY INTAKE OR ACCEPTABLE OPERATOR EXPOSURE LEVEL OR ACUTE DERMAL APPLICATION OR ACUTE DERMAL TOXICITY OR ACUTE EFFECT OR ACUTE EXPOSURE OR ACUTE ORAL TOXICITY OR ACUTE REFERENCE DOSE
- L3 QUE SPE=ON ABB=ON PLU=ON ACUTE TOXICITY OR ADDITIVE TOXICITY OR ADULT MORTALITY OR ADVERSE EFFECT OR ADVERSE EVENT OR AERIAL EXPOSURE OR AIR BLAST OR AIRBLAST OR ANORMAL BEHAVIOUR OR ASSESSMENT(1W)RISK OR AVERSIVE RESPONSE OR BBA MODEL OR BEHAVIOUR
- L4 QUE SPE=ON ABB=ON PLU=ON BEHAVIOURAL ANOMALIES OR BIO MONITORING OR BIOMONITORING OR BIRTH RATE OR BODY ORGANS OR BODY WEIGHT OR BREEDING LOSS OR BYSTANDER OR CARCINOGEN OR CARCINOGENIC OR CARCINOGENICITY OR CHANGE (1W) BODY WEIGHT OR CHEMOSIS OR CHRONIC CONCERN
- L5 QUE SPE=ON ABB=ON PLU=ON CHRONIC EFFECT OR CHRONIC STUDY OR CHRONIC TEST OR CHRONIC TOX OR CHRONIC TOXICITY OR CHRONIC TOXICOLOGICAL STUDY OR CLASTOGENICITY OR CLINICAL SIGN OR CLINICAL SYMPTOM OR CONJUNCTIVAE OR CONJUNCTIVAL CHEMOSIS OR CONJUNCTIVAL SAC
- L6 QUE SPE=ON ABB=ON PLU=ON CONSTIPATIO OR CONSUMER OR CONTACT TOXICITY OR CORNEA OR CORNEAL OPACITY OR CREATININE OR CROP INSPECTION OR CYTOPLASMIC CHANGES(1W)HEPATOCYTES OR DAMAGE TO EYES OR DEAD EMBRYO OR DEAD FETUS OR DEAD PUP OR DEATH OR DECREASE(1W)BODY LENGTH
- L7 QUE SPE=ON ABB=ON PLU=ON DERMAL OR DEVELOPMENTAL TOXICITY OR DIARRHEA OR DIE OR DIED OR DIETARY EXPOSURE OR DIETARY INTAKE OR DIETARY TOXICITY OR DIPPING OR DISLODGEABLE FOLIAR RESIDUE OR DISORIENTING OR DISTURBANCE(1W)VIABILITY OR DOG OR DUST DRIFT
- L8 QUE SPE=ON ABB=ON PLU=ON EFFECT(1W)(BODY WEIGHT OR FOOD CONSUMPTION OR BODY ORGAN) OR EMBRYO OR EMBRYOPATHY OR EMBRYOTOX OR ENDOCRINE(W)DISRUPT? OR ENDOCRINE MODULATION OR ENGINEERING CONTROL OR EPIDEMIOLOGICAL OR EPIDEMIOLOGY OR ERYTHEMA
- L9 QUE SPE=ON ABB=ON PLU=ON ESCHAR OR EUROPOEM OR EXPOSE OR EXPOSURE OR EYE IRRITATION OR FECUNDITY OR FEEDING STUDY OR FERTILITY RATE OR FETOTOX OR FETOTOXICOLOGICAL OR FETOTOXICOLOG Y OR FOETAL CROWN-RUMP LENGTH OR FOETAL DEVELOPMENT OR FOGGING OR FOLIAR DEPOSITION
- L10 QUE SPE=ON ABB=ON PLU=ON FOLIAR DISLODGEABLE RESIDUE OR GAIN(1W)BODY WEIGHT OR GASTROINTESTINAL OR GENOTOX OR GENOTOXIC OR GENOTOXICITY OR GENOTOXICOLOGICAL OR GESTATION OR GROUND BOOM OR GROUNDBOOM OR GUINEA PIG OR HAIR LOSS OR HAND TO MOUTH OR HANDHELD OR HAND-HELD
- L11 QUE SPE=ON ABB=ON PLU=ON HAZARD OR HEALTH RISK OR HEPATOTOXI N OR HERSHBERGER ASSAY OR HUMAN EXPOSURE OR HUMAN HEALTH OR HUMAN MONITORING OR IMMUNOTOXICITY OR IMPLANTATION LOSS OR INCREASE (1W) LIVER WEIGHT OR INDURATION (2W) SKIN OR INFERTILITY
- L12 QUE SPE=ON ABB=ON PLU=ON INHALATION OR INHALATORY ABSORPTION OR INHALATORY EXPOSURE OR INHALATORY RISK OR INTOXICATION OR INTRAPERITONEAL OR INTRAVENOUS OR INTRAVENOUSLY OR IRRITANT OR

- IRRITATING (1W)SKIN OR IRRITATION OR IRRITATION (2W)(IRIS OR SKIN)
- L13 QUE SPE=ON ABB=ON PLU=ON KNAPSACK OR LABORED BREATHING OR LACERATION (2W) SKIN OR LACTATION OR LC50 OR LD50 OR LIGHT-COLORED FECES OR LITTER SIZE OR LITTER WEIGHT OR LIVER OR LIVING PUPS OR LOCAL LYMPH NODE OR LONG-TERM EXPOSURE OR LONGTERM STUDY OR LONG-TERM STUDY
- L14 QUE SPE=ON ABB=ON PLU=ON LONGTERM TOXICOLOGICAL OR LONG-TERM TOXICOLOGICAL OR LOSS (1W)(BODY WEIGHT OR HAIR) OR MALFORMATION OR MAMMAL OR MAMMALIAN OR MARGIN (1W) SAFETY OR MATERNAL TOXICITY OR MATING BEHAVIOUR OR MEDICAL DATA OR METABOLIC PATH OR METABOLIC PATHWAY
- L15 QUE SPE=ON ABB=ON PLU=ON MONKEY OR MORTALITY OR MOUSE OR MRL EXCEEDANCE OR MRL VIOLATION OR MULTIGENERATION OR MUTAGEN OR MUTAGENIC OR MUTAGENICITY OR NECROPSY OR NEUROTOXIC OR NEUROTOXICITY OR NO OBSERVED ADVERSE EFFECT LEVEL OR NO OBSERVED EFFECT LEVEL
- L16 QUE SPE=ON ABB=ON PLU=ON NOAEL SUBCHRONIC DOG OR NON DIETARY EXPOSURE OR NON-DIETARY EXPOSURE OR NO-OBSERVED ADVERSE EFFECT LEVEL OR NURSING OR OBJECT TO MOUTH OR OCCUPATIONAL EXPOSURE OR OEDEMA OR OFFSPRING OR OPACITY OR OPERATOR OR ORAL ABSORPTION OR ORAL TOXICITY
- L17 QUE SPE=ON ABB=ON PLU=ON ORALLY OR OVULATION OR PARENTERAL OR PARTURITION OR PASSIVE DOSIMETRY OR PATHOLOGICAL OR PATHOLOGY OR PATIENT OR PEELING (1W) SKIN OR PENETRATION FACTOR OR PERCUTANEOUS OR PERSONAL PROTECTIVE EQUIPMENT OR PHOTOTOXICITY OR PILOERECTION
- L18 QUE SPE=ON ABB=ON PLU=ON PLACENTAL WEIGHT OR POISON OR POISONING OR POST-MORTEM EXAMINATIONS OR POSTNATAL OR POST-NATAL OR PREGNANCY OR PREGNANT OR PREMATURE BIRTH OR PRENATAL TOX OR PRENATAL TOXICOLOGY OR PRIMATE OR PROTECTIVE CLOTHING OR PROTECTIVE GARMENT
- L19 QUE SPE=ON ABB=ON PLU=ON PROTECTIVE GLOVE OR PUBLIC HEALTH OR RABBIT OR RAT OR RE ENTRY OR REDDENING (1W) TREATMENT AREA OR REDNESS OR REDUCED BODY WEIGHT OR REDUCED BODY WEIGHT GAIN OR REENTRY OR RE-ENTRY OR REFERENCE DOSE OR RELEVANT (2W) REPRODUCTIVE SUCCESS
- L20 QUE SPE=ON ABB=ON PLU=ON REPRODUCTION OR REPRODUCTIVE OR REPROTOX OR RESIDENT OR RESIDENTIAL EXPOSURE OR RESIDUE IN OR RESPIRATORY EXPOSURE OR RESPIRATORY PROTECTIVE EQUIPMENT OR RISK ASSESSMENT OR RISK (2W) (CONSUMER OR OPERATOR) OR SEXUAL
- L21 QUE SPE=ON ABB=ON PLU=ON RISK (1W) SERIOUS DAMAGE (1W) EYES OR SAFE OR SAFETY OR SAFETY ASSESSMENT OR SAFETY PRECAUTION OR SECONDARY EFFECT OR SECONDARY POISONING OR SEEDTROPEX OR SENSITISATION BY SKIN CONTACT OR SENSITISER OR SENSITISING TESTS OR SENSITIZER
- L22 QUE SPE=ON ABB=ON PLU=ON SHORT LONG TERM EXPOSURE OR SHORT-TERM EXPOSURE OR SHORT-TERM TOXICITY OR SHORT-TERM TOXICOLOGICAL OR SIDE EFFECT OR SIGNS (1W)(AGGRESSION OR TOXICITY) OR SKIN IRRITANT OR SKIN IRRITATION OR SKIN SENSITISATION OR SKIN SENSITISING
- L23 QUE SPE=ON ABB=ON PLU=ON SKIN SENSITIZATION OR SKIN SENSITIZING OR SLIGHTLY HARMFUL OR SPASTIC GAIT OR SPERMATOGENESIS OR SPLEEN OR SPRAY DRIFT OR STOMACH LESIONS OR STUNTED FETUS OR SUBACUTE OR SUB-ACUTE OR SUBCHRONIC OR SUB-CHRONIC OR SUBLETHAL OR SUB-LETHAL

- 
- L24 QUE SPE=ON ABB=ON PLU=ON SUBSTANCE-RELATED EFFECT OR SURVIVAL OR SYMPTOMS (1W) TOXICITY OR SYSTEMIC EXPOSURE OR SYSTEMIC INTOLERANCE REACTIONS OR TERATOGEN OR TERATOGENIC OR TERATOGENICITY OR TERATOLOGY OR TESTICULAR DEVELOPMENT
- L25 QUE SPE=ON ABB=ON PLU=ON ACTIVE INGREDIENTS (1W) SAFE OR (COMPOUND OR COMPOSITION OR FUNGICIDE OR INSECTICIDE OR PESTICIDE) (1W)SAFE OR THEORETICAL TOXICITY OR TOPICAL OR TOTAL DIET STUDY OR TOX OR TOXIC OR TOXICITY OR TOXICOGENOMIC OR TOXICOKINETICS OR TOXICOL
- L26 QUE SPE=ON ABB=ON PLU=ON TOXICOLOGICAL OR TOXICOLOGY OR TRACTOR MOUNTED OR TRANSDERMAL OR TRANSFER COEFFICIENT OR TRANSFERABLE RESIDUES OR TREATMENT RELATED EFFECTS OR TUMORIGEN OR TUNNEL TEST OR TWO-GENERATION OR UNACCEPTABLE EFFECTS OR UTEROTROPHIC ASSAY
- L27 QUE SPE=ON ABB=ON PLU=ON VERTEBRATE OR VIABILITY (1W) EMBRYO OR WEANING OR WEIGHT ALTERATION OR WEIGHTS OR WHOLE BODY DOSIMETER OR WHOLE BODY DOSIMETRY OR WORKER
