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VOLUME 3- Annex B (AS)

- Flutolanil -

B.9 Ecotoxicology data

Rapporteur Member State: The Netherlands

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Draft Assessment Report and Proposed decision of the Netherlands prepared in the context of the possible approval of flutolanil under Regulation (EC) 1107/2009

Version history page

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B.9 Ecotoxicology data

Data from the original Annex I inclusion of flutolanil is presented in grey, when necessary. These data have been re-evaluated and a new summary provided. The old summary is included for comparison/completeness.

The following studies have been evaluated generally according to the CRED system (Moermond, et. al. (2016)), with modifications by the Ctgb:

CA 8.1.1.3/01
CA 8.1.1.3/02
CA 8.1.4/01
CA 8.1.4-02
CA 8.1.5-01
CA 8.2.2.1-01
CA 8.2.3-01
CA 8.2.4.2-01
CA 8.2.5.1/01
CA 8.2.5.2-01
CA 8.2.5.3-01
CA 8.2.8-01
CA 8.2.8-02
CA 8.3.1.2-01
CA 8.3.1.3-01
CA 8.4.1-01
CA 8.4.1-02
CA 8.4.1-03
CA 8.4.2.1-01
CA 8.4.2/01
CA 8.7-01
CA 8.8-01

To briefly summarize, the study has been evaluated for both reliability of the study itself and relevance for the risk assessment. The reliability scores have been adapted from Klimisch et. al. (1997). These categories are presented below.

Reliability categories

Score	Description
R1	**Reliable without restrictions:** All critical reliability criteria for this study are fulfilled. The study is well designed and performed, and it does not contain flaws that affect the reliability of the study.
R2	**Reliable with restrictions:** The study is generally well designed and performed, but some minor flaws in the documentation or setup may be present.
R3	**Not reliable:** Not all critical reliability criteria for this study are fulfilled. The study has clear flaws in study design and/or how it was performed.

R4	**Not assignable:** Information needed to make an assessment of the study is missing. This concerns studies which do not give sufficient experimental details and which are only listed in abstracts or secondary literature (books, reviews, etc.), or studies of which the documentation is not sufficient for assessment of reliability for one or more vital parameters.

Relevance categories

Score	Description
C1	**Relevant without restrictions:** The study is relevant for the purpose for which the study is evaluated.
C2	**Relevant with restrictions:** The study has limited relevance for the purpose for which the study is evaluated.
C3	**Not relevant**: The study is not relevant for the purpose for which the study is evaluated.
C4	**Not assignable:** Studies which do not give sufficient details since the result is presented in abstracts or secondary literature (books, reviews, etc.), or studies of which the documentation is not sufficient for assessment of relevance for one or more vital parameters.

B.9.1 Effects on birds and other terrestrial vertebrates

B.9.1.1 Effects on birds

Table 9.1-1 Summary of toxicity effects on birds and other terrestrial vertebrates

Test species	Time scale	Test material	Endpoint [95% CI, lower - upper]	Data point Author, year
Birds				
Bobwhite quail (*Colinus virginianus*)	Acute	Flutolanil Technical	**LD_{50} > 2000 mg a.s./kg bw** NOEL = 2000 mg a.s./kg bw LD_{10} = ND LD_{20} = ND	CA 8.1.1.1-01 [redacted] 1987a
Mallard duck (*Anas platyrhynchos*)	Acute	Flutolanil Technical	LD_{50} > 2000 mg a.s./kg bw NOEL = 2000 mg a.s./kg bw LD_{10} = ND LD_{20} = ND	CA 8.1.1.1-02 [redacted], 1987b
Bobwhite quail (*Colinus virginianus*)	Short-term dietary (5-days)	Flutolanil Technical	LC_{50} > 5243 ppm LD_{50} > 961 mg/kg bw/d	CA 8.1.1.2-01 [redacted], 1987c
Mallard duck (*Anas platyrhynchos*)	Short-term dietary (5-days)	Flutolanil Technical	LC_{50} > 5243 ppm LD_{50} > 1249 mg/kg bw/d	CA 8.1.1.2-02 [redacted] 1987d

Test species	Time scale	Test material	Endpoint [95% CI, lower - upper]	Data point Author, year
Bobwhite quail (*Colinus virginianus*)	Long-term	Flutolanil Technical	**NOEC = 247.8 mg a.s./kg bw/day** EC_{10} * = 525 [ND - 873] mg a.s./kg bw/day EC_{20} = ND EC_{50} = ND	CA 8.1.1.3-01 [redacted] 1993a CA 8.1.1.3-03. [redacted] 2016
Mallard duck (*Anas platyrhynchos*)	Long-term	Flutolanil Technical	NOEC = 267.5 mg a.s./kg bw/day $NOEC_{ecologically\ relevant}$ = 687 mg a.s./kg bw/day EC_{10} = ND EC_{20} = ND EC_{50} = ND	CA 8.1.1.3-02 [redacted] 1996 CA 8.1.1.3-03. [redacted] 2016
Other terrestrial vertebrates				
Rat	Acute oral	Flutolanil 40SC	LD_{50} > 2000 mg/kg bw	CP 7.1.1/01 [redacted] (2007a)
Rat	Acute oral	Flutolanil Technical	**LD_{50} > 2000 mg/kg bw**	CA 5.2.1-03 [redacted], 2009
Rat	Short term oral 28 days	Flutolanil	NOAEL = 180 mg/kg/day (minor reduction in body weight gain with slight liver weight increase at ≥ 916 mg/kg/day)	CA 5.3.1/01 [redacted] 1977
Rat	Short term oral 90 days	Flutolanil technical	NOAEL = 37 mg/kg/day (increased liver and thyroid/parathyroid weight and increased albumin at ≥ 299 mg/kg/day).	CA 5.3.2/01 [redacted] 1986a
Mouse	Short term oral 90 days	Flutolanil technical	NOAEL = 680 mg/kg/day (reduced weight gain with increased liver weight at 8637 mg/kg/day)	CA 5.3.2/02 [redacted] 1987
Dog	Short term oral 90 days	Flutolanil technical	NOAEL = 80 mg/kg/day (increased liver weight with hepatocyte swelling and pallor at 400 mg/kg/day)	CA 5.3.2/03 [redacted] 1986b
Rat	Reproductive	Flutolanil Technical	**$NOAEL_{parental}$ = 160 mg/kg/d for males, 190 mg/kg/d for females** EC_{10} = ND EC_{20} = ND (increased liver weight) $NOAEL_{pup,\ reproduction}$ **=** ≥ 1614 mg/kg bw/d	CA 5.6.1-01 [redacted]., 1991 CA 8.1.2.2-01 [redacted], 2016

Test species	Time scale	Test material	Endpoint [95% CI, lower - upper]	Data point Author, year
Rat	Developmental 6-15 days gestation	Flutolanil Technical	Maternal: NOAEL ≥ 1000 mg/kg bw/day No LOAEL Embryofetal toxicity: NOAEL ≥ 1000 mg/kg bw/day No LOAEL EC_{10} = ND EC_{20} = ND	CA 5.6.2/01 [redacted] 1987, as amended 1992 CA 8.1.2.2-01 [redacted], 2016
Rabbit	Developmental 6-18 days gestation	Flutolanil Technical	**NOAEL = 40 mg/kg bw/d** (resportions and deaths occurring in 5 different litters (out of 13 litters))	CA 5.6.2/02 [redacted] (1987)
Rabbit	Developmental 6-27 days gestation	Flutolanil	Maternal: NOAEL ≥ 1000 mg/kg bw/day No LOAEL Embryofetal toxicity: NOAEL ≥ 1000 mg/kg bw/day No LOAEL EC_{10} = ND EC_{20} = ND	CA 5.6.2/03 [redacted] 2012
Metabolite M-101				
Rat	Acute oral	2-(trifluoromethyl)-benzamide (M-101)	LD_{50} = > 300 mg metabolite/kg bw and < 2000 mg/kg bw	CA 5.8.1/02 [redacted] (2011)
Rat	Short term oral 28 days	2-(trifluoromethyl)-benzamide (M-101)	NOAEL ♂ = 4.2 mg metabolite/kg bw/d (organ weight changes, clinical chemistry) **NOAEL ecotoxicologically relevant ♂ = 17.6 mg metabolite/kg bw/d** (bodyweight decrease♂)	CA 5.8.1/03 [redacted] (2012)
Metabolite M-102				
Rat	Acute oral	2-(trifluoromethyl)-benzoic acid (M-102)	**LD_{50} > 2000 mg metabolite/kg bw**	CA 5.8.1/07 [redacted] (2016)
Rat	Short term oral 28 days	2-(trifluoromethyl)-benzoic acid (M-102)	**NOAEL ♂ = 252 mg metabolite/kg bw/d**	[redacted] (2010) CA 5.8.1/08

Endpoints in **bold** are the agreed endpoints retained for the risk assessment in line with the EFSA Conclusion (2008, 2013)
ND: could not be determined.
CI: Confidence intervals
* Endpoint not considered reliable

B.9.1.1.1 Acute oral toxicity to birds

Study 8.1.1.1-01

Report:	**CA 8.1.1.1-01, [redacted], 1987a**
Title:	The acute oral toxicity (LD_{50}) of flutolanil to the bobwhite quail
Report No.:	NNU 20BT/861565 (W-3003)
Published:	No
GLP:	Yes (OECD/1982, USA/EPA/1983, Japanese MAFF/1984)
Guidelines:	U.S.-E.P.A. Pesticide Assessment Guidelines §71-1 "Avian single dose oral LD_{50} test" (October 1982)
Deviations:	Bird observations should be continuous for the first two hours after dosing according to the guideline. Weight measurements on day 3 are required by OECD 223 guideline.
RMS Comment:	Equivalent to OECD 223 (proposal), 2009. The study has already been reviewed under Uniform Principles for the first approval of flutolanil under Directive 91/414/EEC. (DAR, 2006: B9.1.1). The validity criteria were met and the study deviations are considered to be minor and do not affect the outcome of the study, therefore the study is acceptable.
Endpoint	LD_{50} >2000 mg flutolanil/kg b.w.

Executive Summary

Flutolanil technical was administered to fasted northern bobwhite quail (*Colinus virginianus*) in an acute oral toxicity study. Five quail/sex/dose received single oral doses of 500, 1000 and 2000 mg flutolanil/kg b.w. at a dose volume of 10 mL/kg b.w. in 0.5% w/v carboxymethylcellulose. Birds were observed for clinical signs of toxicity, body weight effects and mortality for 14 days after dosing. No treatment-related mortalities were observed. The acute oral LD_{50} value for northern bobwhite quail exposed to flutolanil by single oral dose was >2000 mg flutolanil/kg b.w. The no mortality dosage was 2000 mg flutolanil/kg b.w. The NOEL was 2000 mg flutolanil/kg b.w. Since no treatment-related effect were observed in this study, LD_{10} and LD_{20} could not be determined.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: Flutolanil technical
Description: White crystalline powder
Lot/Batch #: 543251
Purity: 97.5%

2. Vehicle and/or positive control: 0.5% w/v carboxymethylcellulose

3. Test animals
Species: *Colinus virginianus*
Age: over 16 weeks
Weight at dosing: 171 – 226 g
Source: [redacted]
Acclimation period: 20 days
Diet: Standard HRC pelleted layer diet {Batch 2137), made by Joseph Odam Limited; Eye Mill, Peterborough, Cambridgeshire
Water: Water from the domestic mains supply, *ad libitum*
Housing: Tiered galvanised steel cages measuring 65 x 50 x 40 cm, with a galvanised steel drinking fount and food hopper.

4. Environmental conditions:

Temperature: 25-20°C ± 2°C
Humidity: 84% ± 10%
Photoperiod: 17:7 hour photoperiod

B. STUDY DESIGN AND METHODS

1. In life dates: 15-July-1986 to 12-August-1986

2. Animal assignment and treatment

In an acute toxicity study, northern bobwhite quail (*Colinus virginianus*) were exposed to flutolanil. Flutolanil technical was administered in 0.5% w/v carboxymethylcellulose by oral dose to fasted northern bobwhite quail. Five quail/sex/dose received doses of 500, 1000 and 2000 mg flutolanil/kg b.w. at a dose volume of 10 mL/kg. Levels of flutolanil in the dosing suspensions were found to be within acceptable limits and flutolanil was shown to be physically stable in the vehicle for the duration of the dosing period.

3. Observations

Following test initiation until termination all birds were observed daily for clinical signs of toxicity, and mortality or abnormal behaviour for 14 days before and after dosing. Individual body weights were measured by group at initiation of the test and on days -14, -7, 0, 7 and 14 of the test. Feed consumption was also measured.

4. Statistics

The data were not conducive to calculation of an LD_{10}, LD_{20} and LD_{50} as there were no mortalities present.

II. RESULTS AND DISCUSSION

A. Mortality and symptoms of toxicity

No treatment-related mortalities were observed. No clinical signs of toxicity related to the test item were observed. Feed consumption measurements were variable among the study and control groups, but were not treatment related. There were no test item-related body weight or food intake effects noted. Results are summarised in the tables that follows.

Summary of toxicological responses of bobwhite quail following a single oral dose of flutolanil

Dose (mg/kg b.w.)	Toxicological result	Duration of signs	Time of death	LD_{50} (14 days) mg/kg b.w.
Male birds				
500	**0/0/5**	**-**	**-**	**> 2000**
1000	**0/0/5**	**-**	**-**	
2000	**0/0/5**	**-**	**-**	
Female birds				
500	**0/0/5**	**-**	**-**	**> 2000**
1000	**0/0/5**	**-**	**-**	
2000	**0/0/5**	**-**	**-**	

B. Feed consumption

Feed consumption differences in measurements did not appear to be treatment related.

Dose (mg flutolanil/ kg b.w.)	Number of birds			Estimated feed consumption (g/bird/day)							
				Days -14 to -8		Days -7 to -1		Days 1 to 7		Days 8 to 14	
	M	F	T	M	F	M	F	M	F	M	F
Control	5	5	10	19	18	17	23	19	28	14	22
500	5	5	10	17	23	17	21	19	25	16	23
1000	5	5	10	16	21	16	17	18	28	17	26
2000	5	5	10	17	23	15	24	18	28	17	27

C. Body weight

When compared to the controls, there was no effect on body weight at any of the dosages tested.

Group wise mean body weight (g)

Dose (mg flutolanil/kg b.w.)	Number of birds			Average body weight (g) on day									
				-14		-7		0		7		14	
	M	F	T	M	F	M	F	M	F	M	F	M	F
Control	5	5	10	198	203	197	207	191	192	195	197	187	198
500	5	5	10	188	207	191	216	183	206	192	201	189	209
1000	5	5	10	188	204	187	206	184	194	191	205	192	212
2000	5	5	10	189	200	188	206	181	196	187	201	182	206

D. Toxicity endpoints

Acute oral toxicity to bobwhite quail – Summary of endpoints

Test item	Flutolanil
Test object	Northern bobwhite quail
LD_{50}	>2000 mg flutolanil/kg b.w.
Lowest observed effect level (LOEL)	>2000 mg flutolanil/kg b.w.
Highest tested dose without toxic effect (NOEL)	2000 mg flutolanil/kg b.w.

III. CONCLUSION

The acute oral LD_{50} value for northern bobwhite quail exposed to flutolanil by single oral dose was >2000 mg flutolanil/kg b.w. The no mortality dosage was 2000 mg/kg b.w. The NOEL was 2000 mg flutolanil/kg b.w. Since no treatment-related effect were observed in this study, LD_{10} and LD_{20} could not be determined.

Comments by RMS

The study was conducted according to US-EPA 71-1 (1982) and was in general agreement with OECD 223 (2009). Deviations from OECD 223 have been noted in the summary table above and are not considered to alter the outcome of the study. The study is considered valid and acceptable. Mortality, food consumption, clinical signs and body weight were not statistically significantly affected at any dose.

Reliability of endpoints

There were no effects on behavior nor any clinical effects seen during the test at any dose level. There were no mortalities. EC_{10} and EC_{20} values cannot be calculated.

The LD_{50} of >2000 mg a.s./kg bw/day, may be used for risk assessment.