- L28 QUE SPE=ON ABB=ON PLU=ON MONITORING OR QUECHERS OR TOLERANCE OR CHRONIC EXPOSURE OR METABOLITE OR METABOLISM OR CONSUMER EXPOSURE OR EXPOSURE (1W) CONSUMERS OR DIETARY RISK OR DIETARY RISK ASSESSMENT OR CONSUMPTION OR RESIDUE OR PROCESSING OR PROCESSED COMMODITY
- L29 QUE SPE=ON ABB=ON PLU=ON TRANSFER FACTOR OR PROCESSING FACTOR OR STORAGE OR ENFORCEMENT METHOD
- L30 QUE SPE=ON ABB=ON PLU=ON ADI OR AOEL OR ARFD OR DFR OR I.P. OR I.V. OR LLNA OR NOEL OR P.O. OR PHED OR PPE OR RPE OR S.C. OR UK POEM OR ILV
- L31 QUE SPE=ON ABB=ON PLU=ON ACUTETER OR ALGAL GROWTH OR AMPHIPODA OR APIS OR AQUATIC CRUSTACEANS OR AQUATIC GASTROPOD MOLLUSCS OR AQUATIC INSECTS OR AQUATIC INVERTEBRATES OR AQUATIC ORGANISM OR AQUATIC PEC OR AQUATIC PLANT OR AQUATIC POPULATION OR ASELLUS AQUATICUS
- L32 QUE SPE=ON ABB=ON PLU=ON BEE OR BIOACCUMULATION OR BIOCONCENTRATE RESIDUE OR BIOCONCENTRATED OR BIOCONCENTRATION OR BIRD OR BLACKBIRD OR BLUEGILL SUNFISH OR BOBWHITE OR CAGE TEST OR CAGE TRIAL OR COMMON SHREW OR COMMON VOLE OR CONCENTRATION(1W)NATURAL WATER BODIES
- L33 QUE SPE=ON ABB=ON PLU=ON CONTAMINATED FEED OR CONTAMINATED PREY OR CONTAMINATED WATER OR CRUSTACEA OR DAILY RESIDUE INTAKE OR DAPHNIA OR EARLY LIFE STAGE TEST OR EARTHWORM OR ECOBIOLOGY OR ECOTOX OR ECOTOXICOLOGICAL OR COTOXICOLOGY OR EFFECTS(1W)ARTHROPOD
- L34 QUE SPE=ON ABB=ON PLU=ON EFFECTS (1W)(BIRDS OR SOIL MICRO-ORGANISM) OR EGG PRODUCTION OR EISENIA FETIDA OR ESTIMATED THEORETICAL EXPOSURE OR ESTUARINE ORGANISM OR EXPOSURE OR FATHEAD MINNOW OR FAUNA OR FIELD RESIDUE STUDY OR FISH ACUTE OR FISH-EATER
- L35 QUE SPE=ON ABB=ON PLU=ON FISH-EATING BIRD OR FLORA OR FOLIAGE DWELLING ARTHROPODS OR FOLIAGE DWELLING PREDATORS OR FOOD CHICKS OR GAMMARUS OR GASTROPOD MOLLUSC C OR GREEN ALGAE OR GREENFINCH OR GROUND DWELLING ARTHROPODS OR GROUND DWELLING PREDATORS
- L36 QUE SPE=ON ABB=ON PLU=ON HALF-LIFE(1W)FLOWING WATERS OR HARE OR HATCHING SUCCESS OR HATCHLING HEALTH OR HAZARD QUOTIENT OR HERBIVORE OR HERBIVOROUS BIRDS OR HERBIVOROUS MAMMALS OR HONEYBEE OR INITIAL RESIDUES OR INSECTIVORE OR

- INSECTIVOROUS OR INTAKE RATE
- L37 QUE SPE=ON ABB=ON PLU=ON ISOPODA OR LACTATION INDEX OR LARVAL TOXICITY OR LEMNA OR LIFE CYCLE TEST OR LONG-TERM TER OR LOWEST LETHAL CONCENTRATION OR LOWEST LETHAL DOSE OR LOWEST OBSERVED EFFECT CONCENTRATION OR MACROSCOPIC FINDINGS OR MARINE ORGANISM OR MESOCOSM
- L38 QUE SPE=ON ABB=ON PLU=ON MICROBIAL ACTIVITY OR MICROCOSM OR NEGATIVE INFLUENCE (2W) PLANT SPECIES OR NO OBSERVED EFFECT CONCENTRATION OR NON TARGET OR NON TARGET MACRO ORGANISM OR NUMBER (1W) (SURVIVING ANIMALS OR SURVIVING WORMS)
- L39 QUE SPE=ON ABB=ON PLU=ON OIL MICRO- OR MACRO-ORGANISMS OR ONCORHYNCHUS MYKISS OR PARASITIDS OR PARASITIDS OR PHYTOTOXIC OR PHYTOTOXICITY OR PIMEPHALES PROMELAS OR PREDICTED ENVIRONMENTAL CONCENTRATION OR PSEUDOKIRCHNERIELLA SUBCAPITATA
- L40 QUE SPE=ON ABB=ON PLU=ON QUAIL OR RAINBOW TROUT OR REPRODUCTIVE (1W) WATERFLEAS OR RESIDUE DATA (1W) FISH OR RESIDUE (1W) FEED OR RESIDUE (2W) PESTICIDE OR RHOPALOSIPHIA OR RISK (1W) OFF-CROP AREAS OR RISK REDUCING MEASURES OR RISK (1W) BIRDS
- L41 QUE SPE=ON ABB=ON PLU=ON SEDIMENT DWELLER OR SEDIMENT DWELLERS OR SEEDEATER OR SELEN. CAPRICORNUTUM OR SELENASTRUM CAPRICORNUTUM OR SENSITIVE SPECIES OR SHREW OR SMALL SEEDEATER OR SOIL MICRO (1W) MACROORGANISM OR SOIL MICROORGANISM OR SOIL MICRO-ORGANISMS
- L42 QUE SPE=ON ABB=ON PLU=ON SOIL NON-TARGET MACRO-ORGANISMS OR SOIL NON-TARGET MICRO-ORGANISMS OR TER VALUE OR TERRESTRIAL ECOTOXICOLOGY OR TESTED WITH MUCH HIGHER RATES (3W) REGISTERED OR THRESHOLD EFFECT CONCENTRATION OR TOXICITY (2W) (FISH OR WATERFLEAS)
- L43 QUE SPE=ON ABB=ON PLU=ON VOLE OR WASP OR WATERFLEA OR WORST CASE EXPOSURE SCENARIO OR WORST-CASE TIME-WEIGHTED AVERAGE
- L44 QUE SPE=ON ABB=ON PLU=ON FUNGICIDE RESIDUE OR FUNGICIDES (1W) FRUITS (1W) VEGETABLES OR GROUND WATER OR GROUNDWATER OR HALF-LIFE OR HARVEST TIME RESIDUE OR HERBICIDE RESIDUE OR HERBICIDES (1W) FRUITS (1W) VEGETABLES OR IMPACT (1W) WATER TREATMENT PROCEDURE
- L45 QUE SPE=ON ABB=ON PLU=ON INSECTICIDE RESIDUE OR INSECTICIDES (1W) FRUITS (1W) VEGETABLES OR IPROVALICARB RESIDUE OR LACK (1W) LEACHING OR LANDSCAPE-LEVEL ERA OR LEACHING OR LENTIC WATER OR LOAMY SAND OR (MAXIMUM OR MAXIMAL) (W) RESIDUE SET OR MAXIMUM DAILY RESIDUE
- L46 QUE SPE=ON ABB=ON PLU=ON MAXIMUM RESIDUE OR METABOLISM OR METABOLITE (2W) (ANIMAL OR FOOD OR PLANT OR FRUIT OR VEGETABLE) OR MINIMUM RESIDUE TOLERANCE OR MULTIRESIDUE OR MULTI-RESIDUE OR NON-AGED (W) AGED LEACHING OR PELMO OR PERCENT (1W) APPLIED RADIOACTIVITY
- L47 QUE SPE=ON ABB=ON PLU=ON PERCENTAGE (2W) RADIOACTIVITY OR PERSISTENCE (2W) RESIDUE OR PESTICIDE RESIDUE OR PESTICIDES (1W) FRUITS (1W) VEGETABLES OR PHOTODEGRADATION (1W) SOIL OR PHOTOLYTICAL DEGRADATION OR PLANT METABOLITE OR POLLUTE OR POLLUTED OR POLLUTING
- L48 QUE SPE=ON ABB=ON PLU=ON POLLUTION OR POPULATION MODELLING OR PREDICTED ENVIRONMENTAL CONCENTRATION OR PROBABILISTIC OR QUANTIFY (1W) RESIDUE OR RAT METABOLITE OR READY BIODEGRADABILITY OR RESIDUAL (W) (FUNGICIDE OR HERBICIDE OR INSECTICIDE OR PESTICIDE)
- L49 QUE SPE=ON ABB=ON PLU=ON RESIDUE AMOUNT OR RESIDUE ANAL. OR

- 
- RESIDUE ANALYSIS OR RESIDUE ANALYTICAL DATA OR RESIDUE ANALYTICAL METHOD OR RESIDUE BEHAVIOUR OR RESIDUE CONTENT OR RESIDUE DATA OR RESIDUE DEFINITION OR RESIDUE DETECTION OR RESIDUE(1W)FOOD OR RESIDUE LEVEL
- L50 QUE SPE=ON ABB=ON PLU=ON RESIDUE LIMIT OR RESIDUE MONITORING OR RESIDUE (2W)(FUNGICIDE OR HERBICIDE OR INSECTICIDE OR PESTICIDE OR IPROVALICARB) OR RESIDUE-TOLERANCE OR RESIDUE PERSISTENCE OR RESIDUE POINT(1W)VIEW OR RESIDUE TEST
- L51 QUE SPE=ON ABB=ON PLU=ON RESIDUE TRIALS OR RESIDUE VALUES OR RESIDUE ABOVE (1W)MRL OR RESIDUE (1W) CONTAMINANT OR RESIDUE (1W)(CROPS OR FOOD OR PLANTS OR TREATED PRODUCTS) OR INCURRED RESIDUE
- L52 QUE SPE=ON ABB=ON PLU=ON SEDIMENT SYSTEM OR SEWAGE OR SIMULATION MODEL PELMO OR SLOW MOVING WATER BODIES OR SLOWLY FLOWING WATER BODIES OR SOIL ACCUMULATION TESTING OR SOIL DEGRADATION OR SOIL DISSIPATION OR SOIL METABOLISM STUDY
- L53 QUE SPE=ON ABB=ON PLU=ON SOIL PHOTOLYSIS OR SOIL SCENARIO OR SOIL(W)FOLIAGE DWELLERS OR SPRAY DRIFT RATE OR STATIC WATER BODY OR SURFACE WATER OR TERRESTRIAL AQUATIC FIELD DISSIPATION OR TERRESTRIAL FIELD DISSIPATION OR TERRESTRIAL SEDIMENT FIELD DISSIPATION
- L54 QUE SPE=ON ABB=ON PLU=ON TOXIC RESIDUE OR WATER BODY OR WATER SEDIMENT STUDY OR WATER SEDIMENT SYSTEM OR WATER TREATMENT OR WORST CASE ASSUMPTION OR WORST CASE CONDITION OR WORST CASE SCENARIO OR WORST CASE SITUATION OR WORST CASE USE PATTERNS OR WORST-CASE APPROACH

**B.9.12. REFERENCES RELIED ON**

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.1.1 /01	Jans, D.	2010	Determination of residues of Flufenacet SC 500 g/L in <i>Poecilus cupreus</i> L. (Coleoptera, Carabidae) in an extended laboratory test Bayer CropScience, Report No.: CW09/028, Edition Number: <u>M-368306-01-1</u> Date: 2010-05-05 GLP/GEP: yes, unpublished	N	Y	Information needed for risk assessment	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.1.1 /02	Noss, G.; Diehl, P.	2012	Determination of the residues of flufenacet in/on winter barley and winter wheat after spray application of flufenacet & diflufenican SC 600 in Germany, the Netherlands and Belgium Bayer CropScience, Report No.: 11-2950, Report includes Trial Nos.: 11-2950-01 11-2950-02 11-2950-03 11-2950-04 Edition Number: <u>M-443138-01-1</u> Date: 2012-12-03 GLP/GEP: yes, unpublished	N	Y	Data basis for calculation of DT <sub>50</sub> (for M-451178-01-1)	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.1.1 /03	Scherr, F.; Ellerich, C.	2013	Statement on residue dissipation of flufenacet in treated foliage of monocotyledonous plants: kinetic evaluation - Flufenacet (FOE 5043) Bayer CropScience, Report No.: EnSa-13-0197, Edition Number: <u>M-451178-01-1</u> Date: 2013-04-05 GLP/GEP: no, unpublished	N	Y	Kinetic evaluation to derive DT <sub>50</sub> as refinement for bird and mammals RA	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.1.1.1 /01	██████████ ██████	1992	Technical FOE 5043: An acute oral LD50 with bobwhite quail ████████████████████ ██████████ ██████████ Report No.: 102642, Edition Number: <u>M-003866-01-1</u> EPA MRID No.: 43441113 Date: 1992-05-12 GLP/GEP: yes, unpublished	Y	Y		Bayer CropScience	The study was evaluated in the DAR (1997)

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.1.1.1 /02	██████████ ██████████ ██████████	1997	FOE 5043 technical: An acute oral LD50 with mallards ██████████ ██████████, Report No.: 107700, Edition Number: <u>M-003851-01-1</u> EPA MRID No.: 44246801 Date: 1997-03-11 GLP/GEP: yes, unpublished	Y	Y	Study needed for risk assessment	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.1.1.1 /03	██████████	2013	Toxicity of flufenacet technical during an acute oral LD50 with the canary (Serinus canaria) ██████████ ██████████, Report No.: 07SRLS13C5, Edition Number: <u>M-468210-01-1</u> EPA MRID No.: 49244202 Date: 2013-10-25 GLP/GEP: yes, unpublished	Y	Y	Study required by US EPA on passerine bird. Reveals lowest acute bird endpoint.	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.1.1.2 /01	██████████ █	1993	FOE 5043 technical: A subacute dietary LC50 with mallard duck ██████████ ██████████, Report No.: 103814, Edition Number: <u>M-003864-01-1</u> EPA MRID No.: 43441115 Date: 1993-03-30 GLP/GEP: yes, unpublished	Y	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.1.1.2 /02	██████████	1994	FOE 5043 technical: A subacute dietary LC50 with northern bobwhite ██████████ ██████████, Report No.: 106583, Edition Number: <u>M-003859-01-1</u> EPA MRID No.: 43441114 Date: 1994-06-08 GLP/GEP: yes, unpublished	Y	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.1.1.3 /01	██████████ ██ ██████████ ██████████	1994	Effect of technical FOE 5043 on mallard reproduction ██████████ ██████████, Report No.: 106594, Edition Number: <u>M-003858-01-1</u> EPA MRID No.: 43441120 Date: 1994-07-08 GLP/GEP: yes, unpublished	Y	Y		Bayer CropScience	The study was evaluated in the DAR (1997)

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.1.1.3 /02		1994	Effects of a subchronic dietary exposure of FOE 5043 techn. on bobwhite quail including effects on reproduction and health [REDACTED] [REDACTED], Report No.: SXR/REP 03, Edition Number: <u>M-003861-01-1</u> EPA MRID No.: 43441119 Date: 1994-04-30 GLP/GEP: yes, unpublished	Y	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.1.2.2 /01	Diesing, L.	2014	Flufenacet -Toxicity endpoint for the wild mammal reproductive risk assessment Bayer CropScience, Report No.: <u>M-476600-01-1</u> , Edition Number: <u>M-476600-01-1</u> Date: 2014-02-12 GLP/GEP: n.a., unpublished	N	N		Bayer CropScience	-
KCA 8.1.2.2 /02	Diesing, L.	2014	Trifluoroacetate (TFA) - Toxicity endpoints for terrestrial vertebrate risk assessments Bayer CropScience, Report No.: <u>M-477154-01-1</u> , Edition Number: <u>M-477154-01-1</u> Date: 2014-02-18 GLP/GEP: n.a., unpublished	N	N		Bayer CropScience	-
KCA 8.2.1 /01		1995	Acute Toxicity of FOE 5043 technical to the rainbow trout ( <i>Oncorhynchus mykiss</i> ) under static-renewal conditions [REDACTED] [REDACTED], Report No.: 106673, Edition Number: <u>M-002379-01-1</u> EPA MRID No.: 43850007 Date: 1995-01-18 GLP/GEP: yes, unpublished	Y	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.2.1 /02		1995	Acute toxicity of FOE 5043 technical to the bluegill ( <i>Lepomis macrochirus</i> ) under static-renewal conditions [REDACTED] [REDACTED], Report No.: 106674, Edition Number: <u>M-002378-01-1</u> EPA MRID No.: 43595501 Date: 1995-01-20 GLP/GEP: yes, unpublished	Y	Y		Bayer CropScience	The study was evaluated in the DAR (1997)

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.2.1 /03	██████████ ██████████	1995	FOE 5043 Sulfonic acid - Acute toxicity (96 hours) to rainbow trout (Oncorhynchus mykiss) in a static test ██████████ ██████████ ██████████ Report No.: DOM 95031, Edition Number: <u>M-004932-01-1</u> Date: 1995-09-28 GLP/GEP: yes, unpublished	Y	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.2.1 /04	██████████ ██████████ ██████████	1994	Identification of radioactive residues of Phenyl-(14C) FOE 5043 in bluegill sunfish (Lepomis macrochirus) ██████████ ██████████ Report No.: 106577, Edition Number: <u>M-003804-01-1</u> EPA MRID No.: 43441139 Date: 1994-07-13 GLP/GEP: yes, unpublished ...also filed: KCA 6.2.5 /01	Y	Y	New data requirement (fish metabolism)	Bayer CropScience	The study was evaluated in the Addendum (2003) to the DAR ( 1997)
KCA 8.2.1 /05	██████████ ██████████ ██████████	1994	Acute toxicity of FOE 5043 to the sheepshead minnow (Cyprinodon variegatus) under static renewal conditions ██████████ ██████████ ██████████ Report No.: 106421, Edition Number: <u>M-002422-01-1</u> EPA MRID No.: 43441122 Date: 1994-03-10 GLP/GEP: yes, unpublished	Y	Y	US Study, not submitted to EU so far	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.1 /06	██████████ ██████████ ██████████	1998	Acute toxicity of Thiadone to the rainbow trout (Oncorhynchus mykiss) under static conditions ██████████ ██████████ Report No.: 108738, Edition Number: <u>M-005388-01-1</u> Date: 1998-12-18 GLP/GEP: yes, unpublished	Y	Y		Bayer CropScience	The study was evaluated in the Addendum (2000 and 2003) to the DAR ( 1997)