Study 8.1.1.1-02

Report:	**CA 8.1.1.1-02, [redacted], 1987b**
Title:	The acute oral toxicity (LD_{50}) of flutolanil to the mallard duck
Report No.:	NNU 21BT/861566 (W-3004)
Published:	No
GLP:	Yes (OECD/1982, USA/EPA/1983, Japanese MAFF/1984)
Guidelines:	U.S.-E.P.A. Pesticide Assessment Guidelines §71-1 "Avian single dose oral LD_{50} test" (October 1982)
Deviations:	Bird observations should be continuous for the first two hours after dosing according to the guideline. Weight measurements on day 3 are required by OECD 223 guideline.
RMS Comment:	Equivalent to OECD 223 (proposal), 2009 The study has already been reviewed under Uniform Principles for the first approval of flutolanil under Directive 91/414/EEC. (DAR, 2006: B9.1.1). The validity criteria were met and the study deviations are considered to be minor and do not affect the outcome of the study, therefore the study is acceptable.
Endpoint	LD_{50} >2000 mg flutolanil/kg b.w.

Executive Summary

Flutolanil technical was administered to the mallard duck (*Anas platyrhynchos*) in an acute oral toxicity study. Five duck/sex/dose received single oral doses of 500, 1000 and 2000 mg flutolanil/kg b.w. at a dose volume of 10 mL/kg b.w. in 0.5% w/v carboxymethylcellulose. Birds were observed for clinical signs of toxicity, body weight effects and mortality for 14 days after dosing. No treatment-related mortalities were observed. The acute oral LD_{50} value for mallard duck exposed to flutolanil by single oral dose was greater than 2000 mg flutolanil/kg b.w. The no mortality dosage was 2000 mg flutolanil/kg b.w. The NOEL was 2000 mg flutolanil/kg b.w. Since no treatment-related effect were observed in this study, LD_{10} and LD_{20} could not be determined.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: Flutolanil technical
Description: White crystalline powder
Lot/Batch #: 543251
Purity: 97.5%

2. Vehicle and/or positive control: 0.5% w/v carboxymethylcellulose

3. Test animals
Species: *Anas platyrhynchos*
Age: over 16 weeks
Weight at dosing: 830 – 1195 g
Source: [redacted]
Acclimation period: over 30 days

	Diet:	Standard HRC pelleted layer diet {Batch 2137), made by Joseph Odam Limited; Eye Mill, Peterborough, Cambridgeshire
	Water:	Water from the domestic mains supply, *ad libitum*
	Housing:	Galvanised steel cages with a wire mesh floor measuring 1.5 x 1.2 x 1.75 m, with an automatic drinker and a food hopper.
4.	**Environmental conditions:**	
	Temperature:	22-20°C ± 2°C
	Humidity:	72% ± 6%
	Photoperiod:	17:7 hour photoperiod

B. STUDY DESIGN AND METHODS

1. In life dates: 12-June-1986 to 03-July-1986

2. Animal assignment and treatment

In an acute toxicity study, mallard duck (*Anas platyrhynchos*) were exposed to flutolanil. Flutolanil technical was administered in 0.5% w/v carboxymethylcellulose by oral dose to fasted mallard duck. Five duck/sex/dose received doses of 500, 1000 and 2000 mg flutolanil/kg b.w. at a dose volume of 10 mL/kg. Levels of flutolanil in the dosing suspensions were found to be within acceptable limits and flutolanil was shown to be physically stable in the vehicle for the duration of the dosing period.

3. Observations

Following test initiation until termination all birds were observed daily for clinical signs of toxicity, and mortality or abnormal behaviour for 14 days before and after dosing. Individual body weights were measured by group at initiation of the test and on days -14, -7, 0, 7 and 14 of the test. Feed consumption was also measured.

4. Statistics

The data were not conducive to calculation of an LD_{10}, LD_{20} and LD_{50} as there were no mortalities present.

II. RESULTS AND DISCUSSION

A. Mortality and symptoms of toxicity

No treatment-related mortalities were observed. No clinical signs of toxicity related to the test item were observed. Feed consumption measurements were variable among the study and control groups, but were not treatment related. There were no test item-related body weight or food intake effects noted. Results are summarised in the table that follows.

Summary of toxicological responses of mallard duck following a single oral dose of flutolanil

Dose (mg/kg b.w.)	Toxicological result	Duration of signs	Time of death	LD_{50} (14 days) mg/kg b.w.
Male birds				
500	0/0/5	-	-	> 2000
1000	0/0/5	-	-	
2000	0/0/5	-	-	

Dose (mg/kg b.w.)	Toxicological result	Duration of signs	Time of death	LD_{50} (14 days) mg/kg b.w.
Female birds				
500	0/0/5	-	-	> 2000
1000	0/0/5	-	-	
2000	0/0/5	-	-	

B. Feed consumption

Feed consumption differences in measurements did not appear to be treatment related.

Dose (mg flutolanil/ kg b.w.)	Number of birds			Estimated feed consumption (g/bird/day)							
				Days -14 to -8		Days -7 to -1		Days 1 to 7		Days 8 to 14	
	M	F	T	M	F	M	F	M	F	M	F
Control	5	5	10	91	100	57	74	91	103	86	89
500	5	5	10	149	97	66	83	91	129	89	106
1000	5	5	10	103	151	83	86	123	103	103	80
2000	5	5	10	80	94	51	171	74	111	60	109

C. Body weight

When compared to the controls, there was no effect on body weight at any of the dosages tested.

Group wise mean body weight (g)

Dose (mg flutolanil/kg b.w.)	Number of birds			Average body weight (g) on day									
				-14		-7		0		7		14	
	M	F	T	M	F	M	F	M	F	M	F	M	F
Control	5	5	10	1015	1064	1067	1118	1003	1054	1013	1108	1014	1111
500	5	5	10	1026	1045	1080	1132	1010	1031	1070	1104	1069	1077
1000	5	5	10	1050	1036	1084	1071	1043	959	1093	1027	1108	1050
2000	5	5	10	1034	1049	1045	1029	983	954	995	1000	984	1026

D. Toxicity endpoints

Acute oral toxicity to mallard duck – Summary of endpoints

Test item	Flutolanil
Test object	Mallard duck
LD_{50}	>2000 mg flutolanil/kg b.w.
Lowest observed effect level (LOEL)	>2000 mg flutolanil/kg b.w.
Highest tested dose without toxic effect (NOEL)	2000 mg flutolanil/kg b.w.

III. CONCLUSIONS

The acute oral LD_{50} value for mallard duck exposed to flutolanil by single oral dose was >2000 mg flutolanil/kg b.w. The no mortality dosage was 2000 mg/kg b.w. The NOEL was 2000 mg flutolanil/kg b.w. Since no treatment-related effect were observed in this study, LD_{10} and LD_{20} could not be determined.

Comments by RMS

The study was conducted according to US-EPA 71-1 (1982) and was in general agreement with OECD 223 (2009). Deviations from OECD 223 have been noted in the summary table above and are not considered to alter the outcome of the study. The study is considered valid and acceptable. Mortality, food consumption, clinical signs and body weight were not statistically significantly affected at any dose.

Reliability of endpoints

There were no effects on behavior nor any clinical effects seen during the test at any dose level. There were no mortalities. EC_{10} and EC_{20} values cannot be calculated.

The LD_{50} of >2000 mg a.s./kg bw/day, may be used for risk assessment.

B.9.1.1.2 Short-term dietary toxicity to birds

Study 8.1.1.2-01

Report:	**CA 8.1.1.2-01, [redacted], 1987c**
Title:	The subacute dietary toxicity (LC_{50}) of flutolanil to the bobwhite quail
Report No.:	NNU 22BT/861567 (W-3006)
Published:	No
GLP:	Yes (OECD/1982, USA/EPA/1983, Japanese MAFF/1984)
Guidelines:	Directive 96/12/EC, EPA 71-2: Avian dietary LC_{50} test (October 1982)
Deviations:	Minor environmental.
RMS Comment:	Equivalent to OECD 205, 1984. The study has already been reviewed under Uniform Principles for the first approval of flutolanil under Directive 91/414/EEC. (DAR, 2006: B9.1.2). The validity criteria were met and deviations are considered to be minor. Therefore, it is not expected to adversely affect the overall outcome of the study and the study is considered to be acceptable.
Endpoint	LD_{50} > 5243 ppm LD_{50} > 819.8 mg/kg bw/d

Executive Summary

In a short-term dietary study, bobwhite quail (*Colinus virginianus*) received flutolanil in the feed at concentrations of 0, 500, 800, 1280, 2048, 3277, and 5243 ppm for 5 days. Birds were observed for a total of 11 days for abnormal behaviour, mortality, signs of toxicity, and changes in body weight and food intake. At termination 10 birds surviving from the highest dose group (5243 ppm) were examined for gross pathological changes and one bird from control which dies from drowning. No treatment-related mortalities were observed. All birds were normal in appearance and behaviour throughout the study. The short-term dietary LC_{50} for bobwhite quail exposed to flutolanil in the diet for 5 days was > 5243 ppm. The no mortality dosage was > 5243 ppm. The short-term dietary NOEC was 5243 ppm.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Flutolanil technical

Description: White crystalline powder

Batch no.: 543251

Purity: 97.5%

2. Vehicle and/or positive control: Diet

3. Test animals

Species: Bobwhite quail (*Colinus virginianus*)

Age: over 8 days

Weight at dosing: 13.7 – 15.4 g

Source: [redacted]

Acclimation period: 10 days

Diet: Standard HRC chick diet (Batch 4055), until 11 days old

Water: Water from the domestic mains supply, *ad libitum*

Housing: Wooden pens 80 x 50 x 60 cm, wood shavings used as bedding with a galvanised steel drinking font and food hopper at each pen.

4. Environmental conditions:

Temperature: 27-23° C ± 1° C

Humidity: 61% ± 4%

Photoperiod: continuous artificial lighting (300-watt infra-red lamp)

B. STUDY DESIGN AND METHODS

1. In life dates: 22-July-1986 to 25-July-1986

2. Animal assignment and treatment

Groups of ten bobwhite quail (*Colinus virginianus*) chicks were assigned to each of the treatment and control groups. The test consisted of a geometric series of six test concentrations and three control groups. Nominal dietary concentrations used were 0, 500, 800, 1280, 2048, 3277, and 5243 ppm. Each group was fed the appropriate test or control diet for 5 days. Following the five-day exposure period all groups were given untreated feed for three days. The test diets were prepared by mixing the test substance into the basal diet. Levels of flutolanil in basal diet were found to be within acceptable limits the results also indicated that flutolanil could be homogeneously blended with the avian diet and that it was stable in this matrix over 7 days.

3. Observations

Birds were observed for a total of 8 days for abnormal behaviour, mortality, signs of toxicity, and changes in body weight and food intake.

4. Statistics

The data did not warrant statistical analysis.

II. RESULTS AND DISCUSSION

A. Mortality and symptoms of toxicity

No treatment-related mortalities were observed. No clinical signs of toxicity were observed. All surviving birds appeared normal throughout the study. There were no test item-related body weight or food intake effects noted.

Summary of toxicological responses of bobwhite quail following a single oral dose of flutolanil technical

Concentration (ppm flutolanil)	Toxicological results [a]	Duration of clinical signs [b]	Time of death
Control	1/0/30 [c]	-	-
500	0/0/10	-	-
800	0/0/10	-	-
1280	0/0/10	-	-
2048	0/0/10	-	-
3277	0/0/10	-	-
5243	0/0/10	-	-

[a] Number of animals which died/number of animals with clinical signs related to the test item/number of animals used
[b] Actual number of animals with treatment related clinical signs not reported
[c] One animal drowned in drinker

B. Feed consumption

When compared to the controls, there was no effect on feed consumption at any concentration tested.
Food consumption (g/bird/day)

Dose (ppm flutolanil)	Estimated feed consumption (g/bird/day)							
	Days -3 to -1	1	2	3	4	5	1 to 5	6 to 8
Control*	19.1	5.1	4.5	4.5	2.8	3.9	4.2	5.4
500	19.5	6.2	4.5	4.9	2.9	3.1	4.3	5.9
800	21.2	7.1	5.0	4.3	4.0	4.1	4.9	5.4
1280	19.1	6.6	6.0	5.4	3.3	4.9	5.2	5.5
2048	28.6	6.3	4.8	5.6	3.9	4.5	5.0	5.0
3277	16.1	4.6	4.3	4.3	3.2	3.3	3.9	4.5
5243	15.7	5.1	4.9	4.7	2.9	4.0	4.3	5.4

*Mean value for 3 control groups

C. Body weight

When compared to the controls, there was no effect on body weight gain at any concentration tested.

Group wise mean body weight (g)

Dose (ppm flutolanil)	Average body weight (g)			
	Day -3	Day 0	Day 5	Day 8
Control[b]	14.1	19.0	28.3[a]	35.3[a]
500	14.2	19.3	28.0	33.8
800	15.4	20.5	30.9	36.5
1280	15.4	20.8	30.7	37.6
2048	14.4	19.9	29.6	34.9
3277	13.7	19.2	28.4	35.1
5243	14.2	19.4	27.5	33.3

[a]Total of 29 animals
[b]Mean value for 3 control groups

D. Toxicity endpoints

The subacute dietary toxicity (LC_{50}) for bobwhite quail exposed to flutolanil for 5 days was greater than 5243 ppm. The no mortality dosage was >5243 ppm. The short-term dietary NOEC was 5243 ppm.

Acute oral toxicity to the bobwhite quail – Summary of endpoints

Test item	Flutolanil technical
Test object	Bobwhite quail
LC_{50}	>5243 ppm
Lowest observed effect concentration (LOEC)	>5243 ppm
Highest tested dose without toxic effect (NOEC)	5243 ppm

III. CONCLUSIONS

The short-term dietary LC_{50} for the bobwhite quail exposed to flutolanil in the diet for 5 days was >5243 ppm (equivalent to 961 mg/kg/day). The no mortality dosage was 5243 ppm. The short-term dietary NOEC was 5243 ppm.

Comments by RMS

The study was conducted according to US-EPA 71-2 (1982) and was in general agreement with OECD 205 (1984). A deviation from the OECD 205 guideline was the age of the birds (i.e. >8 days instead of 10-17 days old) but this is not considered to invalidate the study as the validity criteria of the study were met (mortality in the control did not exceed 10% at the end of the test, the test item was maintained in the diet at $\geq$ 80% and there were no toxic signs or mortalities at the lowest tested dose). The study is considered valid and acceptable.

Mortality, food consumption, clinical signs and body weight were not statistically significantly affected at any dose. It is not clear how the notifier converted the dose in ppm to mg/kg bw/day. The evaluator has used the bodyweight at day 5 (27.5 g) and the day 0-5 food consumption (22.5449 mg/d) to calculate a value of 819.8 mg a.s./kg bw/day for the highest dose of 5243 ppm, based on the values reported in the tables above.

Reliability of endpoints

There were no effects on behavior nor any clinical effects seen during the test at any dose level. There were no mortalities. EC_{10} and EC_{20} values cannot be calculated.

The NOEC of 5243 ppm, equivalent to 819.8 mg a.s./kg bw/day, may be used for risk assessment.

Study 8.1.1.2-02

Report:	**CA 8.1.1.2-02. [redacted], 1987b**
Title:	The subacute dietary toxicity (LC_{50}) of flutolanil to the mallard duck
Report No.:	NNU 23BT/861568 (W-3005)
Published:	No
GLP:	Yes (USA/EPA)
Guidelines:	Directive 96/12/EC, US-EPA 71-2: Avian dietary LC_{50} test.
Deviations:	Minor environmental.
RMS Comment:	Equivalent to OECD 205, 1984. The study has already been reviewed under Uniform Principles for the first approval of flutolanil under Directive 91/414/EEC. (DAR, 2006: B9.1.2). The validity criteria were met and deviations are considered to be minor. Therefore, it is not expected to adversely affect the overall outcome of the study and the study is considered to be acceptable.
Endpoint	LD_{50} > 5243 ppm LD_{50} > 929.3 mg/kg bw/d

Executive Summary

In a short-term dietary study, mallard ducks (*Anas platyrhynchos*) received flutolanil technical in the feed at concentrations of 0, 500, 800, 1280, 2048, 3277 and 5243 ppm for 5 days. Birds were observed for a total of 8 days for abnormal behaviour, mortality, signs of toxicity, and changes in body weight and food intake. All birds were examined for gross pathological changes.

Results of the analysed test diet indicated that flutolanil was homogeneously blended with avian diet and stable over 7 days. At 500 and 5243 ppm, the measured concentrations of flutolanil ranged between 104% and 107% of nominal at Day 0 and between 95% and 97% of nominal at Day 7 of the test period.