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.2.1 /07	██████████ ██████████ ██████████	1999	Acute toxicity of thiadone to the sheephead minnow (Cyprinodon variegatus) under static conditions ██████████ ██████████, Report No.: 108809, Edition Number: <u>M-009684-01-1</u> Date: 1999-03-08 GLP/GEP: yes, unpublished	Y	Y	US Study, not submitted to EU so far	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.1 /08	██████████ ██████████	1999	Acute toxicity of Thiadone, a metabolite of FOE 5043, to the bluegill (Lepomis macrochirus) ██████████ ██████████, Report No.: 108455, Edition Number: <u>M-016583-01-1</u> Date: 1999-03-22 GLP/GEP: yes, unpublished	Y	Y	US Study, not submitted to EU so far	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.1 /09	██████████	2010	Acute toxicity of flufenacet (tech.) to fish (Cyprinus carpio) under static-renewal conditions - Amendment 2 from 2012-04-05 ██████████, Report No.: EBFOL149, Edition Number: <u>M-361666-03-1</u> Date: 2010-01-15 <b>...Amended: 2012-04-05</b> GLP/GEP: yes, unpublished	Y	Y		Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.1 /10	██████████ ██████████ ██████████ ██████████ ██████████	1992	The acute toxicity of sodium trifluoroacetate to the zebra fish Brachydanio rerio ██████████ ██████████, Report No.: C047202, Report includes Trial Nos.: C.SOL.51.040 Edition Number: <u>M-247889-01-1</u> Date: 1992-07-20 GLP/GEP: yes, unpublished	Y	Y	Required study for new soil metabolite	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.2.1 /01	Ulhaq, M.; Carlsson, G.; Oern, S.; Norrgrén, L.	2013	Comparison of developmental toxicity of seven perfluoroalkyl acids to zebrafish embryos . Year:2013, Report No.: <u>M-462660-01-1</u> , Edition Number: <u>M-462660-01-1</u> GLP/GEP: n.a., published	N	N			New submitted article for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.2.2.1 /01	Ulhaq, M.; Carlsson, G.; Oern, S.; Norrgren, L.	2013	Comparison of developmental toxicity of seven perfluoroalkyl acids to zebrafish embryos . Year:2013, Report No.: <a href="#">M-462660-01-1</a> , Edition Number: <a href="#">M-462660-01-1</a> GLP/GEP: n.a., published	N	N			New submitted article for renewal of a.s.
KCA 8.2.2 /01	[REDACTED]	1995	Early life stage toxicity of FOE 5043 technical to the Rainbow Trout (Oncorhynchus mykiss) under flow-through conditions [REDACTED] [REDACTED], Report No.: 106978, Edition Number: <a href="#">M-002357-01-1</a> EPA MRID No.: 43795301 Date: 1995-08-17 GLP/GEP: yes, unpublished	Y	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.2.2.1 /01	Ulhaq, M.; Carlsson, G.; Oern, S.; Norrgren, L.	2013	Comparison of developmental toxicity of seven perfluoroalkyl acids to zebrafish embryos . Year:2013, Report No.: <a href="#">M-462660-01-1</a> , Edition Number: <a href="#">M-462660-01-1</a> GLP/GEP: n.a., published	N	N			New submitted
KCA 8.2.2.1 /02	[REDACTED]	2013	Early life stage toxicity of flufenacet technical to the sheepshead minnow (Cyprinodon variegatus) under flow-through conditions [REDACTED] [REDACTED] [REDACTED] [REDACTED], Report No.: EBFOL244, Edition Number: <a href="#">M-464909-01-1</a> EPA MRID No.: 49244201 Date: 2013-08-14 GLP/GEP: yes, unpublished	Y	Y	Study required by US EPA on saltwater fish. Reveals lowest ELS endpoint	Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.2.2.2 /01	██████████ ██████████	2002	Fathead minnow (Pimephales promelas) fish life cycle test with flufenacet (FOE 5043 technical) ████████████████████ ██████████ ██████████, Report No.: 109767, Edition Number: <u>M-082934-01-1</u> Date: 2002-10-28 GLP/GEP: yes, unpublished	Y	Y	US Study, not submitted to EU so far	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.2.3 /01	██████████ █	1994	Uptake, depuration and bioconcentration of 14C-FOE 5043 technical by bluegill (Lepomis macrochirus) under flow-through conditions ████████████████████ ██████████ ██████████, Report No.: 106760, Edition Number: <u>M-003803-01-1</u> EPA MRID No.: 43441127 Date: 1994-07-08 GLP/GEP: yes, unpublished ...also filed: <b>KCA 6.2.5 /02</b>	Y	Y	New data requirement (fish metabolism)	Bayer CropScience	The study was evaluated in the the DAR ( 1997)
KCA 8.2.4.1 /01	Bowers, L. M.	1994	Acute toxicity of FOE 5043 technical to the waterflea (Daphnia magna) under static conditions Bayer Corporation, Kansas City, MO, USA Bayer CropScience, Report No.: 106597, Edition Number: <u>M-003805-01-1</u> EPA MRID No.: 43441118 Date: 1994-06-24 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.2.4.1 /02	Heimbach, F.	1995	Acute toxicity of FOE 5043-sulfonic acid to water fleas (Daphnia magna) Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: HBF/DM 145, Edition Number: <u>M-004930-01-1</u> Date: 1995-10-09 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.2.4.1 /03	Bowers, L. M.; Lam, C. V.	1998	Acute toxicity of Thiadone (a metabolite of FOE 5043) to the waterflea Daphnia magna under static conditions Bayer Corporation, Stilwell, KS, USA Bayer CropScience, Report No.: 108464, Edition Number: <u>M-005390-01-1</u> Date: 1998-12-18 GLP/GEP: yes, unpublished	N	Y	US Study, not submitted to EU so far	Bayer CropScience	The study was evaluated in the Addendum (2000 and 2003) to the DAR (1997)
KCA 8.2.4.1 /04	Groenevald, A. H. C.; de Kok, H. A. M.; van den Berg, G.	1992	The acute toxicity of sodium trifluoroacetate to Daphnia magna Solvay Duphar, Netherlands; Bayer CropScience, Report No.: C047203, Report includes Trial Nos.: C.SOL.51.039 Edition Number: <u>M-247890-01-1</u> Date: 1992-07-20 GLP/GEP: yes, unpublished	N	Y	New major metabolite	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.4.2 /01	Bowers, L. M.	1995	Acute toxicity of FOE 5043 (technical) to Hyalella azteca under static conditions Bayer Corporation, Stilwell, KS, USA Bayer CropScience, Report No.: 106908, Edition Number: <u>M-002374-01-1</u> EPA MRID No.: 43595503 Date: 1995-03-08 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.2.4.2 /02	Palmer, S. J.; Krueger, H. O.	1998	Thiadone metabolite of FOE 5043: A 96-hour flow-through acute toxicity test with the saltwater mysid (Mysidopsis bahia) Wildlife International, Ltd., Easton, MD, USA Bayer CropScience, Report No.: 108488, Edition Number: <u>M-005110-01-1</u> Date: 1998-08-12 GLP/GEP: yes, unpublished	N	Y	US Study, not submitted to EU so far	Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.2.4.2 /03	Claude,, M. B.; Martin, K. H.; Gallagher, S. P.; Krueger, H.O.	2013	Flufenacet: A 96-hour static acute toxicity test with the saltwater mysid ( <i>Americamysis bahia</i> ) Wildlife International, Ltd., Easton, MD, USA Bayer CropScience, Report No.: 149A-248, Edition Number: <u>M-452205-01-1</u> EPA MRID No.: 49119001 Date: 2013-04-26 GLP/GEP: yes, unpublished	N	Y	New US study, so far not submitted to EU	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.5 /01	Gagliano, G. G.; Bowers, L. M.	1994	Chronic toxicity of FOE 5043 technical to the waterflea ( <i>Daphnia magna</i> ) under static renewal conditions Bayer Corporation, Kansas City, MO, USA Bayer CropScience, Report No.: 106762, Edition Number: <u>M-003795-01-1</u> EPA MRID No.: 43441126 Date: 1994-07-06 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.2.5.2 /01	Claude,, M. B.; Martin, K. H.; Gallagher, S. P.; Krueger, H.O.	2013	Flufenacet: A flow-through life-cycle toxicity test with the saltwater mysid ( <i>Americamysis bahia</i> ) Wildlife International, Ltd., Easton, MD, USA Bayer CropScience, Report No.: 149A-246, Edition Number: <u>M-452207-01-1</u> EPA MRID No.: 49119002 Date: 2013-04-26 GLP/GEP: yes, unpublished	N	Y	New US study, so far not submitted to EU	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.5.3 /01	Bruns, E.	2010	Chironomus riparius 28-day chronic toxicity test with flufenacet (tech.) in a water-sediment system using spiked water Bayer CropScience, Report No.: EBFOL153, Edition Number: <u>M-372857-01-1</u> Date: 2010-06-16 GLP/GEP: yes, unpublished	N	Y	New data requirement	Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.2.6.1 /01	Bowers, L. M.	1995	Toxicity of 14C-FOE 5043 to the Green Alga <i>Selenastrum capricornutum</i> Bayer Corporation, Stilwell, KS, USA Bayer CropScience, Report No.: 107114, Edition Number: <a href="#">M-002348-02-1</a> EPA MRID No.: 438344-02 Date: 1995-10-19 ...Amended: 1998-09-09 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.2.6.1 /02	Anderson, J. P. E.	1997	Growth of the Green Alga, <i>Pseudokirchneriella subcapitata</i> (formerly <i>Selenastrum capricornutum</i> ), during and after exposure to high concentrations of FOE 5043 Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: AJO/157097, Edition Number: <a href="#">M-002343-01-1</a> Date: 1997-07-14 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.2.6.1 /03	Dorgerloh, M.	1998	FOE 5043-Methylsulfide - Influence on the Growth of the Green Alga, <i>Pseudokirchneriella subcapitata</i> (formerly <i>Selenastrum capricornutum</i> ) Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: DOM 98011, Edition Number: <a href="#">M-002341-01-1</a> Date: 1998-06-16 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	The study was evaluated in the Addendum (2000 and 2003) to the DAR (1997)
KCA 8.2.6.1 /04	Anderson, J. P. E.	1995	Influence of FOE 5043-sulfonic acid on the growth of the green alga, <i>Scenedesmus subspicatus</i> Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: AJO/132495, Edition Number: <a href="#">M-004931-01-1</a> Date: 1995-10-06 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.2.6.1 /05	Dorgerloh, M.	1998	Toxicity of 14C-FOE 5043 to the green alga <i>Selenastrum capricornutum</i> Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: DOM 98092, Edition Number: <a href="#">M-086475-01-1</a> Date: 1998-09-09 GLP/GEP: no, unpublished	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.2.6.1 /06	Hall, A. T.; Lam, C. V.	1999	Toxicity of 14C-Thiadone, a metabolite of FOE 5043, to the green alga <i>Selenastrum capricornum</i> Bayer Corporation, Kansas City, MO, USA Bayer CropScience, Report No.: 108823, Edition Number: <a href="#">M-009214-01-1</a> EPA MRID No.: 45796115 Date: 1999-03-22 GLP/GEP: yes, unpublished	N	Y	US Study, not submitted to EU so far	Bayer CropScience	The study was evaluated in the Addendum (2000 and 2003) to the DAR (1997)
KCA 8.2.6.1 /07	Berends, A. G.; Molenaar, J. A.	1993	The toxicity of sodium trifluoroacetate to the alga <i>Selenastrum capricornutum</i> at low concentrations Solvay Duphar B.V., Netherlands; Bayer CropScience, Report No.: C047121, Report includes Trial Nos.: C.SOL.51.045 Edition Number: <a href="#">M-247818-02-1</a> Date: 1993-04-15 GLP/GEP: yes, unpublished	N	Y	New major metabolite	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.6.1 /08	Bruns, E.	2009	<i>Pseudokirchneriella subcapitata</i> growth inhibition test with flufenacet-oxalate Bayer CropScience, Report No.: EBFOL137, Edition Number: <a href="#">M-358823-01-1</a> Date: 2009-11-06 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.6.1 /09	Bruns, E.	2010	<i>Pseudokirchneriella subcapitata</i> growth inhibition test with flufenacet (tech.) Bayer CropScience, Report No.: EBFOL150, Edition Number: <a href="#">M-363891-03-1</a> Date: 2010-02-19 ...Amended: 2011-02-19 GLP/GEP: yes, unpublished	N	Y	Study performed for Japan; necessary for refined risk assessment	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.6.1 /10	Bruns, E.	2010	<i>Pseudokirchneriella subcapitata</i> growth inhibition test with flufenacet-methylsulfone Bayer CropScience, Report No.: EBFOL146, Edition Number: <a href="#">M-364591-01-1</a> Date: 2010-02-26 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.2.6.1 /12	Groeneveld, A. H. C.; de Kok, H. A. M.; van den Berg, G.	1992	The toxicity of sodium trifluoroacetate to the alga <i>Selenastrum capricornutum</i> Solvay Duphar B.V., Netherlands; Bayer CropScience, Report No.: C047124, Report includes Trial Nos.: C.SOL.51.038 Edition Number: <u>M-247820-01-1</u> Date: 1992-10-15 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.6.1 /13	Berends, A. G.; Keetelaar-Jansen, W. A. J.; van Dijk, N. R. M.	1995	A comparison of the toxicity of sodium trifluoroacetate, sodium difluoroacetate, sodium monofluoroacetate and sodium fluoride to the alga <i>Scenedesmus supspicatus</i> Solvay Duphar B.V., Netherlands; Bayer CropScience, Report No.: C047129, Report includes Trial Nos.: C.SOL.51.074 Edition Number: M-247825-01-1 Date: 1995-02-01 GLP/GEP: yes, unpublished	N	Y	required study for new soil metabolite	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.6.1 /14	Berends, A. G.	1996	The toxicity of sodium trifluoroacetate to algae Third Draft Solvay Duphar B.V., Netherlands; Bayer CropScience, Report No.: C047126, Edition Number: <u>M-247822-01-1</u> Date: 1996-01-01 GLP/GEP: no, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.6.1 /15	Bruns, E.	2012	<i>Pseudokirchneriella subcapitata</i> growth inhibition test with BCS-CU62474 - limit test Bayer CropScience, Report No.: EBFOP017, Edition Number: <u>M-444217-01-1</u> Date: 2012-12-21 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer Crop Science	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.2.6.1 /16	Bruns, E.	2011	Desmodesmus subspicatus growth inhibition test with flufenacet (tech.) Bayer CropScience, Report No.: EBFOL114/3, Edition Number: <a href="#">M-415813-01-1</a> Date: 2011-10-04 GLP/GEP: yes, unpublished	N	Y	Additional algae species needed for refinement of risk assessment	Bayer Crop Science	New study submitted for renewal of a.s.