No treatment-related mortalities were observed. All birds were normal in appearance and behaviour throughout the study. The short-term dietary LC_{50} for mallard ducks exposed to flutolanil technical in the diet for 5 days was >5243 ppm. The short-term dietary NOEC was 5243 ppm.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Flutolanil technical
Description: White crystalline powder
Lot/Batch #: 543251
Purity (according to CoA): 97.5%
Stability of test compound: Shown to be stable under the conditions of the test

2. Vehicle and/or positive control: Diet

3. Test animals

Species: *Anas platyrhynchos*

Age: 6 days at test start

Weight at dosing: 39.7 – 40.6 g

Source: [redacted]

Acclimation period: 3 days

Diet: Test diet was prepared by mixing flutolanil technical into the diet.

Water: Tap water *ad libitum*

Housing: Pen of galvanised steel wire and sheeting, floor: 80 x 40cm, height: 20cm

4. Environmental conditions:

Temperature: 28 ± 1° C

Humidity: 47%

Photoperiod: Continuous

B. STUDY DESIGN AND METHODS

1. In life dates: 04 Aug to 09 Aug 1987

2. Animal assignment and treatment

A 5-day range finding study exposing groups of six mallard ducks (8 days old) to nominal dietary concentrations of 100 to 6000 ppm was performed. No mortality was recorded at all concentrations tested. Based on these results, a definitive test employed a geometric series of six nominal dietary concentrations of 500, 800, 1280, 2048, 3277 and 5243 ppm. Groups of ten mallard duck (*Anas platyrhynchos*) chicks, which were assigned to each of the treatment and three control groups by random draw. Each group was fed the appropriate test or control diet for 5 days. Birds were given untreated feed for three days prior to the test and three days following the five-day exposure period.

3. Observations

Birds were observed for a total of 8 days for abnormal behaviour, mortality, signs of toxicity, and changes in body weight and food intake.

4. Statistics

The data did not warrant statistical analysis.

II. RESULTS AND DISCUSSION

A. Analytical verification

Results of the analysed test diet indicated that flutolanil was homogeneously blended with avian diet and stable over 7 days. At 500 and 5243 ppm, the measured concentrations of flutolanil ranged between 104% and 107% of nominal at Day 0 and between 95% and 97% of nominal at Day 7 of the test period.

Measured concentrations of flutolanil at Day 0 and Day 7 of the test period

Test item nominal (ppm)	Day 0		Day 7	
	Mean measured (ppm)	% Nominal	Mean measured (ppm)	% Nominal
500	535	107%	483	97%
5243	5450	104%	4940	95%

B. Mortality and symptoms of toxicity

No treatment-related mortalities were observed. No clinical signs of toxicity were observed. All surviving birds appeared normal throughout the study. There were no test item-related body weight or food intake effects noted.

Summary of toxicological responses of mallard duck following a short-term dietary intake of flutolanil technical

Concentration (ppm flutolanil)	Toxicological results[a]	Duration of clinical signs	Time of death
0 (control)	0/0/30	-	-
500	0/0/10	-	-
800	0/0/10	-	-
1280	0/0/10	-	-
2048	0/0/10	-	-
3277	0/0/10	-	-
5243	0/0/10	-	-

[a] Number of animals which died/number of animals with clinical signs related to the test item/number of animals used

C. Feed consumption

When compared to the controls, there was no effect on feed consumption at any concentration tested.

Food consumption (g/bird/day)

Dose (ppm flutolanil)	Feed consumption (g/bird/day)	
	Exposure days 0-5	Days 6-8
0 (control)	22.3*	39.1*
500	20.3	36.0
800	20.6	35.7
1280	19.8	32.5
2048	22.4	41.2
3277	22.3	36.2
5243	19.0	34.1

*Mean value for 3 control groups

D. Body weight

When compared to the controls, there was no effect on body weight gain at any concentration tested.

Group wise mean body weight (g)

Dose (ppm flutolanil)	Average body weight (g)		
	Day 0	**Day 5**	**Day 8**
0 (control)	61.2*	130.5*	197.0*
500	57.0	119.4	180.4
800	57.4	113.3	176.1
1280	55.0	113.8	174.7
2048	59.2	123.7	183.8
3277	58.1	122.3	192.1
5243	52.3	107.2	163.4

*Mean value for 3 control groups

E. Endpoints

The short-term dietary LC_{50} for mallard ducks exposed to flutolanil technical in the diet for 5 days was >5243 ppm. The no mortality dosage was >5243 ppm, equivalent to 2000 mg a.s./kg bw. The short-term dietary NOEC was 5243 ppm, equivalent to 1249 mg/kg bw/day.

Acute oral toxicity to the mallard duck – Summary of endpoints

Test item	Flutolanil technical
Test object	Mallard ducks
LC_{50}	>5243 ppm
Lowest observed effect concentration (LOEC)	>5243 ppm
Highest tested dose without toxic effect (NOEC)	5243 ppm

III. CONCLUSIONS

The short-term dietary LC_{50} for the mallard duck exposed to flutolanil in the diet for 5 days was >5243 ppm, equivalent to 1249 mg/kg bw/day. The short-term dietary NOEC was 5243 ppm, equivalent to 1249 mg/kg bw/day.

Comments by RMS

The study was conducted according to US-EPA 71-2 (1982) and was in general agreement with OECD 205 (1984). A deviation from the OECD 205 guideline was the age of the birds (i.e. 6 days instead of 10-17 days old) but this is not considered to invalidate the study as the validity criteria of the study were met (mortality in the control did not exceed 10% at the end of the test, the test item was maintained in the diet at $\geq$ 80% and there were no toxic signs or mortalities at the lowest tested dose). The study is considered valid and acceptable.

Mortality, food consumption, clinical signs and body weight were not statistically significantly affected at any dose. It is not clear how the notifier converted the dose in ppm to mg/kg bw/day. The evaluator has used the bodyweight at day 5 (107.2 g) and the day 0-5 food consumption (99.617 mg/d) to calculate a value of 929.3 mg a.s./kg bw/day for the highest dose of 5243 ppm, based on the values reported in the tables above.

Reliability of endpoints

There were no effects on behavior nor any clinical effects seen during the test at any dose level. There were no mortalities. EC_{10} and EC_{20} values cannot be calculated.

The NOEC of 5243 ppm, equivalent to 929.3 mg a.s./kg bw/day, may be used for risk assessment.

B.9.1.1.3 Sub-chronic toxicity and reproduction to birds

Study 8.1.1.3-01

Report:	**CA 8.1.1.3-01. [redacted] 1993a**
Title:	Flutolanil technical: a one-generation reproduction study with the bobwhite (*Colinus virginianus*)
Report No.:	244-108 (W-3025)
Publication:	No
GLP:	Yes
Guidelines:	U.S. EPA 71-4, ASTM E1062-86 (1986)
Deviations:	Minor deviations occurred, most of which were related to the environmental conditions. According to the study director, these deviations had no impact on the outcome of the study. Maximum recommended concentration was 1000 ppm as per guideline – this was exceeded.
RMS Comment:	Equivalent to OECD 206, 1984. The study has already been reviewed under Uniform Principles for the first approval of flutolanil under Directive 91/414/EEC. (DAR, 2006: B9.1.3). The validity criteria were met and deviations are considered to be minor. Therefore, it is not expected to adversely affect the overall outcome of the study and the study is considered to be acceptable.
Endpoint	NOEC = 247.8 mg a.s./kg bw/day EC_{10}^{*} = 525 [ND - 873] mg a.s./kg bw/day EC_{20} = ND EC_{50} = ND

Executive Summary

In a one-generation reproduction study bobwhite quail (*Colinus virginianus*) received flutolanil in the feed at concentrations of 0, 400, 960, 1920 or 4800 ppm for 21 weeks. Birds were observed daily for abnormal behaviour, mortality, signs of toxicity, and changes in body weight and food intake. All birds were examined for gross pathological changes at the end of the study. No treatment-related mortalities were observed. Except for incidental clinical findings, all birds appeared normal appearance and behaviour throughout the study. At the 4800 ppm test concentration there was a slight reduction in the number of eggs laid that was also reflected in the numbers of hatchlings and 14-day old survivors as percentages of the maximum number of eggs set. Based upon the slight reduction in laid eggs at the 4800 ppm test concentration, the no observed effect concentration (NOEC) for northern bobwhite exposed to flutolanil technical in the diet during this study was 1920 ppm (247 mg/kg bw/day). The test guideline followed by this study has serious limitation for the derivation of reliable ED_{10}, ED_{20} and ED_{50}. Since there was an apparent reduction of greater than 10%

in the number of eggs produced in the 4800 ppm treatment group, a three parameter cumulative-normal (Bruce-Versteeg) model was fit to this data (see CA 8.1.1.3-03). An ED_{10} estimate of 4083 ppm was obtained. However only the upper 95% confidence bound for the ED_{10} (6897 ppm) was obtained, indicating little confidence in the reliability of ED_{10} estimate. Therefore, the original conclusion of the study that the NOEC was 1920 ppm (equivalent to 247 mg/kg bw/day) is appropriate.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Flutolanil technical

Description: White powder

Lot/Batch no.: 91208

Purity: not indicated, treated as 100%

Stability of test compound: Stable in the diet under conditions of administration.

2. Vehicle and/or positive control: Diet

Treatment: 0 (control), 400, 960, 1920 and 4800 ppm (nominal)Estimated intake for northern bobwhite during the study:
0 (control), 53, 124, 247, 594 mg/kg bw/day

3. Test animals

Species: Bobwhite quail (*Colinus virginianus*)

Age: 19 weeks of age at the initiation of the test

Weight at dosing: 193 – 197 g

Source: [redacted]

Acclimation period: 4 weeks

Diet: ≥ 27% protein, ≥ 2.5% fat, ≤ 5% fiber, 5% (w/w) limestone. Test diet was prepared by mixing flutolanil into the diet with corn oil, acetone and ration

Water: Easton public water *ad libitum*

Housing: Indoor pens of galvanised wire grid and sheeting, floor: 30 x 51 cm, height: 21-26 cm

4. Environmental conditions:

Temperature: 19.6 ± 1.2° C

Humidity: 33 ± 13%

Photoperiod: 8 h light and 16 h darkness cycle (~ 375 lux) for the first 7 weeks, 17 h of light and 7h of darkness until the end of the test.

B. STUDY DESIGN AND METHODS

1. In life dates: 24-October-1991 to 28-April-1992

2. Animal assignment and treatment

Groups of sixteen pairs of bobwhite quail (*Colinus virginianus*) were assigned to each of the treatment and control groups by random draw, with one male and one female per pen.

3. Diet preparation and analysis

Test diets were prepared by mixing flutolanil into acetone and incorporating corn oil and ration. This mixture was then used to create a premix for weekly preparation of diet. The control diet contained acetone, corn oil and ration equivalent to the highest amounts used in the treated diets. Homogeneity of the test substance was evaluated by collecting six samples from each treatment on Day 0 of Week 1. Samples were collected from the left and right sides of the mixing vessel at the top, middle and bottom. Control and test concentration diets were prepared weekly and presented to the birds on day 7 of each week. Analysis was also performed to verify the presence of the test substance under actual test conditions. Samples were analysed using a sufficiently validated HPLC method (overall procedural recovery at 30, 300, 1200 and 6000 ppm was 92%, RSD 9%, n = 21 (6 at 30 ppm, 7 at 300 ppm, 2 at 1200 ppm and 6 at 6000 ppm).

4. Observations

Reproductive parameters were measured at the onset of egg laying and include: Eggs laid, eggs cracked, eggs set, viable embryos, live three-week embryos, hatchlings, body weight of hatchlings, 14-day old survivors, body weight of 14-day old survivors, and egg shell thickness. Adult birds were observed daily for abnormal behaviour, mortality, signs of toxicity, and changes in body weight and food intake. All birds were examined for gross pathological changes.

5. Statistics

Dunnett's method was used to determine statistically significant differences between control and treatment. Percentage data were examined using Dunnett's method following arcsine transformation. The pens in which mortality occurred were not used in statistical comparisons of the reproductive data. The test guideline followed by this study has serious limitation for the derivation of reliable ED_{10}, ED_{20} and ED_{50}. A case by case evaluation of the utility of ED_x estimates was performed (see CA 8.1.1.3-03).

II. RESULTS AND DISCUSSION

A. Biological data

There was no treatment related mortality at any of the concentrations tested. Two incidental mortalities occurred in the 960 ppm treatment group. No mortalities occurred in the control group, or in the 400, 1920 or 4800 ppm treatment groups.

There was no treatment related mortality overt signs of toxicity or treatment related effects on body weight or feed consumption at any concentration tested. In addition, there were no apparent treatment related effects upon the reproductive parameters measured. Two incidental mortalities occurred in the 960 ppm treatment group at week 18 and 19. None of the mortalities were treatment related. There were no apparent treatment related effects on adult body weights at any concentration level tested or the controls (Table CA 8.1.1.3/01-1). Feed consumption was variable between pens and some

statistical significance was calculated, however these differences were not considered to be treatment related and comparing to the control groups the differences were slight and not concentration dependent (Table CA 8.1.1.3/01-2).

Table CA 8.1.1.3/01-1 Mean body weight (g)

Test group	Sex	Week of Study					
		0	2	4	6	8	Term.
control	Male	194	196	197	202	206	206
	Female	195	196	197	201	201	235
400 ppm	Male	197	199	201	205	208	212
	Female	193	193	195	198	201	228
960 ppm	Male	194	194	197	202	203	204
	Female	194	195	198	204	206	234
1920 ppm	Male	194	196	198	203	206	206
	Female	194	195	197	201	203	233
4800 ppm	Male	194	193	195	201	202	204
	Female	196	195	197	204	205	232

Table CA 8.1.1.3/01-2 Mean feed consumption (g/bird/day)

Weeks	0 ppm	400 ppm	960 ppm	1920 ppm	4800 ppm
1	19	20	22*	22*	20
2	18	22**	19	19	17
3	22	23	23	22	20
4	17	20**	20**	21**	16
5	18	18	16	18	17
6	21	22	22	22	21
7	19	22	21	21	19
8	21	22	21	20	21
9	21	25**	23	23	23
10	21	23	22	23	26**
11	24	26	25	24	25
12	25	27	26	27	27
13	27	30	30	30	28
14	31	31	32	31	29
15	30	29	29	29	27
16	33	33	32	33	30
17	33	33	31	33	30
18	34	34	34	33	30
19	31	32	30	31	31

Weeks	0 ppm	400 ppm	960 ppm	1920 ppm	4800 ppm
20	34	34	34	34	32
21	32	32	30	32	30
Mean	25.3	26.6	25.8	26.1	24.7

*Difference from the control statistically significant at $p < 0.05$

**Difference from the control statistically significant at $p < 0.01$

Reproductive parameters: There were no apparent treatment related effects upon reproductive performance at any concentration tested (Tables CA 8.1.1.3/01-3A and -3B).

While not statistically significant, there appeared to be a slight reduction in the number of eggs laid in the 4800 ppm treatment group. In control and all other treatments no more than two hens laid a small number of eggs (< 20), however in the 4800 ppm group five hens laid less than 20 eggs. A slight reduction in egg production at the 4800 ppm test concentration was also observed, but it did not show to be statistically significant ($p > 0.05$) when compared with the control group.

There was no apparent treatment related effect on egg shell thickness at any concentrations tested when compared to the control group (Table CA 8.1.1.3/01-4).

There was no apparent treatment related effect on the body weights of hatchlings or 14-day old survivors at any of the concentrations tested (Table CA 8.1.1.3/01-5).