KCA 8.2.6.2 /01	Bowers, L. M.; Dobbs, M. G.	1995	Acute toxicity of 14C-FOE 5043 to the freshwater diatom (Navicula pelliculosa) Bayer Corporation, Stilwell, KS, USA Bayer CropScience, Report No.: 107113, Edition Number: <a href="#">M-002355-01-1</a> EPA MRID No.: 43834401 Date: 1995-10-18 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.2.6.2 /02	Hughes, J. S.; Alexander, M. M.	1993	Acute toxicity of FOE 5043 (technical) to Anabaena flos-aquae Malcolm Pirnie, Tarrytown, NY, USA Bayer CropScience, Report No.: 105199, Edition Number: <a href="#">M-002423-01-1</a> EPA MRID No.: 43441131 Date: 1993-12-17 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	The study was evaluated in the Addendum (2000 and 2003) to the DAR (1997)
KCA 8.2.6.2 /04	Bruns, E.	2011	Synechococcus leopoliensis growth inhibition test with flufenacet (tech.) Bayer CropScience, Report No.: EBFOL114, Edition Number: <a href="#">M-415814-01-1</a> Date: 2011-10-07 GLP/GEP: yes, unpublished	N	Y	Additional algae species needed for refinement of risk assessment	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.6.2 /05	Bruns, E.	2011	Chlorella vulgaris growth inhibition test with flufenacet (tech.) Bayer CropScience, Report No.: EBFOL114/4, Edition Number: <a href="#">M-416169-01-1</a> Date: 2011-10-17 GLP/GEP: yes, unpublished	N	Y	Additional algae species needed for refinement of risk assessment	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.6.2 /06	Sobczyk, H.	2011	Chlamydomonas terricola growth inhibition test with flufenacet (tech.) Bayer CropScience, Report No.: EBFOL114/1, Edition Number: <a href="#">M-418627-01-1</a> Date: 2011-11-29 GLP/GEP: yes, unpublished	N	Y	Additional algae species needed for refinement of risk assessment	Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.2.7 /01	Hughes, J. S.; Alexander, M. M.	1994	Acute toxicity of FOE 5043 (technical) to Lemna gibba G3 Malcolm Pirnie, Tarrytown, NY, USA Bayer CropScience, Report No.: 105198, Edition Number: <u>M-002418-02-1</u> EPA MRID No.: 43441132 Date: 1994-01-04 ...Amended: 1998-09-01 GLP/GEP: yes, unpublished	N	Y	-	Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.2.7 /02	Dorgerloh, M.	1995	FOE 5043-Sulfonic acid - Toxicity (14 days) to Lemna gibba G3 Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: DOM 95072, Edition Number: <u>M-004929-01-1</u> Date: 1995-11-28 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.2.7 /03	Dorgerloh, M.	1998	Acute toxicity of FOE 5043 (technical) to Lemna gibba G3 Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: DOM 98091, Edition Number: <u>M-086479-01-1</u> Date: 1998-09-01 GLP/GEP: no, unpublished	N	Y	According to new guideline	Bayer CropScience	The study was evaluated in the DAR (1997). New recalculation was provided according to new guideline
KCA 8.2.7 /04	Smyth, D. V.; Thompson, R. S.; Gillins, E.	1993	Sodium trifluoroacetate: toxicity to the duckweed (Lemna gibba) Zeneca Ltd., Brixham Environmental Laboratory, Brixham, United Kingdom Bayer CropScience, Report No.: C047215, Report includes Trial Nos.: SP91-18.7 W907/B Edition Number: <u>M-247900-01-1</u> Date: 1993-05-12 GLP/GEP: yes, unpublished	N	Y	New major metabolite	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.7 /05	Bruns, E.	2009	Lemna gibba G3 Growth inhibition test with flufenacet-oxalate under static conditions Bayer CropScience, Report No.: EBFOL138, Edition Number: <u>M-359515-02-1</u> Date: 2009-11-24 ...Amended: 2009-12-08 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.2.7 /06	Bruns, E.	2010	Lemna gibba G3 - Growth inhibition test with flufenacet-methylsulfone (BCS-CO62475) under static conditions Bayer CropScience, Report No.: EBFOL145, Edition Number: <u>M-369703-01-1</u> Date: 2010-05-21 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.7 /07	Bruns, E.	2010	Lemna gibba G3 - Growth inhibition test with flufenacet-methylsulfide under static conditions Bayer CropScience, Report No.: EBFOL143, Edition Number: <u>M-393709-01-1</u> Date: 2010-10-27 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.7 /08	Bruns, E.	2010	Lemna gibba G3 - Growth inhibition test with flufenacet-thiadone under static conditions Bayer CropScience, Report No.: EBFOL144, Edition Number: <u>M-393718-01-3</u> Date: 2010-10-27 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.7 /10	Weyers, A.	2013	Lemna gibba G3 - Growth inhibition test with BCS-CU62474 (potassium salt of trifluoroethanesulfonic acid, metabolite of flufenacet) under static conditions Bayer CropScience, Report No.: EBFOP018, Edition Number: <u>M-445884-01-1</u> Date: 2013-01-25 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.7 /11	Bruns, E.	2013	Lemna gibba G3 - Growth inhibition test with flufenacet (technical substance) under static conditions Bayer CropScience, Report No.: EBFON004, Edition Number: <u>M-451198-01-1</u> Date: 2013-03-13 GLP/GEP: yes, unpublished	N	Y	New 7 day study performed as old US-EPA study was performed over 14 days	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.7 /12	Bruns, E.	2013	Lemna gibba G3 - Growth inhibition test with flufenacet tech. under peak exposure conditions Bayer CropScience, Report No.: EBFOL234, Edition Number: <u>M-452567-01-1</u> Date: 2013-04-16 GLP/GEP: yes, unpublished	N	Y	Higher tier study, necessary for refinement	Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.2.7 /13	Sowig, P.	2014	Flufenacet rationale for the replacement of the old 14-day Lemna growth inhibition study (Hughes & Alexander 1993; <u>M-002418-02-1</u> ) with the new 7-day Lemna study (Bruns 2013; <u>M-451198-01-1</u> ) Bayer CropScience, Report No.: <u>M-478762-01-1</u> , Edition Number: <u>M-478762-01-1</u> Date: 2014-02-28 GLP/GEP: n.a., unpublished	N	N		Bayer CropScience	-
KCA 8.2.7 /14	Hanson, M. L.; Solomon, K. R.	2012	Haloacetic acids in the aquatic environment. Part I: macrophyte toxicity Report No.: <u>M-455787-01-1</u> , Edition Number: <u>M-455787-01-1</u> GLP/GEP: n.a., published	N	N			New study submitted for renewal of a.s.
KCA 8.2.8 /01	Wheat, J.; Evans, J.	1993	Acute effects of FOE 5043 (technical) on new shell growth of the eastern oyster (Crassostrea virginica) Toxikon Environmental Sciences, Jupiter, FL, USA Bayer CropScience, Report No.: 105181, Edition Number: <u>M-002427-01-1</u> EPA MRID No.: 43441123 Date: 1993-09-28 GLP/GEP: yes, unpublished	N	Y	US Study, not submitted to EU so far	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.8 /02	Palmer, S. J.; Krueger, H. O.	1998	Thiadone metabolite of FOE 5043: A 96-hour shell deposition test with the eastern oyster (Crassostrea virginica) Wildlife International, Ltd., Easton, MD, USA Bayer CropScience, Report No.: 108489, Edition Number: <u>M-005108-01-1</u> Date: 1998-08-12 GLP/GEP: yes, unpublished	N	Y	US Study, not submitted to EU so far	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.2.8 /03	Bruns, E.	2009	Statement on the suitability of the microcosm study "The fate and biological effects of Flufenacet WG 60 in aquatic indoor microcosms" for the use in higher tier risk assessments with special focus on algal species and aquatic macrophytes Bayer CropScience, Report No.: <u>M-329959-01-1</u> , Edition Number: <u>M-329959-01-1</u> Date: 2009-02-27 GLP/GEP: n.a., unpublished	N	N		Bayer CropScience	-

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.2.8 /04	Banman, C. S.; Alexander, T. M.; Moore, S.	2013	Acute toxicity of flufenacet technical to the African clawed frog ( <i>Xenopus laevis</i> ) under static conditions SynTech Research Laboratory Services, LLC, Stilwell, KS, USA Bayer CropScience, Report No.: EBFON083, Edition Number: <u>M-471899-01-1</u> Date: 2013-12-04 GLP/GEP: yes, unpublished	N	Y	Study required by US EPA on African clawed frog	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.3.1.1.1 /01	Nengel, S.	1995	Assessment of side effects of FOE 5043 (tech.) to the honey bee, <i>Apis mellifera</i> L. in the laboratory following the EPPO guideline No. 170 GAB Biotechnologie GmbH, Niefern-Oeschelbronn, Germany Bayer CropScience, Report No.: 94137/01-BLEU, Edition Number: <u>M-004919-01-1</u> Date: 1995-08-30 GLP/GEP: yes, unpublished ...also filed: KCA 8.3.1.1.2 /02	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.3.1.1.1 /02	Tornier, I.	1995	Results of the screening test on the honey bee <i>Apis mellifera</i> L. test substance: FOE 5043 (techn.) GAB Biotechnologie GmbH, Niefern-Oeschelbronn, Germany Bayer CropScience, Report No.: B-958264, Edition Number: <u>M-004920-01-1</u> Date: 1995-02-08 GLP/GEP: no, unpublished ...also filed: KCA 8.3.1.1.2 /03	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.3.1.1.1 /03	Schmitzer, S.	2011	Effects of flufenacet tech. (acute contact and oral) on honey bees ( <i>Apis mellifera</i> L.) in the laboratory IBACON GmbH, Rossdorf, Germany Bayer CropScience, Report No.: 67681035, Edition Number: <u>M-421687-01-1</u> Date: 2011-12-15 GLP/GEP: yes, unpublished	N	Y	Study performed according to most recent guideline	Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.3.1.1.2 /02	Nengel, S.	1995	Assessment of side effects of FOE 5043 (tech.) to the honey bee, <i>Apis mellifera</i> L. in the laboratory following the EPPO guideline No. 170 GAB Biotechnologie GmbH, Niefern-Oeschelbronn, Germany Bayer CropScience, Report No.: 94137/01-BLEU, Edition Number: <u>M-004919-01-1</u> Date: 1995-08-30 GLP/GEP: yes, unpublished ...also filed: KCA 8.3.1.1.1 /01	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.3.1.1.2 /03	Tornier, I.	1995	Results of the screening test on the honey bee <i>Apis mellifera</i> L. test substance: FOE 5043 (techn.) GAB Biotechnologie GmbH, Niefern-Oeschelbronn, Germany Bayer CropScience, Report No.: B-958264, Edition Number: <u>M-004920-01-1</u> Date: 1995-02-08 GLP/GEP: no, unpublished ...also filed: KCA 8.3.1.1.1 /02	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.3.1.1.2 /04	Vergé, E.	2014	Flufenacet (tech.): Acute contact toxicity to the bumble bee <i>Bombus terrestris</i> L. (Hymenoptera, Apidae) under laboratory conditions Eurofins-GAB GmbH, Niefern-Oeschelbronn, Germany Bayer CropScience, Report No.: S13-01762, Edition Number: <u>M-478564-01-1</u> Date: 2014-02-04 GLP/GEP: yes, unpublished	N	Y	Study according to new guideline	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.3.1.2 /01	Kling, A.	2014	Flufenacet (tech.) - Assessment of chronic effects to the honeybee, <i>Apis mellifera</i> L., in a 10 days continuous laboratory feeding limit test eurofins-GAB GmbH, Niefern-Oeschelbronn, Germany Bayer CropScience, Report No.: S13-00145, Edition Number: <u>M-477339-01-1</u> Date: 2014-01-29 GLP/GEP: yes, unpublished	N	Y	required to fulfill new data requirements	Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.3.1.3 /01	Hecht-Rost, S.	2012	Flufenacet SC 508.8: A honeybee brood feeding study to evaluate the effects on brood development of the honeybee, <i>Apis mellifera</i> L. (Hymenoptera: Apidae) Innovative Environmental Services (IES) Ltd, Witterswil, Switzerland Bayer CropScience, Report No.: 20110057, Edition Number: <u>M-456504-01-1</u> Date: 2012-08-12 GLP/GEP: yes, unpublished	N	Y	New data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.1 /01	Nienstedt, K. M.	1999	FOE 5043-Oxalate: A 14-day acute toxicity test with the earthworm ( <i>Eisenia fetida</i> ) Springborn Laboratories AG, Horn, Switzerland Bayer CropScience, Report No.: 1022.006.630, Edition Number: <u>M-008793-01-1</u> Date: 1999-07-19 GLP/GEP: yes, unpublished	N	Y		Bayer Crop Science	The study was evaluated in the DAR (1997)
KCA 8.4.1 /02	Heimbach, F.	1995	Toxicity of FOE 5043 T (tech.) to earthworms Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: 107310, Edition Number: <u>M-004876-01-2</u> Date: 1995-01-05 GLP/GEP: yes, unpublished	N	Y		Bayer Crop Science	The study was evaluated in the DAR (1997)
KCA 8.4.1 /03	Nienstedt, K. M.	1999	FOE 5043-Sulfonic acid Na-salt: A 14-day acute toxicity test with the earthworm ( <i>Eisenia fetida</i> ) Springborn Laboratories AG, Horn, Switzerland Bayer CropScience, Report No.: 99-005-1022, Edition Number: <u>M-008794-01-1</u> Date: 1999-07-15 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.4.1 /05	Leicher, T.	2009	Flufenacet (FOE 5043)-sulfonic acid Na-salt: Effects on survival, growth and reproduction on the earthworm <i>Eisenia fetida</i> tested in artificial soil with 5% peat Bayer CropScience, Report No.: LRT-RG-R-64/09, Edition Number: <u>M-358264-01-1</u> Date: 2009-11-02 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.4.1 /06	Leicher, T.	2010	Flufenacet (FOE 5043) Methylsulfone: Effects on survival, growth and reproduction on the earthworm <i>Eisenia fetida</i> tested in artificial soil with 5 % peat Bayer CropScience, Report No.: LRT-RG-R-68/09, Edition Number: <u>M-362081-01-1</u> Date: 2010-01-21 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.1 /07	Leicher, T.	2010	FOE 5043-oxalate: Effects on survival, growth and reproduction on the earthworm <i>Eisenia fetida</i> tested in artificial soil with 10 % peat Bayer CropScience, Report No.: LRT-RG-R-93/10, Edition Number: <u>M-398163-01-1</u> Date: 2010-12-20 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.1 /08	Kratz, M. A.	2012	Flufenacet-thiadone (AE 1258593, BCS-AA 41715): Effects on survival, growth and reproduction on the earthworm <i>Eisenia fetida</i> tested in artificial soil Bayer CropScience, Report No.: KRA-RG-R-136/12, Edition Number: <u>M-442579-01-1</u> Date: 2012-11-28 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.1 /09	Luehrs, U.	2005	Effects of AE C502988 00 1B99 0001 on reproduction and growth of earthworms <i>Eisenia fetida</i> in artificial soil IBACON GmbH, Rossdorf, Germany Bayer CropScience, Report No.: C048065, Edition Number: <u>M-251328-01-1</u> Date: 2005-05-03 GLP/GEP: yes, unpublished	N	Y	New data requirements; major metabolite	Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.4.1 /10	Kratz, M.A.	2012	Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474): Effects on survival, growth and reproduction on the earthworm <i>Eisenia fetida</i> tested in artificial Bayer CropScience, Report No.: KRA-RG-R-131/12, Edition Number: <u>M-436340-01-1</u> Date: 2012-08-08 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.1 /11	Leicher, T.	2008	Flufenacet SC 500: effect on the earthworm fauna of a grassland area within one year Bayer CropScience, Report No.: LRT/RG-F-4/08, Edition Number: <u>M-307211-01-1</u> Date: 2008-09-19 GLP/GEP: yes, unpublished	N	Y	Earthworm field study, needed for refinement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.2.1 /01	Kratz, M.-A.	2009	Flufenacet-methylsulfone: Influence on mortality and reproduction on the soil mite species <i>Hypoaspis aculeifer</i> tested in artificial soil with 5 % peat Bayer CropScience, Report No.: KRA-HR-17/09, Edition Number: <u>M-357707-01-1</u> Date: 2009-10-15 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.2.1 /02	Frommholz, U.	2010	Flufenacet a.s.: Influence on the reproduction of the collembola species <i>Folsomia candida</i> tested in artificial soil with 5 % peat Bayer CropScience, Report No.: FRM-COLL-79/10, Edition Number: <u>M-363896-01-1</u> Date: 2010-02-23 GLP/GEP: yes, unpublished	N	Y	New data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.2.1 /03	Kratz, M.