Table CA 8.1.1.3/01-3A Summary of reproductive effects of flutolanil on bobwhite quail

Reproductive parameter	**Test group (dietary concentration in ppm)**				
	0	**400**	**960**	**1920**	**4800**
Number of replicates	16	16	14	16	16
Total eggs laid/group	620	633	607	618	516
Eggs cracked	11	33	33	19	4
Eggs set	544	531	513	535	456
Viable embryos	490	493	485	521	407
Live 3-week embryos	485	490	485	515	405
Hatchlings	453	460	466	479	377
14-day-old survivors	405	385	407	436	316
Eggs laid/hen	39	40	43	39	32
Eggs laid/hen/day[a]	0.53	0.54	0.59	0.53	0.44
14-day-old survivors/hen	25	24	29	27	20

[a] Based on 73 days of egg production

Table CA 8.1.1.3/01-3B Summary of reproductive effects of flutolanil on bobwhite quail

Reproductive parameter	Test group (dietary concentration in ppm)				
	0	**400**	**960**	**1920**	**4800**
Number of replicates	16	16	14	16	16
Total number eggs laid [a]	620	633	607	618	516
Eggs laid/maximum laid (%)[a]	58	59	65	58	48
Eggs cracked/eggs laid (%)[a]	2	5	7	4	0
Viable embryos/eggs set (%)[a]	85	91	95	97	84
Live three-week embryos/viable embryos (%)[a]	99	98	100	99	100
Hatchlings/live 3-week embryos (%)[a]	93	93	97	94	91
14-Day old survivors/hatchlings (%)[a]	88	81	88	90	83
Hatchlings/eggs set (%)[a]	79	85	91	90	76
14-Day-old survivors/eggs set (%)[a]	71	70	81	81	64
Hatchlings/maximum set (%)[a]	49	50	57	52	41
14-Day-old survivors/maximum set (%)[a]	44	41	50	47	34

[a] Not significantly different from the control (Dunnett's method, $p < 0.05$)

Table CA 8.1.1.3/01-4 Egg shell thickness (mm)

	Control	400 ppm	960 ppm	1920 ppm	4800 ppm
No. of Eggs Measured	59	61	56	62	52
Mean Egg Shell Thickness (mm)	0.218	0.220	0.217	0.215	0.208
± standard deviation	0.021	0.022	0.015	0.015	0.016

Table CA 8.1.1.3/01-5 Body weight data (g) - Hatchlings and 14-day survivors

	Hatchlings					14-Day Survivors				
	Control	400 ppm	960 ppm	1920 ppm	4800 ppm	Control	400 ppm	960 ppm	1920 ppm	4800 ppm
No. of chicks Weighed	453	459	466	479	377	405	385	407	436	316
Mean Body Weight (g)	5.6±0.6	5.6±0.7	5.9±0.6	5.7±0.6	5.5±0.5	21±3	21±3	21±4	22±3	19±3

B. Analytical verification

Analysis of homogeneity samples from the 400, 960, 1920 and 4800 ppm diets showed means and standard deviations of 379 ± 13.3 ppm, 1010 ± 24.3 ppm, 1850 ± 48.3 ppm and 4590 ± 93.5 ppm, respectively. Coefficients of variation for each treatment group were 3.51%, 2.41%, 2.61% and 2.04%, respectively. Analysis of stability samples collected during the course of the study demonstrated that the test substance was stable in the diet under conditions of administration (recoveries 95-111% of initial at all doses).

C. Toxicity Endpoints

Sub-chronic and reproduction toxicity in bobwhite quail exposed to flutolanil - Summary of endpoints

Test item	Flutolanil
Test object	Bobwhite quail
Lowest observed effect concentration (LOEC)	4800 ppm
Highest tested dose without toxic effect (NOEC)	1920 ppm

III. CONCLUSIONS

Based upon the slight reduction in egg production at the 4800 ppm test concentration, the no observed effect concentration (NOEC) for northern bobwhite exposed to flutolanil technical in the diet during this study was 1920 ppm (247 mg flutolanil/kg bw/day). The test guideline followed by this study has serious limitation for the derivation of reliable ED_{10}, ED_{20} and ED_{50}. Since there was an apparent reduction of greater than 10% in the number of eggs produced in the 4800 ppm treatment group, a three parameter cumulative-normal (Bruce-Versteeg) model was fit to this data (see CA 8.1.1.3-03). An ED_{10} estimate of 4083 ppm was obtained. However only the upper 95% confidence bound for the ED_{10} (6897 ppm) was obtained, indicating little confidence in the reliability of ED_{10} estimate. Therefore, the original conclusion of the study that the NOEC was 1920 ppm (equivalent to 247 mg/kg bw/day) is appropriate.

Comments by RMS

The study was conducted according to U.S. EPA 71-4 (1982) and was in general agreement with OECD 206 (1984). A deviation from the OECD 206 guideline was that the age of the birds (i.e. 19 weeks old instead of 20-24 weeks) but this is not considered to invalidate the study as the validity criteria of the study were met (mortality in the control did not exceed 10% at the end of the test, the average number of 14-day-old survivors per hen in the control was at least 12 and the average eggshell thickness for the control group was at last 0.19 mm). The study is considered valid and acceptable.

Mortality, adult body weight and reproductive parameters were not statistically significantly affected at any dose. Food consumption was statistically significantly increased at incidental samplings in all doses. As these effects were not dose related, they were not considered to be related to the treatment.

As the reduction in number of eggs laid in the highest treatment group was higher than 10% (17 %), derivation of an ED_{10} value was considered. It should be noted that, according to EFSA Supporting publication 2015:EN-924, the test guideline has serious limitation for the derivation of reliable ED_{10} estimations and the NOEC should be retained as primary endpoint.

In the summary, the applicant referred to CA 8.1.1.3-03, in which a three parameter cumulative-normal (Bruce-Versteeg) model was fit to the data on egg production and an ED_{10} estimate of 4083 ppm was obtained. However, only the upper 95% confidence bound for the ED_{10} (6897 ppm) was obtained, indicating little confidence in the reliability of ED_{10} estimate. Therefore, the original conclusion of the

study that the NOEC was 1920 ppm (equivalent to 247.8 mg/kg bodyweight/day, as calculated by the RMS using a food intake rate of 50.1 mg/day and a bodyweight of 202.2 g) is appropriate. This is considered acceptable.

Reliability of endpoints

According to Regulation (EU) No 283/2013, the statistical power of the NOEC shall be assessed in case no EDx values can be derived. However, this is not possible as long as default type I and II errors have not been fixed, default variance for any response variable have not been established and typical detectable effect sizes of each parameter have not been calculated (EFSA Supporting publication 2015:EN-924). The NOEC was based on a slight but non-statistically significant reduction in the number of eggs laid (17%) at the highest tested dose, and set at the next lower level. This is considered a conservative approach, which is sufficiently protective.

The NOEC of 1920 ppm, equivalent to 247.8 mg a.s./kg bw/day, may be used for risk assessment.

[redacted]	1993a	Flutolanil technical: a one-generation reproduction study with the bobwhite (*Colinus virginianus*)	Report no 244-108 (W-3025)
Reliability			
General information			
Is a guideline method or modified guideline used?*			Yes
Is the test performed under GLP conditions?*			Yes
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?			Yes
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?			Yes
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?			Yes
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?			Yes
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?			Not applicable

[redacted]	1993a	Flutolanil technical: a one-generation reproduction study with the bobwhite (*Colinus virginianus*)	Report no 244-108 (W-3025)
Test organism			
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?			Yes
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?			Yes
Exposure conditions			
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?			Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?			Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?			Not applicable
Is a correct spacing between exposure concentrations applied?			Yes
Is the exposure duration defined?			Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?			Yes
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?			Yes
Statistical Design and Biological Response			
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?			Yes
Are appropriate statistical methods used?			Yes
Is a concentration-response curve observed? Is the response statistically significant?			Yes
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?			Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints			
Exposure Relevance			

[redacted]	1993a	Flutolanil technical: a one-generation reproduction study with the bobwhite (*Colinus virginianus*)	Report no 244-108 (W-3025)

Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Yes
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes, a reliable NOEC was derived
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable

Concluding weight of evidence/proposed action	Fully acceptable
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable
Consideration/concluding score	Fully acceptable

Study 8.1.1.3-02

Report:	**CA 8.1.1.3-02. [redacted] 1996b**
Title:	Flutolanil technical: A one-generation reproduction study with the mallard (*Anas platyrhynchos*)
Report No.:	244-109 (W-3026)
Published:	No
GLP:	Yes (US-EPA)
Guidelines:	OECD 206 (1984); U.S. EPA 71-4 (1982)
Deviations:	Minor deviations occurred, most of which were related to the environmental conditions. According to the study director, these deviations caused no apparent adverse impact on the outcome of the study. Maximum recommended concentration was 1000 ppm as per guideline, but this was exceeded.
RMS Comment:	The study has already been reviewed under Uniform Principles for the first approval of flutolanil under Directive 91/414/EEC. (DAR, 2006: B.9.2.3.1). The validity criteria of the OECD guideline No. 206 (1984) were met and deviations are considered to be minor. Therefore, it is not expected to adversely affect the overall outcome of the study and the study is considered to be acceptable.
Endpoint	NOEC = 267 mg a.s./kg bw/day $NOEC_{ecologically\ relevant}$ = 687.2 mg a.s./kg bw/day EC_{10} = ND EC_{20} = ND EC_{50} = ND

Executive Summary

A one-generation mallard duck (*Anas platyrhynchos*) reproduction study was conducted with flutolanil technical. Sixteen pairs of mallards received flutolanil in the feed at concentrations of 0, 400, 960, 1920 and 4800 ppm for 19 weeks. Reproductive parameters were measured at the onset of egg laying. Adult birds were observed for abnormal behaviour, mortality, signs of toxicity, and changes in body weight and food intake. All birds were examined for gross pathological changes.

There was no treatment related mortality, overt signs of toxicity or treatment related effects on body weight or feed consumption at any concentration tested. There was no apparent treatment related effect upon any of the reproductive parameters up to 1920 ppm, however, a slight reduction in egg shell thickness was statistically significant and biologically relevant at the 4800 ppm test concentration. The NOEC for mallards exposed to flutolanil in the diet for 19 weeks was 1920 ppm (267 mg/kg bw/day). The test guideline followed by this study has serious limitation for the derivation of reliable ED_{10}, ED_{20} and ED_{50}. Furthermore, the differences between the control and the high treatment group were less than 10% and there is no evident downward trend in eggshell thickness in treated groups (see CA 8.1.1.3-03). Thus, an estimate of ED_{10} would be an extrapolated value and the estimation of

ED_{10}, ED_{20} or ED_{50} for this endpoint would be inappropriate. Therefore, the original conclusion of the study that the NOEC was 1920 ppm (equivalent to 267 mg/kg bw/day) is appropriate.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: Flutolanil technical
Lot/Batch no.: 91208
Purity: Not reported, treated as 100%
Description: White powder
Stability of test compound: Shown to be stable under the conditions of the test

2. Vehicle and/or positive control: Diet
Treatment: 0 (control), 400, 960, 1920 and 4800 ppm (nominal)
Estimated intake for mallards during the study:
0 (control), 57, 151, 267, 684 mg/kg bw/day

3. Test animals
Species: *Anas platyrhynchos*
Age: 21 weeks
Weight at dosing: 1085 – 1242 g
Source: [redacted]
Acclimation period: 4 weeks
Diet: ≥27% protein, ≥2.5% fat, ≤5% fiber, 5% (w/w) limestone
Water: Tap water provided *ad libitum*
Housing: Pens: 75 cm x 90 cm, height 45 cm

4. Environmental conditions adults:
Temperature: 17.8 ± 1.8°C
Humidity: 47 ± 15%
Photoperiod: 8 hours light/16 hours dark for 8 weeks, 17 hours light/7 hours dark at Week 9 (approximately 300 lux)

B. STUDY DESIGN AND METHODS

1. In life dates: 24 Oct 1991 to 23 April 1992

2. Animal assignment and treatment

A one-generation mallard (*Anas platyrhynchos*) reproduction study was conducted with flutolanil. Sixteen pairs of mallards received flutolanil in the feed at concentrations of 0, 400, 960, 1920 and 4800 ppm in the diet for 19 weeks.

3. Diet preparation and analysis

Test diets were prepared by mixing flutolanil into acetone and adding this mixture to corn oil. This mixture was then used as premix for weekly preparation of the final diet. Homogeneity of the test

substance in the diet was evaluated by collecting six samples from each of the treatment groups on Day 0 of Week 1. Samples were collected from the top, middle, and bottom of the left and right sections of the mixing vessel. Samples were analysed using a sufficiently validated HPLC method (overall procedural recovery at 30, 300, 1200 and 6000 ppm was 92%, RSD 9%, N = 21 (6 at 30 ppm, 7 at 300 ppm, 2 at 1200 ppm and 6 at 6000 ppm).

4. Observations

Reproductive parameters were measured at the onset of egg laying. Reproductive parameters observed included: eggs laid, eggs cracked, eggs set, viable embryos, live three-week embryos, hatchlings, body weight of hatchlings, 14-day old survivors, body weight of 14-day old survivors and egg shell thickness. Adult birds were observed for abnormal behaviour, mortality, signs of toxicity, and changes in body weight and food intake.

5. Statistics

Dunnett's method was used to determine statistically significant differences between the control group and each treatment group. The test guideline followed by this study has serious limitation for the derivation of reliable ED_{10}, ED_{20} and ED_{50}. A case by case evaluation of the utility of ED_x estimates was performed (see CA 8.1.1.3-03).

II. RESULTS AND DISCUSSION

A. Biological data

There were no treatment related mortalities, overt signs of toxicity or treatment related effects on body weight or feed consumption at any concentration tested (Tables CA 8.1.1.3/02-1 and CA 8.1.1.3/02-2). In addition, there were no apparent treatment related effects upon any of the reproductive parameters up to 1920 ppm (Tables CA 8.1.1.3/02-3A, 3B, 4 and 5). However, a slight reduction in egg shell thickness was statistically significant at the 4800 ppm test concentration (Table CA 8.1.1.3/02-4). While the numerical difference with the control group was small, the egg shell thickness values of 25% of the pens were below 0.357 mm (historical control mean of 0.385 mm minus 2 × SD of ± 0.014 mm). Hence, the reduction in egg shell thickness was considered to be biologically meaningful and the no observed effect concentration for mallards in the diet was found to be 1920 ppm (267 mg/kg bw/day).

Table CA 8.1.1.3/02-1 Mean body weight (g)

Test group	Sex	Week of Study					
		0	2	4	6	8	Term.
control	Male	1242	1260	1248	1229	1207	1229
	Female	1131	1135	1140	1125	1123	1270
400 ppm	Male	1233	1240	1238	1237	1230	1278
	Female	1131	1152	1139	1129	1118	1270
960 ppm	Male	1229	1228	1217	1217	1206	1277
	Female	1085	1063	1059	1064	1057	1224

Test group	Sex	Week of Study					
		0	2	4	6	8	Term.
1920 ppm	Male	1227	1256	1227	1230	1205	1239
	Female	1091	1102	1075	1084	1072	1240
4800 ppm	Male	1228	1239	1230	1231	1225	1247
	Female	1111	1146	1147	1156	1149	1275

Table CA 8.1.1.3/02-2 Mean feed consumption (g/bird/day)

Weeks	0 ppm	400 ppm	960 ppm	1920 ppm	4800 ppm
1	137	146	138	127	101
2	132	144	139	138	152
3	135	141	145	128	135
4	128	123	137	122	129
5	141	143	152	135	139
6	116	115	123	112	124
7	122	124	126	115	126
8	136	133	142	122	131
9	111	112	121	91	125
10	152	164	165	144	142
11	147	178	194**	181	179
12	177	207	203	178	202
13	189	202	218	191	216
14	198	205	225	205	217
15	209	211	237	221	218
16	217	218	245	218	222
17	225	217	245	224	228
18	195	205	226	199	218
19	195	202	231	191	197
20	209	219	250	220	231
Mean	164	170	183	163	172

**Difference from the control statistically significant at $p < 0.01$

Table CA 8.1.1.3/02-3A Summary of reproductive effects of flutolanil on mallard duck

Reproductive parameter	Test group (dietary concentration in ppm)				
	0	400	960	1920	4800
Number of replicates	16	16	16	16	16
Total eggs laid/group	728	807	747	755	707
Eggs cracked	13	10	8	16	10
Eggs set	646	725	668	673	625
Viable embryos	606	680	628	651	528
Live three-week embryos	601	662	604	641	523
Hatchlings	482	534	482	521	424
14-day-old survivors	475	511	465	511	408
Eggs laid/hen	46	50	47	47	44
Eggs laid/hen/day[a]	0.68	0.75	0.70	0.70	0.66
14-day-old survivors/hen	30	32	29	32	26

[a] Based on 67 days of egg production

Table CA 8.1.1.3/02-3B Summary of reproductive effects of flutolanil on mallard duck

Reproductive parameter	Test group (dietary concentration in ppm)				
	0	400	960	1920	4800
Number of replicates	16	16	16	16	16
Total number eggs laid	728	807	747	755	707
Eggs laid/maximum laid (%)	69	76	71	71	67
Eggs cracked/eggs laid (%)	2	1	1	2	1
Viable embryos/eggs set (%)	93	94	94	97	86
Live three-week embryos/viable embryos (%)	99	97	96*	98	98
Hatchlings/live 3-week embryos (%)	80	81	81	80	80
14-Day old survivors/hatchlings (%)	98	96	97	98	96
Hatchlings/eggs set (%)	74	74	73	76	67
14-Day-old survivors/eggs set (%)	73	70	70	75	64
Hatchlings/maximum set (%)	50	56	50	54	44
14-Day-old survivors/maximum set (%)	49	53	48	53	43

* Statistically significant difference ($p < 0.05$) from control due to accidental damage and no apparent to a treatment related effect. When accidental loss was taken into account, both treatment groups 400 and 960 ppm increased to 99% and comparable to the control.

Table CA 8.1.1.3/02-4 Effect of flutolanil on egg shell thickness of mallard duck

Reproductive parameter	Test group (dietary concentration in ppm)				
	0	**400**	**960**	**1920**	**4800**
Number of eggs measured	63	67	68	63	66
Mean egg shell thickness (mm) ± standard deviation	0.403 ± 0.024	0.383 ± 0.023	0.398 ± 0.022	0.396 ± 0.022	0.382* ± 0.026

* Difference from control statistically significant ($p < 0.05$)

Table CA 8.1.1.3/02-5 Body weight data (g) - Hatchlings and 14-day survivors

	Hatchlings					14-Day Survivors				
	control	400 ppm	960 ppm	1920 ppm	4800 ppm	control	400 ppm	960 ppm	1920 ppm	4800 ppm
No. of Ducklings Weighed	479	531	482	518	422	475	511	465	511	408
Mean Body Weight (g)	36±4	37±4	38±3	37±3	37±4	285±38	288±35	276±33	282±33	277±40

B. Toxicity Endpoints

Sub-chronic toxicity and reproduction in mallard duck exposed to flutolanil - Summary of endpoints

Test item	Flutolanil technical
Test object	Mallard ducks
Lowest observed effect concentration (LOEC)	4800 ppm
Highest tested dose without toxic effect (NOEC)	1920 ppm

III. CONCLUSIONS

There was no mortality, overt signs of toxicity, or treatment related effects upon body weight or feed consumption at any of the concentrations tested.

There was no apparent treatment related effect upon any of the reproductive parameters up to 1920 ppm. However, a slight reduction in egg shell thickness was statistically significant and biologically relevant at the 4800 ppm test concentration.

The NOEC for mallards exposed to flutolanil in the diet for 19 weeks was 1920 ppm (equivalent to 267 mg/kg bw/day). The test guideline followed by this study has serious limitation for the derivation of reliable ED_{10}, ED_{20} and ED_{50}. Furthermore, the differences between the control and the high treatment group were less than 10% and there is no evident downward trend in eggshell thickness in treated groups (see CA 8.1.1.3-03). Thus, an estimate of ED_{10} would be an extrapolated value and the estimation of ED_{10}, ED_{20} or ED_{50} for this endpoint would be inappropriate. Therefore, the original conclusion of the study that the NOEC was 1920 ppm (equivalent to 267 mg/kg bw/day) is appropriate.

Comments by RMS

The study was conducted according to U.S. EPA 71-4 (1982) and was in general agreement with OECD 206 (1984). A deviation from the OECD 206 guideline was the age of the birds (i.e. 21 weeks old instead of ca. 40 weeks) but these are not considered to invalidate the study as the validity criteria of the study were met (mortality in the control did not exceed 10% at the end of the test, the average number of 14-day-old survivors per hen in the control was at least 14 and the average eggshell thickness for the control group was at least 0.34 mm). The study is considered valid and acceptable. Mortality, adult body weight and reproductive parameters were not statistically significantly affected at any dose. Eggshell thickness was slightly but statistically significantly reduced at the highest tested dose (by 5.2%).

The applicant referred to CA 8.1.1.3-03 which states that, as the reduction in eggshell thickness is < 10% and there is no evident downward trend in eggshell thickness in treated groups, it is inappropriate to estimate ED_{10}, ED_{20} or ED_{50} for this endpoint. Therefore, the original conclusion of the study that the NOEC was 1920 ppm (equivalent to 267 mg/kg bodyweight/day) is appropriate. This is acceptable. The RMS has checked the endpoints considering a bodyweight of 313.15 mg/day and a bodyweight of 1.17 kg, which resulted in a very slightly higher endpoint (267.5), therefore, this endpoint was considered acceptable.

However, within the EFSA Birds & Mammals guidance (2009), eggshell thinning is cited as an example of an endpoint that could be considered not biologically relevant if it has no effect upon hatchability or number of surviving chicks. This is also stated in the draft Guidance on Biological Relevance (EFSA March 6, 2017), which refers to information from the EFSA Guidance (2009) and Blus, L. 2003[1], "It is believed that the biological [sic] relevant percentage of egg shell thinning starts with 18%." A graph is presented which suggests that increased egg cracking only occurs at more than 18% egg shell thinning and that even higher than this is the relevant endpoint value (considering the % of cracked eggs which is relevant to have an population level effect). The graph is reproduced below, taken from Figure 5 of Annex K of the draft Guidance on Biological Relevance (2017).

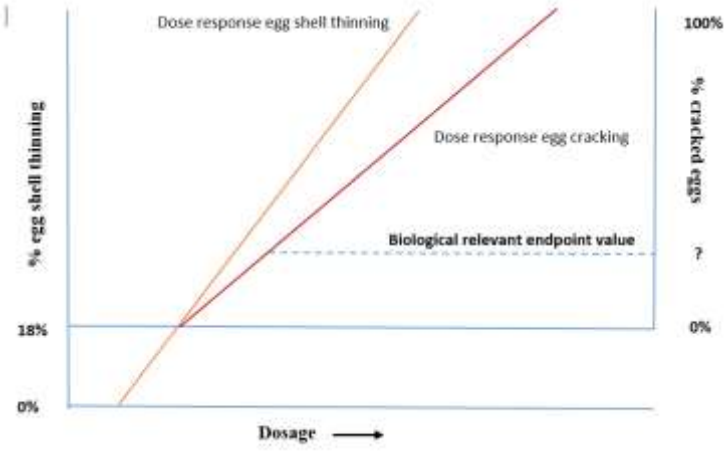


Figure 5: Relation between egg shell thickness (orange line) and cracked eggs (red line). The dashed line is the line for effecting the reproduction of a bird species (e.g. when is the number of cracked eggs too much for maintaining a stable population).

[1] Blus, L., 2003. Handbook of ecotoxicology: Organochlorine pesticides. Chapter 13. 2nd ed. CRC Press LLC, Boca Raton.

Considering this, the biologically relevant endpoint from this study could be considered to be the highest tested dose of 4800ppm (687 mg/kg bw/d, calculated by the RMS using the same bodyweight and food consumption as mentioned above). It is noted that the effect on eggshell thickness could be exacerbated by lower availability of calcium in the wild, and that, of course, effects on other species could be higher. However, considering the relatively low level of effect (about 5%) seen, and the fact that no effect was seen in the bobwhite quail reproduction study, the RMS considers it acceptable to use the highest tested dose of 687 mg/kg bw/day as the ecologically relevant endpoint for use in the risk assessment.

Reliability of endpoints

According to Regulation (EU) No 283/2013, the statistical power of the NOEC shall be assessed in case no EDx values can be derived. However, this is not possible as long as default type I and II errors have not been fixed, default variance for any response variable have not been established and typical detectable effect sizes of each parameter have not been calculated (EFSA Supporting publication 2015:EN-924). Considering the level of effect on which the NOEC was based (i.e. a 5.2 % reduction of eggshell thickness at the next higher level) the NOEC is considered sufficiently protective. **The NOEC is 1920 ppm, equivalent to 267 mg a.s./kg bw/day. The biologically relevant NOEC is 4800 ppm, equivalent to 687 mg/kg bw/day.**

[redacted]	1996b	Flutolanil technical: a one-generation reproduction study with the mallard (*Anas platyrhynchos*)	Report no 244-109 (W-3026)
Reliability			
General information			
Is a guideline method or modified guideline used?*	Yes		
Is the test performed under GLP conditions?*	Yes		
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?	Yes		
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?	Yes		
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?	Yes		

Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	Yes
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	Not applicable
Test organism	
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	Yes
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	Yes
Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Not applicable
Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	Yes
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	

Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	Yes
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Yes
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes, a reliable NOEC was derived
Are appropriate life-stages studied?	Yes

Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable
Concluding weight of evidence/proposed action	Fully acceptable
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable
Consideration/concluding score	Fully acceptable

Study 8.1.1.3-03

Report:	**CA 8.1.1.3-03. Palmer, D.A., 2016**
Title:	EAG Laboratories letter – To whom it may concern
Report No.:	Test code: 244-108 l 18-7606-6837
Published:	No
GLP:	No
Guidelines:	N/A
Deviations:	N/A
RMS Comment:	Response to questions concerning EC_x calculations for avian reproduction studies: [redacted] Project No. 244-108 (W-3025) Flutolanil technical: a one generation reproduction study with the bobwhite (*Colinus virginianus*) [redacted] 1993a; [redacted] Project No. 244-109 (W-3026) Flutolanil technical: a one generation reproduction study with the mallard (*Anas platyrhynchos*) U.S. EPA 71-4 (1988), [redacted]1996b.
Endpoint:	Not relevant.

Executive summary

We agree fully with the opinion given in the EFSA document on the limitations of the design of avian reproductions to support calculation of EC_x values. It is sometimes impossible to provide meaningful EC_x values for endpoints from the studies, but it is also true that reasonable EC_x estimates can sometimes be obtained. Therefore, although we agree that the EC_{10} and EC_{20} estimates should not be routinely provided in avian reproduction study reports, if there are questions regarding the reliability of a NOEC, and an EC_x estimate is requested by regulatory authorities, such requests must be judged on a case-by-case basis. One cannot make a blanket statement that EC_x estimates will not be reliable. The two studies identified above provide examples of the need for case by case evaluation of the

utility of EC_x estimates.

In the case of the mallard study (244-109) identified above, eggshell thickness appeared to be the most sensitive endpoint, and in the original analysis, eggshell thickness in the 4800 ppm treatment group was found to be significantly different from eggshell thickness in the control group ($p < 0.05$). However, the differences between the control and the high treatment group were less than 10%. Thus an estimate of EC_{10} would be an extrapolated value. Furthermore, there is no evident downward trend in eggshell thickness in treated groups, and it is therefore inappropriate to estimate EC_{10}, EC_{20} or EC_{50} for this endpoint. Therefore, the original conclusion of the study that the NOEC was 1920 ppm (equivalent to 267 mg/kg bodyweight/day) is appropriate.

In the case of the bobwhite study (244-108) identified above, the most sensitive endpoint appeared to be egg production. There was an apparent reduction of greater than 10% in the number of eggs produced in the 4800 ppm treatment group. A three parameter cumulative-normal (Bruce-Versteeg) model was fit to this data, an EC_{10} estimate of 4083 ppm was obtained. However only the upper 95% confidence bound for the EC_{10} (6897 ppm) was obtained, indicating little confidence in the reliability of EC_{10} estimate. Therefore, the original conclusion of the study that the NOEC was 1920 ppm (equivalent to 247 mg/kg bodyweight/day) is appropriate.

B.9.1.2 Effects on terrestrial vertebrates other than birds

B.9.1.2.1 Acute oral toxicity to mammals

Please refer to the mammalian toxicology section of this dossier M-CA 5.2.1.

B.9.1.2.2 Long-term and reproduction toxicity to mammals

Please refer to the mammalian toxicology section of this dossier M-CA 5.6.1.

Study 8.1.2.2-01

Report:	**CA 8.1.2.2-01. Thomas, G.D., Myers, D.P., 2016**
Title:	Request to determine reliable ECx values
Report No.:	17 October 2016
Published:	No
GLP:	No
Guidelines:	N/A
Deviations:	N/A
RMS Comment:	Response to request to determine reliable EC_x values for T-3043 FLUTOLANIL: TERATOLOGY STUDY IN THE RAT (AMENDMENT TO FINAL REPORT First Amendment to: LSR Report No. 871NHH0231554) and, T-3069 A TWO GENERATION REPRODUCTION STUDY IN RATS WITH FLUTOLANIL (PROJECT NO. 69-3417)
Endpoint:	Not relevant.

Executive summary

The reliability of EC_x values was evaluated in the two-generation reproduction study in rats (CA 5.6.1/01) and in the teratology study in the rat (CA 5.6.2/01). Due to the limited number of dose levels tested and potentially high variability, combined with the fact that the studies were not designed to

determine reliable EC_x values, it is not possible to fit a reliable model to the endpoints of the studies mentioned in order to calculate the required $EC_{10, 20, 50}$ values. Usually for such a calculation data are available for at least 7 concentrations for a relevant endpoint and then an appropriate model can be fit to the data in order to obtain $EC_{10, 20, 50}$ estimates. The lack of ecotoxicologically relevant endpoints in the studies also makes this determination inappropriate and there would be very little to gain by performing such an exercise.

Comments RMS:

We agree with the conclusion of the notifier that derivation of ECx values for these studies is not possible/useful.

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

This point is addressed in the risk assessment for birds and mammals in M-CP 9.1.1. Studies on bioconcentration in fish are found under point (M-CA) 9.2.2.3, below.

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

The data point is addressed by the Amphibian Metamorphosis Assay performed under GLP and any further information found in the open literature.

Study 8.1.4-01

Report:	**CA 8.1.4-01, [redacted], 2011**
Title:	Flutolanil: Amphibian Metamorphosis assay for the detection of Thyroid Active Substances
Report no.:	397A-149 (W-3073)
Published:	No
GLP:	Yes
Guidelines:	OECD 231 (2009), US EPA-OPPTS Number 890.1100 (2009)
Deviations:	Minor protocol deviations; none that have affected the integrity of the study. A minor guideline deviation was noted in the water temperature on Day 15 for a short duration in one control replicate.
RMS Comment:	The validity criteria were met and the study is considered to be acceptable.
Endpoint	NOEC = 0.13 mg a.s./L (not to be used for risk assessment). No indications of thyroid activity.

Executive Summary

The effects of flutolanil on the normal function of the hypothalamic-pituitary-thyroid (HPT) axis of the African clawed frog (*Xenopus laevis)* were determined in a 21-day exposure period under flow-through test conditions. Tadpoles at Nieuwkoop and Faber (NF) stage 51 were exposed to 0.015, 0.15 and 1.5 mg a.s./L (nominal), a dilution water control and a solvent control using dimethylformamide (20 µL/L). Groups of 20 tadpoles were impartially assigned to four test chambers in each treatment

and control group. Behavioural observations were made throughout the test. The endpoints evaluated to determine if the test substance might impact the hypothalamic-pituitary-thyroid (HPT) axis of tadpoles were survival, total length, wet weight, snout-to-vent length, normalized hind limb length, developmental stage, and thyroid histopathology.

Analysis of the test solutions for the active substance flutolanil was performed in samples collected weekly during the test. The mean measured test concentrations for this study were 0.015, 0.13 and 1.1 mg a.s./L, representing 100, 87 and 73% of nominal concentrations, respectively. The results of the study were based on the mean measured concentrations.

Statistically significant treatment-related effects were observed on Day 21 in the high (1.1 mg a.s./L) treatment group and included decreases in total length, wet weight and snout-to-vent length in comparison to the solvent control. Although the study report does not provide a NOEC value, a value of 0.13 mg a.s./L was derived. The effects on growth suggest non-thyroidal toxicity rather than a dysfunction of the thyroid, since there were no statistically significant treatment-related effects on thyroid histology, developmental stage or hind-limb length at any concentration tested.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test material:**	Flutolanil Technical
	Batch no.:	30116
	Purity:	98.7%
2.	**Test organism:**	African Clawed Frog (*Xenopus laevis*)
	Age:	Tadpoles at NF Stage 51 at test initiation
	Source:	[redacted]
	Feeding:	From four days post-fertilisation, tadpoles were fed Sera Micron® three times daily during the acclimation period. The Sera Micron® was delivered to the tanks as a suspension in dilution water (30 mg/mL) and amounts were increased to approx. 15 mg/animal/day before test initiation. During the test, tadpoles were fed three times a day during the test and once on the last day of the test. Feeding rates were adjusted for growth of the tadpoles.
3.	**Treatment:**	Control, Solvent control (DMF), 0.015, 0.15 and 1.5 mg a.s./L (nominal) or 0.015, 0.13, 1.1 mg a.s./L (measured)
4.	**Test vessels:**	12 L Glass aquaria filled with approx. 10 L of test water
	Test water:	Filtered, aerated and UV-sterilised well water
5.	**Environmental conditions**	
	Temperature:	21.2-22.2°C
	pH:	7.9 – 8.3
	Dissolved oxygen:	3.7 – 8.4 (mg/L)
	Photoperiod:	12 h light : 12 h darkness

B. STUDY DESIGN AND METHODS

1. In-life phase: 08 July to 04 August 2011

2. Test organism assignment and treatment

Xenopus laevis tadpoles were exposed to a series of three test concentrations (nominally 0.015, 0.15 and 1.5 mg a.s./L as flutolanil), a negative (dilution water) control and a solvent control (20 µL/L dimethylformamide) under flow-through conditions. Groups of 20 tadpoles at Nieuwkoop and Faber (NF) stage 51 were impartially assigned to four test chambers in each treatment and control group for 21 days.