-A.	2010	Flufenacet-oxalate: Influence on mortality and reproduction on the soil mite species <i>Hypoaspis aculeifer</i> tested in artificial soil with 5 % peat Bayer CropScience, Report No.: KRA-HR-38/10, Edition Number: <u>M-393634-01-1</u> Date: 2010-10-26 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.4.2.1 /04	Frommholz, U.	2010	Flufenacet-oxalate: Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil Bayer CropScience, Report No.: FRM-COLL-95/10, Edition Number: <u>M-394712-01-1</u> Date: 2010-11-15 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.2.1 /05	Frommholz, U.	2010	Flufenacet-sulfonic acid Na-salt: Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil. Bayer CropScience, Report No.: FRM-COLL-98/10, Edition Number: <u>M-396039-01-1</u> Date: 2010-11-29 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.2.1 /06	Frommholz, U.	2012	Trifluoroacetic acid Na-salt (BCS-AZ56567): Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil Bayer CropScience, Report No.: FRM-COLL-132/12, Edition Number: <u>M-436127-01-1</u> Date: 2012-08-02 GLP/GEP: yes, unpublished	N	Y	New data requirements; major metabolite	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.2.1 /07	Frommholz, U.	2012	Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474): Influence on the reproduction of the Collembolan species Folsomia candida tested in artificial soil Bayer CropScience, Report No.: FRM-COLL-123/12, Edition Number: <u>M-436128-01-1</u> Date: 2012-08-02 GLP/GEP: no, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.2.1 /08	Kratz, M.A.	2012	Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474): Influence on mortality and reproduction on the soil mite species Hypoaspis aculeifer tested in artificial soil Bayer CropScience, Report No.: KRA-HR-57/12, Edition Number: <u>M-436315-01-1</u> Date: 2012-08-07 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.4.2.1 /09	Kratz, M.A.	2012	Trifluoroacetic acid Na-salt (BCS-AZ56567): Influence on mortality and reproduction on the soil mite species Hypoaspis aculeifer tested in artificial soil Bayer CropScience, Report No.: KRA-HR-58/12, Edition Number: <u>M-436326-01-1</u> Date: 2012-08-07 GLP/GEP: yes, unpublished	N	Y	New data requirements; major metabolite	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.2.1 /10	Frommholz, U.	2012	Flufenacet-thiadone (BCS-AA41715): Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil Bayer CropScience, Report No.: FRM-COLL-137/12, Edition Number: <u>M-440372-01-1</u> Date: 2012-10-24 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.2.1 /11	Kratz, M. A.	2012	Flufenacet-thiadone (BCS-AA41715): Influence on mortality and reproduction on the soil mite species Hypoaspis aculeifer tested in artificial soil Bayer CropScience, Report No.: KRA-HR-68/12, Edition Number: <u>M-442897-01-1</u> Date: 2012-11-20 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.2.1 /12	Kratz, M. A.	2013	Flufenacet a.s.: Influence on mortality and reproduction of the soil mite species hypoaspis aculeifer tested in artificial soil Bayer CropScience, Report No.: kra-HR-83/13, Edition Number: <u>M-455214-01-1</u> Date: 2013-05-31 GLP/GEP: yes, unpublished	N	Y	New data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.4.2.1 /13	Kratz, M. A.	2013	Flufenacet-sulfonic acid Na-salt (BCS-AZ23374): Influence on mortality and reproduction of the soil mite species Hypoaspis aculeifer tested in artificial soil Bayer CropScience, Report No.: kra-HR-88/13, Edition Number: <u>M-455654-01-1</u> Date: 2013-05-31 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.4.2.1 /14	Frommholz, U.	2010	Flufenacet-methylsulfone (BCS-CO62475): Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil Bayer CropScience, Report No.: FRM-COLL-96/10, Edition Number: <u>M-392345-01-1</u> Date: 2010-10-19 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.5 /01	Anderson, J. P. E.	1994	Influence of FOE 5043 on microbial mineralization of nitrogen in soils Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: AJO/124594, Edition Number: <u>M-003871-01-2</u> <u>Amedmend (recalculation)</u> <u>Ernst G., CroderS., 2016</u> Date: 1994-11-08 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.5 /02	Anderson, J. P. E.	1994	Influence of FOE 5043 on glucose stimulated respiration in soil Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: 107670, Edition Number: <u>M-003872-01-2</u> Date: 1994-11-08 GLP/GEP: yes, unpublished	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.5 /03	Lechelt-Kunze, C.	2005	Metabolite Flufenacet-Sulfonic acid Na-salt: Determination of effects on nitrogen transformation in soil Bayer CropScience, Report No.: LKC-N-41/05, Edition Number: <u>M-250265-01-1</u> Date: 2005-04-25 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.5 /04	Lechelt-Kunze, C.	2005	Metabolite flufenacet-oxalate hydrate: determination of effects on nitrogen transformation in soil Bayer CropScience, Report No.: LKC-N-45/05, Edition Number: <u>M-250511-01-1</u> Date: 2005-05-02 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.5 /05	Frommholz, U.	2010	Metabolite flufenacet-methylsulfone (BCS-CO62475): Determination of effects on nitrogen transformation in soil Bayer CropScience, Report No.: FRM-N-151/10, Edition Number: <u>M-398568-01-1</u> Date: 2010-12-23 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.5 /06	Schulz, L.	2013	Trifluoroacetic acid Na-salt (BCS-AZ56567): Effects on the activity of soil microflora (nitrogen transformation test) BioChem agrar GmbH, Gerichshain, Germany Bayer CropScience, Report No.: 12 10 48 080 N, Edition Number: <u>M-444423-01-1</u> Date: 2013-01-07 GLP/GEP: yes, unpublished	N	Y	New data requirements; major metabolite	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.5 /07	Schulz, L.	2013	Flufenacet-thiadone (BCS-AA41715): Effects on the activity of soil microflora (nitrogen transformation test) BioChem agrar GmbH, Gerichshain, Germany Bayer CropScience, Report No.: 13 10 48 078 N, Edition Number: <u>M-457326-01-1</u> Date: 2013-06-11 GLP/GEP: yes, unpublished	N	Y	Major metabolite; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.5 /08	Schulz, L.	2013	Flufenacet-trifluoroethanesulfonic acid Na-salt (BCS-CU62474): Effects on the activity of soil microflora (nitrogen transformation test) BioChem agrar GmbH, Gerichshain, Germany Bayer CropScience, Report No.: 13 10 48 079 N, Edition Number: <u>M-457331-01-1</u> Date: 2013-06-11 GLP/GEP: yes, unpublished	N	Y	New metabolite which needs to be addressed; new data requirement	Bayer CropScience	New study submitted for renewal of a.s.
KCA 8.7 /01	Oehrle, N. W.; Green, L. S.; Karr, D. B.; Emerich, D. W.	2012	The HFC/HCFC breakdown product trifluoroacetic acid (TFA) and its effects on the symbiosis between Bradyrhizobium japonicum and soybean (Glycine max) Report No.: <u>M-455785-01-1</u> , Edition Number: <u>M-455785-01-1</u> GLP/GEP: n.a., published	N	N			New study submitted for renewal of a.s.

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 8.7 /02	Smit, M.; Van Heerden, P.; Pienaar, J.; Weissflog, L.; Strasser, R.; Kruger, G.	2009	Effect of trifluoroacetate, a persistent degradation product of fluorinated hydrocarbons, on Phaseolus vulgaris and Zea mays Report No.: <u>M-455801-01-1</u> , Edition Number: <u>M-455801-01-1</u> Date: 2009-01-01 GLP/GEP: no, published	N	N			New study submitted for renewal of a.s.
KCA 8.7 /03	Benesch, J. A.; Gustin, M. S.; Cramer, G. R.; Cahill, T. M.	2012	Investigation of effects of trifluoroacetate on vernal pool ecosystems Report No.: <u>M-455780-01-1</u> , Edition Number: <u>M-455780-01-1</u> GLP/GEP: n.a., published	N	N			New study submitted for renewal of a.s.
KCA 8.8 /01	Kanne, R.	1989	Oxygen consumption test with activated sludge FOE 5043 Bayer AG, Leverkusen, Germany Bayer CropScience, Report No.: BA-898271, Report includes Trial Nos.: 89238067 Edition Number: <u>M-004740-02-1</u> Date: 1989-12-08 ...Amended: 2006-03-20 GLP/GEP: no, unpublished	N	Y		Bayer CropScience	The study was evaluated in the DAR (1997)
KCA 8.8 /02	Weyers, A.	2007	Flufenacet TC - Toxicity to bacteria Bayer Industry Services GmbH, Leverkusen, Germany Bayer CropScience, Report No.: 2006/0171/01, Edition Number: <u>M-283846-01-1</u> Date: 2007-02-05 GLP/GEP: yes, unpublished	N	Y	New data requirement	Bayer CropScience	New study submitted for renewal of a.s.
-	Hills, M.	2009	Evaluation of the pre-emergence biological activity of FOE 5043-Oxalate (code: BCS-AB16305) a metabolite of Flufenacet. Study No. PP09022, Reference BCS No: M-353844-01-1	N	Y	Additional data	Bayer CropScience	New study submitted for renewal of a.s.
-	Dahmen, P.	2004	Screening and Efficacy Data for WAK6222 (metabolite of FOE5043) Report No: PF-F-HB-WAK6222-01, Reference BCS No: M-089475-01-1	N	Y	Additional data	Bayer CropScience	New study submitted for renewal of a.s.
	Jans, D.	2013	Evaluation of the post emergence herbicidal activity of trifluoroethansulfonic acid sodium-salt (metabolite of flufenacet) in comparison with flufenacet Report No: RF13/035, Reference BCS No: M-460341-01-1	N	Y	Additional data	Bayer CropScience	New study submitted for renewal of a.s.

---

Annex point / reference number	Author(s)	Year	Title Source (where different from company) Company name, Report No., Date, GLP status (where relevant), published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
	S. Nöding	2013	Evaluation of the pre-emergence herbicidal activity of flufenacet and its metabolite BCS-AZ 56567 Study No: FFS135016, Reference BCS No: M-461398-01-1	N	Y	Additional data	Bayer CropScience	New study submitted for renewal of a.s.