3. Dose preparation

All test solutions were adjusted to 100% active ingredient during preparation, based on the reported test substance purity (98.7%). A primary stock solution was prepared in dimethylformamide (DMF) at a nominal concentration of 75 mg a.s./mL, which was serially diluted in DMF to produce two secondary stock solutions at 7.5 and 0.75 mg a.s./mL (nominal). The stock solutions were held under refrigerated conditions in amber bottles and fresh aliquots were placed in the syringe pump weekly during the test. The stock solutions were pumped into the diluter mixing chambers at a target rate of 5.6 µL/min, and were mixed with well water delivered at a target rate of 280 mL/min, to achieve the nominal test concentrations of 0.015, 0.15 and 1.5 mg a.s./L. The mixing chambers were stirred continuously and were protected from light. The test solutions were pumped from the mixing chambers into the test chambers at a target rate of 66 mL/min. The negative control received dilution water only. The solvent control was prepared by delivering DMF to the mixing chamber for the solvent control, resulting in a concentration of 20 µL DMF/L equivalent to all flutolanil treatment groups.

4. Measurements and observations

During the test, survival and general observations were made daily. Any mortalities, external abnormalities (such as lesions or malformations), abnormal behaviour (such as floating, lying on the bottom of the tank, inverted or irregular swimming, lack of surfacing activity, or being non-responsive to stimulus) were noted. On Day 7 of the test, five tadpoles were randomly selected from each test chamber of each treatment and control group. The tadpoles selected on Day 7, and all surviving tadpoles in each test chamber at test termination, were euthanized with MS-222 (tricaine methanesulfonate). The developmental stage, total length, wet weight, snout-to-vent length, and hind limb length, were evaluated. All surviving tadpoles in each test chamber at test termination were fixed in formalin and five per replicate were evaluated for thyroid gland histology.

5. Statistics

To evaluate differences between treatment and control groups, statistical analyses were performed for each biological endpoints, using SAS (6) software at confidence level of $\alpha = 0.05$. The multi-quantile Jonkheere-Terpstra trend test was applied in a step-down procedure to identify concentration responsive trends among the treatment groups. All endpoints except for survival and developmental stage data were evaluated for normality using Shapiro-Wilk's test and for homogeneity of variance using Levene's, then analysed by performing pair-wise comparisons using Dunnett's multiple

comparison test. Survival data were analysed by performing pair-wise comparisons using the Fishers exact test. Developmental stage data were analysed using the Jonckheere-Terpstra trend analysis developed by T. Springer and J. Green as recommended by the guidelines.

II. RESULTS AND DISCUSSION

A. Biological data

Survival and general observations

There were no statistically significant treatment-related effects on survival of tadpoles during the 21-day test in comparison to the control ($p > 0.05$).

Tadpoles in the control groups and in the 0.015, 0.13 and 1.1 mg a.s./L treatment groups generally appeared normal throughout the test. One tadpole in the 0.13 mg a.s./L treatment group was periodically noted as small with a misshapen head, and another was noted as weak. At test termination, one tadpole in the 0.13 mg a.s./L treatment group was noted to have an extra front leg. All other surviving tadpoles appeared normal throughout the test. Incidence of tail curvature ranged from 0 to 14% among the groups by test termination and was comparable to the controls.

Growth and Development – Day 7 and at Termination Day 21

The use of DMF as a solvent resulted in slight increases in the stage of development, length, and weight measurements relative to the negative control. These slight increases were attributed to greater primary productivity, therefore, the solvent control was believed to be the appropriate control group to be used in data evaluation.

Total length – On Day 7, there was no significant trend in the data (Jonckheere-Terpstra trend test, $p > 0.05$). A slight statistically significant increase was noted at 0.13 mg a.s./L, but was not considered to be treatment-related. On Day 21, there was no significant trend in the mean body length data (Jonckheere-Terpstra trend test, $p > 0.05$). However, there was a statistically significant decrease in length in the 1.1 mg a.s./L treatment group in comparison to the solvent control (Dunnett's test, $p \leq 0.05$) that was considered to be treatment related.

Wet weight – On Day 7, there were no statistically significant effects on wet weights in any treatment group in comparison to the solvent control ($p > 0.05$). On Day 21, there was no significant trend in the data (Jonckheere-Terpstra trend test, $p > 0.05$). However, there was a statistically significant decrease in weight in the 1.1 mg a.s./L treatment group in comparison to the solvent control (Dunnett's test, $p \leq 0.05$) that was considered to be treatment related.

Snout-to-vent length – On Day 7, there were no statistically significant effects on snout-to-vent lengths in any treatment group in comparison to the solvent control ($p > 0.05$). On Day 21, there was no significant trend in the data (Jonckheere-Terpstra trend test, $p > 0.05$). However, there was a statistically significant decrease in snout-to-vent length in the 1.1 mg a.s./L treatment group in comparison to the solvent control (Dunnett's test, $p \leq 0.05$) that was considered to be treatment related.

Normalised hind limb length – There were no statistically significant effects on normalised hind limb length in any treatment group in comparison to the solvent control ($p > 0.05$) on Day 7 or Day 21.

Developmental Stage – There were no statistically significant effects on developmental stage in any treatment group in comparison to the solvent control ($p > 0.05$) on Days 7 or 21.

Histopathology

There was no treatment related effects observed by histopathological examination of the thyroids of tadpoles in any treatment group at termination of the 21-day test.

B. Analytical verification

All test solutions appeared clear and colourless, with no evidence of precipitation observed in the test chambers or diluter mixing chambers during the test. Measured concentrations of the pre-test samples ranged from 88.1 to 95.9% of nominal concentrations. Measured concentrations of the samples collected from the 0.015 and 0.15 mg a.s./L treatment groups on Days 0, 7, 14 and 21 ranged from 88.2 to 103% of nominal concentrations. Measured concentrations of the samples collected on Days 0, 7, 14 and 21 from the 1.5 mg a.s./L treatment group ranged from 48.8 to 95.3% of nominal concentrations. The variability in the measured concentrations at 1.5 mg a.s./L was attributed to testing at the limit of solubility in the test system, although no precipitates were visible. The measured concentrations of the stock solution samples collected on Day 0 ranged from 100 to 103% of nominal concentrations, confirming that the correct concentrations were being delivered.

The mean measured test concentrations for this study were 0.015, 0.13 and 1.1 mg a.s./L, representing 100, 87 and 73% of nominal concentrations, respectively. The results of the study were based on the mean measured concentrations.

C. Toxicity Endpoints

The endpoints evaluated to determine if the test substance might impact the hypothalamic-pituitary-thyroid (HPT) axis of tadpoles were survival, total length, wet weight, snout-to-vent length, normalized hind limb length, developmental stage, and thyroid histopathology. Statistically significant treatment-related effects were observed on Day 21 in the highest treatment group (1.1 mg a.s./L mean measured) and included treatment-related decreases in total length, wet weight and snout-to-vent length in comparison to the solvent control. A NOEC value of 0.13 mg a.s./L was derived from the study.

Toxicity endpoints for the test item flutolanil technical

21 Days	**Effect concentration** (mg a.s./L)
LC_{50}	>1.1
21-d NOEC	0.13

III. CONCLUSION

African clawed frog (*Xenopus laevis*) tadpoles were exposed to flutolanil at mean measured concentrations of 0.015, 0.13 and 1.1 mg a.s./L for 21 days. Statistically significant treatment-related effects were observed on Day 21 in the high (1.1 mg a.s./L) treatment group and included decreases in total length, wet weight and snout-to-vent length in comparison to the solvent control. A NOEC value of 0.13 mg a.s./L was derived from the study. The effects on growth suggest non-thyroidal

toxicity rather than a dysfunction of the thyroid, since there were no statistically significant treatment-related effects on thyroid histology, developmental stage or hind-limb length at any concentration tested.

([redacted] 2011)

The results of the amphibian metamorphosis assay could be considered to be uncertain, since slightly significant effects between the controls and the solvent controls were observed. Although some effects were observed in the highest exposure level of 1.1 mg/L, there were no statistically significant differences between the control groups for survival. The NOEC derived for this study is considered to be between 1.1 and 0.13 mg/L which includes the lower endpoint derived from the fish reproductive study (0.23 mg/L). Since there were no additional levels tested in-between and the separation of the two levels exceeds the factor of 3.2 therefore, the fish study is still considered to be the most sensitive and protective endpoint for the aquatic risk assessment. The effects seen in this amphibian metamorphosis assay were not considered to be related to flutolanil treatment (USEPA, 2015). Therefore, these results do not suggest a potential for interaction with the thyroid hormone pathway in amphibians (Odum J., *et al.* 2016).

Comments by RMS

The study was conducted in accordance with the OECD 231 test guideline and all validity criteria were met. The test guideline contains a list of performance criteria, which can be used as guidance for determining the quality of the test performed and the performance of the control organisms. Three out of ten criteria were not met:

1. At the highest test concentration, actual test concentrations were not maintained at ≤20% CV over the test period. This was attributed to the solubility of flutolanil, which had been established to be lower than 2.0 mg a.s./L in test medium at the test facility. The NOEC of this study was however found at the next lower level, where the concentration was maintained within 20% CV. Therefore, the excess of the CV at the highest test concentration is not considered to affect the outcome of the study.
2. Inter-replicate/inter-treatment water temperature differentials should not exceed 0.5°C. This criterion was exceeded in the solvent control (differential 0.6 °C) and at 0.015 mg a.s./L (differential 0.9 °C). However, as temperatures were similar across treatments and all validity criteria were met, this is not considered to have affected the outcome of the study.
3. In the statistical analysis, flutolanil treated groups were compared to the solvent control, as statistically significant differences were detected between the two control groups for developmental stages on days 7 and 21 and for total length, wet weight and snout-to-vent length on day 21. The authors of the report attributed these differences to the use of DMF as a solvent, as this can result in slight increases in the stage of development, length and weight due to greater primary production in solvent tanks, leading to increased microbial growth and more food for the tadpoles. The OECD guideline states that developmental stage, snout-to-vent length and wet weight are relevant endpoints for consideration among controls, as these can be affected through non-thyroidal toxicities, and that if statistically significant differences are detected in these

endpoints between the clean water control and solvent control groups, study endpoints should be determined using the clean water control. In the current study however, the use of the solvent did not lead to toxicity but rather to promoted growth of the tadpoles, and comparison with the clean water control would possibly have led to a higher NOEC than the one derived by comparison with the solvent control. Therefore, the statistical analysis based on comparison between treatments and the solvent control is considered acceptable.

The study is considered valid and reliable.

Results were based on mean measured concentrations, which is acceptable. The analytical method used for verification of test concentrations was sufficiently validated (HPLC-UV, mean recovery at fortification levels of 0.015 and 1.5 mg a.s./L 102%, RSD 3.95%, n=5 at both levels).

For histological analysis, tadpoles were selected such that they were stage-matched to the clean water control (median stage 57). The median developmental stage in both the solvent control and all treatments was 59. However, as all tadpoles were stage-matched with the clean water control, stages were also matched between solvent control and treatments. Therefore, stage-matching to the clean water control is considered acceptable. Thyroid hypertrophy, increased colloid area, increased follicle size and increased follicle atrophy were statistically significantly different in the solvent control compared to the clean water control. Therefore, histological results for the flutolanil treatments were statistically compared with both controls (separately), which is acceptable. Severity was graded according to the following scale: 0 = unremarkable, 1 = mild, 2 = moderate, 3 = severe.

Results are shown in the Tables below.

Table 8.1.4/01-01 Results for apical endpoints

	Survival (%)	**Median developmental stage**	**Mean total length (mm)**	**Mean total weight (mg)**	**Mean snout-to-vent length (mm)**	**Mean normalised hind limb lenth (mm)**
Day 7						
Control	98	53	36 ± 1.9	225 ± 28	14.5 ± 0.61	0.15 ± 0.016
Solvent control	100	55	39 ± 2.5	289 ± 55	16.0 ± 1.1	0.15 ± 0.017
0.015 mg a.s./L	98	55	41 ± 1.3	316 ± 30	16.6 ± 0.92	0.14 ± 0.007
0.13 mg a.s./L	99	55	43 ± 2.1*	348 ± 62	17.1 ± 0.97	0.14 ± 0.006
1.1 mg a.s./L	99	54	39 ± 1.6	256 ± 21	15.3 ± 0.40	0.15 ± 0.014
NOEC (mg a.s./L)	1.1	1.1	1.1	1.1	1.1	1.1
Day 21						
Control	98	52	52 ± 1.7	729 ± 59	21.9 ± 0.41	0.43 ± 0.030
Pooled control	100	59	60 ± 1.3	965 ± 75	24.0 ± 0.82	0.54 ± 0.078
0.015 mg a.s./L	96	59	58 ± 0.6	873 ± 39	23.1 ± 0.35	0.55 ± 0.038
0.13 mg a.s./L	99	59	60 ± 0.6	983 ± 31	23.9 ± 0.17	0.54 ± 0.023
1.1 mg a.s./L	96	59	56 ± 0.9*	755 ± 40*	22.1 ± 0.61*	0.53 ± 0.039
NOEC (mg a.s./L)	1.1	1.1	0.13	0.13	0.13	1.1

* Significantly different from solvent control at 5% level

Table 8.1.4/01-02 Results for histological assessment

	Thyroid atrophy	Thryoid hypertrophy	Colloid area decreased	Colloid area increased	Gland size decreased	Gland size increased	Gland asymmetry increased	Follicle size decreased	Follicle size increased	Follicle asymmetry increased	Peripheral vacuolization absent
% of tadpoles with observation											
Control	5	10	0	0	5	10	0	0	0	0	0
Solvent-control	0	70	0	45	0	70	5	0	60	25	0
0.015 mg a.s./L	5	40	5	40	5	40	5	5	40	15	0
0.13 mg a.s./L	5	50	5	50	5	50	5	5	50	10	5
1.1 mg a.s./L	5	25	5	20	5	25	0	5	20	5	0
Mean severity of observation											
Control	0.05	0.10	0	0	0.05	0.10	0	0	0	0	0
Solvent-control	0	1.20	0	0.45	0	1.30	0.05	0	0.60	0.25	0
0.015 mg a.s./L	0.10	0.90	0.05	0.50	0.10	0.90	0.05	0.05	0.55	0.15	0
0.13 mg a.s./L	0.05	0.90	0.05	0.50	0.05	0.95	0.05	0.05	0.50	0.10	0.05
1.1 mg a.s./L	0.05	0.40	0.05	0.25	0.05	0.45	0	0.05	0.25	0.10	0
NOEC (control)	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
NOEC (solvent-control)	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1

Based on comparison with the solvent control, total length, total weight and mean snout-to-vent weight were statistically significantly reduced at the highest tested concentration (by 7, 22 and 8%, respectively), while reductions were not observed at the lower concentrations. Effects on thyroid gland histopathology were not observed at any treatment level, either when compared with the solvent control or the clean water control.

Based on the lack of advanced development, asynchronous development, delayed development and changes in thyroid histology, it is concluded that flutolanil was thyroid inactive. The NOEC from the present study was 0.13 mg a.s./L, based on reduced total length, wet weight and snout-to-vent length (i.e. growth).

Reliability of endpoints

The current study does not allow for the calculation of ECx values. According to Regulation (EU) No 283/2013, the statistical power of the NOEC shall be assessed in case no ECx values can be derived. However, this is not possible as long as default type I and II errors have not been fixed, default variance for any response variable have not been established and typical detectable effect sizes of each parameter have not been calculated (EFSA Supporting publication 2015:EN-924). Considering the level of effect on which the NOEC was based (i.e. a 7%, 22% and 8% reduction in total length, total weight and snout-to-vent length, respectively, compared to the solvent control in the highest

tested concentration) and the complete absence of effects at the next lower level (i.e. the NOEC), the NOEC is considered sufficiently protective.

The NOEC of flutolanil in an amphibian metamorphosis assay was 0.13 mg a.s./L and may be used for risk assessment. Flutolanil was concluded to be thyroid inactive.

[redacted]	2011	Flutolanil: Amphibian metamorphosis assay for the detection of thyroid active substances	Report no 397A-149 (W-3073)
Reliability			
General information			
Is a guideline method or modified guideline used?*		yes	
Is the test performed under GLP conditions?*		Yes	
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?		Yes	
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?		Yes	
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?		Yes	
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?		Yes	
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?		Not applicable	
Test organism			
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?		Yes	
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?		Yes	
Exposure conditions			

Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Yes, except for the highest concentration, which was however above the level of the NOEC
Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	Yes
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	Yes
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes

Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Yes
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable
Concluding weight of evidence/proposed action	Fully acceptable
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable
Consideration/concluding score	Fully acceptable

Study 8.1.4-02

Report:	**CA 8.1.4-02, Millot, F., Berny, P., Decors, A., Bro, E., 2015**
Title:	Little field evidence of direct acute and short-term effects of current pesticides on the grey partridge
Report no.:	Not applicable
Published:	Yes
GLP:	No
Guidelines:	None
Deviations:	Not applicable
RMS Comment:	Ecotoxicology and Environmental Safety 117:41-61 Institutions: National Game and Wildlife Institute (ONCFS), Research Department, Saint Benoist, 78610 Auffargis, France. College of Veterinary Medicine, Toxicology, Ecole Nationale Vétérinaire de Lyon, 1, av Bourgelat, 69280 Marcy l'étoile, France.
Endpoint:	Not relevant.

Executive Summary

Pesticides may affect farmland avian populations either directly, through lethal or sublethal poisoning, or indirectly, especially by reducing food resource. Since the restriction of the use of plant protection products (PPP's) which are known to be directly toxic to avian populations in Europe, it is unknown

how the use of plant protection products may contribute to declining farmland avian populations. This large-scale auto-ecological study combined radiotelemetry, farmer surveys, residue analyses on carcasses and modelling to assess the unintentional effects of pesticides on terrestrial birds:

- Radiotracking study
- Farmer questionnaire to record plant protection products (PPP's) use
- Spatio-temporal analysis using a GIS to cross-check bird habitat use and PPPs application at the field-day scale to identify the potential exposure of each bird to PPP's, and quantify associated mortality rates
- Necropsies and residue analyses on carcasses
- Modelling

The grey partridge, *Perdix perdix*, was chosen as a case study as it is typical of European cereal ecosystems, its biology and population dynamics are extensively known and it is exposed to pesticides. The acute and short-term impacts of pesticides on adult mortality during spring and summer were assessed in a one-substance approach for a large variety of active substances (a.s.) actually used in cultivated farmland. The study was conducted in France, where agriculture is one of the most intensive in Europe, both in terms of yields and tonnages of pesticides used. The fate and the location of radiotransmittered 529 partridges were monitored twice a day from early March to late August 2010 and 2011 on 12 sites (14,500 ha). Their daily potential exposure to 183 active substances was determined by overlapping birds' habitat use and daily pesticide application data. Based on this procedure, mortality rates within 10 days following a potential exposure for 157 different active substances were calculated.

During spring–summer, the grey partridge is highly exposed to a variety of active substances, including flutolanil.

Of the 261 mortalities recorded (N = 529), 94 carcasses were in suitable condition for residue analyses. Furthermore, modelling results showed that these lethal pesticide-related poisonings decreased the population growth rate by less than 1%.

In conclusion, direct acute and short-term effects of pesticides currently used by farmers during the breeding season had no significant acute or short term effect on the grey partridge.

Flutolanil was used on 33.3% of the 12 sites, on an area of 0.7% of the total study area, by 4.4% of farmers who participated in the survey taken. The target crop for flutolanil, potatoes, was considered a main crop in this study. Through modelling, it was concluded that only 5 birds were potentially exposed to flutolanil (n = 529). Of the 5 birds potentially exposed, the statistically derived mortality rate was null. Of 94 carcases analysed, no flutolanil was detected using GC-MS (limit of detection = 0.1 µg/g). Flutolanil was determined to be of low risk to the Grey Partridge through potential exposure and no mortality as a direct result of flutolanil exposure was established.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Sites:

	Location:	Northern-Central France
	Number:	12
	Total area:	145 km^2
	Partridge density:	5 to 60 pairs/km^2 in spring 2010-2011
	Dominant crop:	Winter Wheat
	Other major crops:	Winter Barley
		Rapeseed
	Other minor crops:	Sugar beet
		Alfalfa
		Pastures
		Maize
		Potato
		Linseed
	Typical Harvest:	6.5 to 9.5 T/ha Winter Wheat
2.	**Test Species:**	Grey Partridge (*Perdix perdix*)
	Habitat:	Cereal farmland
	Background:	Representative focal species
		Population decline
		Forage in crops
		Diet (adult): Opportunistic omnivorous diet: Leaves, buds, crop grains, weed seeds, invertebrates
	Diet (chick):	Mainly insectivorous diet (up to 2 weeks)

B. STUDY DESIGN AND METHODS

1. Publication: Received 12 September 2014, Accepted 18 March 2015

2. Test Sites

All habitat features (hedgerows, woods, copses, bushes, set-aside plots, crop fields, roads, etc.) of each study site were mapped on a GIS (QGIS 1.7.4) using a nomad PDA-GPS (Trimble JunoSB, D3E Electronique – France/Software Windows mobile 6 and ArpentGIS).

3. Radio-tracking survey

A radio-tracking survey of grey partridges over the 12 study sites in spring and summer 2010–2011 was undertaken. From late February to the end of March, partridges were caught at night with a hand-held net while they were temporarily dazed with a strong light. Birds were tagged with a metal leg ring and fitted with a transmitter. The sex of each bird was determined using feather criteria and their body mass recorded using a steelyard. Handling time did not exceed 5 min. Necklace radio-transmitters including a 30-cm antenna were used. The transmitter package was ca. 2.5% of the body mass of lightest birds. Transmitters were equipped with a motion-sensitive mortality circuit that transmitted a mortality signal after 42 h of motionless. A total of 529 partridges (62 males and 467 females) were fitted with transmitters and monitored (alive, dead or “missing”) twice daily. The location of living birds

was determined via triangulation. When a bird was determined as dead, its carcass was retrieved as soon as possible, in order to collect it in an as freshly condition as possible to allow an adequate field diagnosis of the cause of death and a necropsy. The delay was usually <12 h after death. The epidemiological and ecological context was recorded at the time of the carcass discovery: The surrounding area was observed to establish the putative proximal cause of death (predation, collision, disease, intoxication or other causes) according to the following key.

Cause of death	**Criteria**
Mammal predation	Feathers cut
Raptor predation	Feathers plucked
Collision	Intact carcass found < 25-30 m from road
Suspected disease or poisoning	Intact carcass with no sign of predation or collision
Unidentified cause	Insufficient remains or absence of signs

4. Necropsies and Analysis for Pesticide Residue

Necropsy of carcasses (except those with only feather and bone remains) was undertaken at a local veterinary laboratory.

Body condition was roughly scored as “good” or “cachexia”.

A wildlife pathologist performed a macroscopic pathological examination to verify field diagnosis for cause of death (e.g. collisions were confirmed by the presence of fracture and internal haemorrhage). Further examinations (parasitology, bacteriology, virology, histology) were conducted when necessary to determine the aetiology of death or to identify a disease. The necropsy conclusion on the cause of death was accepted as conclusive. After the necropsy, all available organs were systematically analysed for residues at a toxicological laboratory.

5. Pesticide Use

A survey, of 142 farmers, to identify all agricultural practices that farmers carried out on each of their 1000 field plots (total 6500 ha area) was carried out. Farmers were asked to provide information on the PPP’s they used (trade formulations, dates and techniques of application, and doses used). Active substances were identified from trade formulations using the E-PHY database of the French ministry in charge of Agriculture. Additives and biological agents were excluded.

For each active substance, the following details were recorded:

- Registration number (CAS)
- Chemical family and classification (e.g., fungicide, herbicide, insecticide, etc.)
- Avian acute toxicity (lowest LD_{50})
- Bird acute risk assessment (lowest acute Tier 1)
- HD5 (Hazardous Dose at the 5% tail of the species sensitivity distribution of acute toxicity), when available

6. Dead Birds

The potential exposure to PPP’s of each dead bird during its last ten days of life was established. The activity area of each bird as the convex hull of their 10 bi-daily locations before their death was

determined. The field plots included in these activity areas and which PPP's were applied during these 10-day periods were identified. The last 10 days before death were considered as a compromise to investigate acute and short-term effects of PPP's on partridge mortality with a relatively short list of PPP's to be realistic from an analytical and a financial point of view, and to use enough bird locations to determine the best of current activity area. It was considered that it was more ecologically relevant of the whole habitat use. Although partridges were geo-located with a consistent effort (twice a day), data obtained was probably not sufficient to capture in detail the whole daily habitat use. This field knowledge was considered highly valuable considering both the pesticides currently and actually used, and the spatio-temporal heterogeneity of their application.

6. Surviving Birds

Similarly, the potential exposure of surviving birds to PPP's was determined according to their daily habitat use. To do so, it was examined whether the birds that spent time in a field plot at the time of a given PPP application, or during the 10 days after application, was still alive after 11 days. This procedure was repeated for each application of a given PPP on a given field plot.

7. Percentage of Dead Partridges Exposed to a Given Active Substance

Simple descriptive statistics such as the proportion of dead partridges among the ones potentially exposed to a given active substance were calculated. The visit of several field plots on which the same active substance was used the same day was considered as a single exposure. On the contrary, when a same active substance was used on different days, the situation was considered as several exposures. The index of exposure, "Active substance × bird × day" was used. This statistical approach offered a global overview to discuss acute and short-term effects of pesticides on grey partridge survival. Only the effects of an active substance in a univariate way were considered, cocktail effects were not investigated. Analysis was simplified (owing to environmental complexities) using a step-stair approach. Data were collected over 12 sites during 2 years to depict the global situation of pesticide use but site and year effects and possible interactions were not analysed, due to the small sample size at this scale. Spatio-temporal heterogeneity was considered at the partridge individual level.

8. Residue Analysis

Residues analyses related to 20 chemical families served two purposes and were performed on all carcasses when suitable organs (liver, gizzard/ stomach contents, and encephalon) were available according to the following process.

- Could mortality be explained by active substance poisoning?
- Investigation of evidence of actual exposure (tissue analysis of pyrethroids and triazoles the bird was exposed to over the ten days preceding death) *vs* potential exposure
- Screening of dead bird tissue to chemical families, including Sulfonylureas (quantified by GC-MS after extraction in organic solvent, LOD 0.10 µg/g)

All residue analyses were undertaken by the Toxicology Laboratory (VetAgro Sup, Lyon, France):

9. Modelling Structure

A life-cycle model (time step = 1 year) was built to investigate the potential impact of PPP use at the population level which assumed reproductive events to be instantaneous. Environmental stochasticity without demographic stochasticity was modelled. Population dynamics were simulated according to:

$N_{(t+1)}=N_{(t)}\cdot S_{ss}\cdot(1+OFSF)\cdot S_{aw}$

Where:

$N_{(t)}$ = The population size in early spring

S_{ss} = The spring–summer survival rates

S_{aw} = The autumn–winter survival rates

OFSF = The final reproductive success (No. offspring ♀/surviving adult ♀in summer)

T = Indicates time *t*

Estimation of Model Parameters

Survival rates were estimated from the radio-tracking survey. The spring–summer survival rate was estimated as:

$$\hat{SS} = \mathrm{II}_{i=1}^{n}(1-\hat{M}_i)$$

Where:

$\hat{M}$ = The daily mortality rate

N = The number of days of the considered period

Transmitters and signal losses were either censored (“censoring” hypothesis) or considered as mortality cases (“mortality” hypothesis”) or survival cases (“survival” hypothesis). Minimum, mean and maximum estimates were calculated according to these 3 scenarios. S_{ss} estimates were calculated twice, including or not birds dead from pesticide poisoning. Breeding success was defined as the number of male and female offspring per female in summer. It was estimated in August through covey surveys. Fields were searched by slowly driving a car along lanes and across fields at dawn and dusk when the birds were feeding in cereal stubbles or other patches of open grounds. When a covey was detected, binoculars were used to count the number of cocks, hens and offspring. Parameter's S.D. was calculated using parameter estimates across sites * years (n¼24). Hence, spatio-temporal variability was assumed to describe correctly environmental stochasticity (only included stochasticity for S_{ss}). For each time step, S_{ss} was randomly selected within a gaussian distribution with a mean of 0.468, a S.D. of 0.134, a minimum of 0.352 and a maximum of 0.933 (minimum and maximum estimates of S_{ss} across sites and years). For OFSF, the mean of the Gaussian distribution was set to 3.28, S.D. to 1.03, the minimum to 1.20 and the maximum to 5.25.

Population Viability Analysis

Population dynamics were run for 10 years and 10,000 replicates were completed for every MonteCarlo simulation to ensure statistically reliable predictions. A new random seed was selected for each simulation and values of demographic parameters varied randomly at each time step to tackle environmental stochasticity. Demographic stochasticity was not included. Unified Life Model (ULM)

software was used to run the model. The population growth rate and two measures of population persistence (extinction risk and extinction time) were chosen as endpoints.

The stochastic growth rate:

$$\lambda_{stocha} = \exp(r)$$

Where:

r = The average of the logarithmic growth rates of *M* trajectories computed as:

$$r = (\frac{1}{M}) \sum_{j=1}^{M} [\ln(n_j(T)) - \ln(n_j(0))/T]$$

Where:

$n_j(0)$ = The population size of the j^{th} trajectory at time 0

$n_j(T)$ = The population size of the j^{th} trajectory at time T

Intoxication Scenarios

In the 'intoxication' scenario, S_{ss} was assigned to the values estimated in the field, i.e., including casualties attributed both to lethal pesticide poisoning and predation ultimately resulting from non-lethal poisoning (through e.g., behavioural effects). In the 'no intoxication" scenario, S_{ss} was re-estimated by excluding the actual mortality cases attributed to lethal pesticide poisoning. The fact that some non-lethal effects were masked due to differences of rate of analysable carcasses was not totally excluded. Hence, 2%, 5% and 10% of mortality cases attributed to predation cases (hereafter "without 2%, 5% or 10% non-lethal effect" scenario nested within the "without 1% lethal effect" scenario) were fictively removed.

II. RESULTS AND DISCUSSION

A. Pesticide Use

Data on PPP usage between 1 March and 31 August in 2010 and 2011, from 142 farmers for 1000 agricultural fields for an area of 6500 ha (50% of the total arable land area of the 12 field sites in this study) was collected.

186 active substances were recorded as having been used which corresponds to 60% of the 317 active substances listed on the French AGRITOC database (2013).

Of the active substance identified, 91% were applied as sprays, 3% were both sprayed and applied by another method (e.g. seed treatment) and 6% were only applied by another method.

Thus, the principal route of exposure was spraying in vegetation, arthropods or weed seeds.

B. Partridge Mortality

Number of tagged birds	529
Radio failure or transmitter loss	54
Mortality	261
Survival (late August)	268
Mortality rate (estimated)	50%
Mortality due to predation	78.9%

Mortality due to farming(physical tasks)	4.2%
Mortality due to collision	1.9%
Mortality due to disease	5%
Undefined cause of mortality	5%
Mortality due to acute pesticide poisoning	0.4%

Most mortality cases occurred in May, June and July, when females were laying and incubating their eggs.

C. Potential Exposure

The number of birds (dead and alive) potentially exposed to a given active substance within 10 days after its application ranged from 1 to 253. There was no correlation between the acute toxicity or risk of a.s. and the proportion of potentially exposed dead birds.

Data for Flutolanil are given below:

Parameter and units, where applicable	**Reported finding**
Active Substance	Flutolanil
CAS Number	66332-96-5
Chemical Family	Phenylbenzamide
Use	Fungicide
% of Sites (N=12)	33.3
% of Farmers	4.4
% of Area	0.7
Main Crops	Potatoes
Avian LD_{50} (mg/kg)	> 2000
Laboratory Species	*Colinus virginianus*
HD5* 50% (mg/kg)	208.12
Acute Avian TER!	> 29.0
Number of potentially exposed birds	5
% of dead potentially exposed birds	0.0

*Hazardous dose at the 5% tail of the species sensitivity distribution of acute toxicity according to Mineau et al. (2001) and completed with Mineau et al. (2006).
! Lowest value of acute TER for birds in the first tier risk assessment reported in the peer reviews edited by the EFSA.

D. Residues Analysis

Number of carcases analysed = 94
Number with flutolanil detected > LOD = 0

III. DISCUSSION

A great diversity of active substances were used in North-Central France farmland but the majority of them, including flutolanil, did not present a high acute toxicity or risk. Not all carcasses could be analysed – mainly due to predation. Thus, acute pesticide poisoning from active substances, including flutolanil, could be absolutely ruled out.

Five main factors may have affected the results of this study:

- The grey partridge is a relatively large omnivorous bird, compared to other cereal fields birds, partridges may be less vulnerable to pesticide effects than smaller birds but conversely, the

particular diet of the grey partridge may make it more exposed to pesticides used in seed treatments.

- The fact that partridge is a relatively large farmland bird may have allowed to find more carcasses in suitable condition for necropsies and residue analyses.
- No investigation of the effects of mixtures on adult partridge mortality.
- No control sites (in France, in 2013, organic farming represented only 3.9% of arable land)
- Farmers may not have reported all pesticides they used, especially if it was a banned active substance.

IV. CONCLUSION

Flutolanil was used on 33.3% of the 12 sites, on an area of 0.7% of the total study area by 4.4% of farmers who participated in the survey taken. Through modelling, it was concluded that only 5 birds were potentially exposed to flutolanil (n = 529). Of the 5 birds potentially exposed, the statistically derived mortality rate was null. of the 94 carcases analysed, no flutolanil was detected using GC-MS (limit of detection, 0.1 µg/g). Flutolanil was determined to be of low risk to the Grey Partridge through potential exposure and no mortality as a direct result of flutolanil exposure was established.

Comments of RMS

This general, monitoring study was not conducted according to any guideline. The study contains several uncertainties, which compromise the reliability of the conclusion: in addition to the five factors listed in the summary above, flutolanil was not included in the chemical analysis. Indeed, the summary above states that flutolanil was not detected, however it should be noted that the chemical class of flutolanil was not included in the wide screening analysis.

Although the study indicates that pesticide related mortality and effects on the population due to pesticides among partridges were low, this conclusion cannot directly be linked to flutonanil, as use of and exposure to flutolanil was not demonstrated.

Overall, the modelling results showed that the two lethal pesticide-related poisonings (which were actually related to a pesticide other than flutolanil) decreased the population growth rate by less than 1%.

Millot, F., Berny, P., Decors, A., Bro, E.	2015	Little field evidence of direct acute and short-term effects of current pesticides on the grey partridge	Ecotoxicology and Environmental Safety 117 (2015) 41-61
Reliability			
General information			
Is a guideline method or modified guideline used?*		No	
Is the test performed under GLP conditions?*		No	
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?		Not applicable	
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?		No	

* these criteria are of minor importance for study reliability, but may support study evaluation	
Test compound	
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?	No
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	No
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	No
Test organism	
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	No
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	No
Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Not applicable
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Not applicable
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Not applicable
Is a correct spacing between exposure concentrations applied?	Not applicable
Is the exposure duration defined?	Monitoring duration is known
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	No
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Not applicable
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Not applicable
Are appropriate statistical methods used?	Not applicable
Is a concentration-response curve observed? Is the response statistically significant?	Not applicable
Are sufficient data available to check the calculation of endpoints and (if applicable)	No

validity criteria (e.g., control data, concentration-response curves)?	
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	No
Are the reported endpoints appropriate for the regulatory purpose?	Not applicable
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Not applicable
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Not applicable
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Exposure duration was relevant, but no endpoints were derived.
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable
Concluding weight of evidence/proposed action	The study indicates that short-term effects on survival or longer term effects on population growth of partgridge in the general farmland, where different pesticides are used, are not to be expected.
Type of information (Fully acceptable, supporting information, not applicable)	Supporting information for weight of evidence only
Consideration/concluding score	Supporting information for weight of evidence only

B.9.1.5 Potential for endocrine disruption

The data point is addressed by information found in the open literature. The following study report reviews the potential of flutolanil to interact with endocrine systems in humans and vertebrate wildlife.

Report:	**CA 8.1.5-01. Odum J., Roberts, M., Matthiessen, P., 2016**
Title:	Assessment of flutolanil and its potential for endocrine disruption
Report no.:	NIC002_001
Published:	No
GLP:	No
Guidelines:	Not applicable
Deviations:	Not applicable
RMS Comment:	The report was considered to be acceptable.
Endpoint:	The RMS does not find indications of endocrine disruption from exposure to flutolanil.

Executive summary:

The potential of flutolanil and any major metabolites to interact with endocrine systems in mammals and wildlife has been reviewed, to facilitate an assessment of whether flutolanil may be judged to be an endocrine disrupter (ED) within the framework of European legislation. The studies on which this assessment is based are those reviewed in the EC Draft Assessment Report on flutolanil (DAR, 2006), studies conducted for USEPA's Endocrine Disruptor Screening Program (USEPA, 2015), Toxcast *in vitro* studies (USEPA, 2016) and a search of published literature. The evidence shows that flutolanil does not interact with molecular endpoints known to cause endocrine activity. Flutolanil also had no endocrine activity in mammalian assays specific for endocrine disruption and in other regulatory studies with endpoints relevant for endocrine disruption. This weight of evidence indicates that it does not interact with mammalian endocrine systems in studies designed to detect effects relevant for human health. The major mammalian metabolites M-2 and M-4 have also been concluded to have no effect on endocrine systems. In ecotoxicity assays, flutolanil gave equivocal results in the Fish Short-Term Reproduction Assay, most likely due to systemic toxicity. In acute and chronic regulatory ecotoxicity studies in fish there were no endocrine-related effects. There was no evidence of effects on amphibians. In reproduction studies in birds there was no evidence that flutolanil has endocrine activity. The human health studies demonstrate that flutolanil will not cause endocrine effects in wild mammals.

According to the criteria suggested by EFSA (2013), an ED is defined by the presence of i) an adverse effect in an intact organism, or a (sub)population; ii) an endocrine activity; iii) a plausible relationship between the two. Flutolanil does not produce an adverse effect relevant for human health and it does not cause adverse effects in wildlife species at concentrations below those where effects might be expected as a consequence of systemic toxicity. The weight of evidence indicates that it has no endocrine activity.

Overall, flutolanil does not fit either the interim EC criteria, or the recently published (but not yet ratified as final) 2016 EC criteria, with respect to human or environmental health and therefore is not an ED.

Comments RMS

The mammalian data package, bird reproduction, and amphibian metamorphosis studies do not indicate endocrine disrupting properties of flutolanil, however, the same cannot be said for the potential for endocrine disruption in fish (see section 9.2.3, below).

B.9.2 Effects on aquatic organisms

The aquatic toxicity endpoints for flutolanil are given in Table 9.2-1.

Table 9.2-1 Summary of toxicity data on fish, aquatic invertebrates, aquatic algae and macrophytes

Species	Test substance	Time-scale (Test type)	End point		Data point Author, year
Toxicity to Fish					
Oncorhynchus mykiss[1] (Rainbow trout)	Flutolanil Technical	Acute, 96h (static)	LC_{50} NOEC	5.4 mg/L (m.m.) 3.0 mg/L (m.m.)	CA 8.2.1-01 [redacted] 1987a
Lepomis macrochirus (Bluegill sunfish)	Flutolanil Technical	Acute, 96h (static)	LC_{50} NOEC	> 5.4 mg/L (m.m.) 2.5 mg/L (m.m.)	CA 8.2.1-02 [redacted] 1987b
Pimephales promelas (Fathead minnow)	Flutolanil Technical	Acute, 96h (static)	LC_{50} NOEC	**4.8 mg/L (m.m.)** 1.2 mg/L (m.m.)	CA 8.2.1-03 [redacted] 1990
Pimephales promelas (Fathead minnow)	Flutolanil Technical	Long-term, FELS, 30 days (flow-through)	NOEC $EC_{10, wet weight}$ EC_{20} EC_{50} MATC	**0.233 mg/L (m.m.)** 0.601 mg/L (m.m.) ND ND 0.337 mg/L (m.m.)	CA 8.2.2.1-01 [redacted], 1995 CA 8.2.2.1-02 [redacted], 2016
Toxicity to aquatic invertebrates					
Daphnia magna (Water flea)	Flutolanil Technical	Acute, 48h (static)	EC_{50}	> 6.8 mg/L (m.m.)	CA 8.2.4.1-01 Forbis, A.D. *et al*., 1990
Daphnia magna (Water flea)	Flutolanil Technical	Reproduction, 21 days (semi-static)	NOEC EC_{10} (95% CI) EC_{20} (95% CI) EC_{50} (95% CI) MATC	0.29 mg/L (m.m.) 2.03 (1.35-2.45) mg/L (m.m.) 2.37 (1.74-2.75) mg/L (m.m.) 3.18 (2.73-3.58) mg/L (m.m.) 0.76 mg/L (m.m.)	CA 8.2.5.1-01 Blakemore, G.C. & Burgess, D.,1991 CA 8.2.5.1-02 Palmer, D.A., 2016
Mysidopsis bahia (Shrimp)	Flutolanil	Acute, 48h (static)	LC_{50}	**0.13 [3] (0.087-0.16) mg/L (m.m.)**	CA 8.2.4.2-01 Forbis, A.D., 1991
Mysidopsis bahia (Shrimp)	Flutolanil	Life-cycle, 28 days (flow-through)	NOEC EC10 (95%CI) Survial production young/female growth (dry weight) EC20 (95%CI) Survial production young/female growth (dry weight) EC50 (95%CI) Survial production	0.0113 mg/L **0.00397 (0.00241-0.00560) mg/L (m.m.)** 0.0117 (0.0101-0.0129) mg/L (m.m.) 0.0165 (0.0063-0.0252) mg/L (m.m.) 0.00685 (0.00472-0.00896) mg/L (m.m.) 0.0136 (0.0122-0.0147) mg/L (m.m.) 0.0321 (0.0192-0.0430) mg/L (m.m.) 0.0195 (0.0158-0.0238) mg/L (m.m.) 0.0182 (0.0172-0.0191) mg/L (m..m)	CA 8.2.5.2-01 Boeri, R.L., Kowalski, P.L., Ward, T.J., 1995

Species	Test substance	Time-scale (Test type)	End point		Data point Author, year
			young/female growth (dry weight)	0.115 (0.0812-0.237) mg/L (m.m.)	
Chironomus riparius (Chironomid Midge)	Flutolanil	Long-term: Water spiked, 28 days (static)	NOEC EC_{10} EC_{20} EC_{50}	**1.0 mg/L (nom.)** ND ND > 1 mg/L (nom.)	CA 8.2.5.3-01 Desmares-Koopmans, D., 2003
Toxicity to algae					
Pseudokirchneriella subcapitata[2] (Green algae)	Flutolanil Technical	Chronic, 72h (static)	E_rC_{10} E_rC_{25} E_rC_{50} E_bC_{50} NOEC	0.49 mg/L (nom.) 2.30 mg/L (nom.) **> 3.2 mg/L (nom.)** 0.97 mg/L (nom.) 0.18 mg/L (nom.)	CA 8.2.6.1-01 Migchielsen, M.H.J., 2003

[1] Formerly known as *Salmo gairdneri*
[2] Formerly known as *Selenastrum capricornutum*
[3] only for adults, not for juvenile shrimps
ND: Could not be determined
CI: Confidence Intervals
Note: When more than one endpoints are available for a substance for the same taxonomic group and study type, the lowest endpoint is in **bold** and is the one used in the risk assessment

B.9.2.1 Acute toxicity to fish

Study CA 8.2.1-01

Report	CA 8.2.1-01, [redacted] 1987a
Title	Acute toxicity of flutolanil technical to rainbow trout (*Salmo gairdneri*)
Report no	35378 (W-3008)
Guidelines	US EPA 72-1, Directive 96/12/EC, Directive 92/69/EEC Method C.1
GLP	Yes
Previous evaluation	DAR (2006)
RMS comment	The applicant submitted a new, more extensive summary. Considered acceptable for use in risk assessment
Endpoint	

Effect concentration (mg/L)	**24 h**	**48 h**	**96 h**
LC_{50}	5.8	5.8	5.4
96-h NOEC	3.0		

Executive Summary

The effects of flutolanil technical, to the rainbow trout (*Oncorhynchus mykiss*, formerly known as *Salmo gairdneri*), were determined in a 96-hour static system. Groups of ten fish (one replicate) were exposed to nominal concentrations of 0.56, 1.0, 1.8, 3.2, 5.6 and 10 mg/L for 96 hours. Two control groups were exposed to dilution water only and dilution water containing solvent (dimethylformamide). The test fish were observed after 24, 48 and 96 hours for mortality and symptoms of toxicity. The

concentration of flutolanil technical in the test water was measured at the beginning and at the end of the test.

The measured concentration of flutolanil technical in the analysed test medium ranged from 48% to 82% of mean nominal at the start and the end of the test period, respectively. All study results were therefore based on measured concentrations.

No mortalities or treatment-related effects were observed in control groups and at the test concentrations of 0.46 - 3.0 mg/L (measured), during the test period of 96 hours. At the highest concentration of 7.0 mg/L (measured), treatment-related effects were observed after 24, 48 and 96 hours and comprised fish mortality at 70% after 24 and 48 hours and 80% after 96 hours.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test material:**	Flutolanil technical
	Lot no.:	521169
	Purity:	97.8%
	Description:	White powder, stored in the freezer and in the dark
2.	**Test organism:**	Rainbow trout (*Salmo gairdneri*)
	Mean length:	32 mm (range: ± 2 mm)
	Mean weight:	0.44 g (± 0.073g)
	Source:	[redacted]
3.	**Treatment:**	Control, Solvent control (DMF), 0.56, 1.0, 1.8, 3.2, 5.6 and 10 mg/L
4.	**Test vessels:**	Glass aquaria (total capacity of 15 L); Depth was ~29.8 cm
	Test water:	Soft reconstituted water

5. Environmental conditions:

Temperature:	12 – 13°C
pH:	6.5 – 7.4
Dissolved oxygen:	74 – 94% oxygen saturation
Water hardness:	54 – 58 mg/L $CaCO_3$ (measured)
Photoperiod:	16 h light : 8 h darkness (*ca*. 320 lux)

B. STUDY DESIGN AND METHODS

1. In-life phase: Mar 02, 1987 to Mar 06, 1987

2. Test organism assignment and treatment

Fish were in good health and were acclimatised to the dilution water and test temperature for 48-96 hours prior to testing and were last fed 48-96 hours before the test was started. Based on the results of a preliminary test, the definitive test employed nominal exposure concentrations of 0.56, 1.0, 1.8, 3.2, 5.6 and 10 mg/L. Control groups were placed in dilution water alone and dilution water containing

an aqueous solution of dimethylformamide (DMF) at the same concentrations as in the test medium (0.1 mL/L). The fish were allocated in a random order to the vessels containing the prepared test or control media until each contained ten animals within approximately 30 minutes after the test item was added to the water. The fish were not fed during the test. The study was carried out under static exposure conditions.

3. Dose preparation

The definitive test concentrations were obtained by transferring appropriate aliquots of a working standard directly to the test chambers. The working standard was prepared in 50 ml of dimethylformamide (DMF). All test concentrations were corrected for sample purity. The solvent control chamber received a 1.5 ml aliquot of DMF, which was equivalent to the highest amount used in any test solution. Precipitation occurred during the definitive test and was present throughout the 96 hours in the 1.8, 3.2, 5.6 and 10 mg/L (nominal) test concentrations.

4. Measurements and observations

All test organisms were observed at 24, 48 and 96 hours for mortality and abnormal (sub-lethal) effects. Any dead individuals were removed from the test chambers after each 24-hour observation. The temperature, pH and concentration of dissolved oxygen of the contents of each vessel were measured at the start of the test, at 48 and 96 hours. The total hardness of the dilution water control was determined during the test.
For the chemical analysis, 50 mL samples were taken at the start of the test and after 96 hours. Analysis was performed by a GLC system using a Hewlett-Packard 5890 gas liquid chromatograph equipped with an electron capture detector (ECD) and a Hewlett-Packard 1000 mini-computer using Beckman CALS System® software.

Minor environmental deviations were present, which did not affect the integrity of the study according to the study director. Water quality and fish observations for sub lethal effects were not completed at 24 and 72 hrs, deviating from the guidelines.

5. Statistics

Statistical analysis was not performed as there was insufficient mortality for the statistical calculation of 95% confidence limits.

II. RESULTS AND DISCUSSION

A. Mortality and abnormal responses of the fish

No mortalities or treatment-related effects were observed in control groups and at the test concentrations of 0.46 - 3.0 mg/L (measured), during the test period of 96 hours. At the highest concentration of 7.0 mg/L (measured), treatment-related effects were observed after 24, 48 and 96 hours and comprised fish mortality at 70% after 24 and 48 hours and 80% after 96 hours.

Percent mortality of fish exposed to flutolanil

Nominal Concentration (mg/l)	Mean measured Concentration* (mg/l)	% Mortality			LC_{50} value
		24 hr	48 hr	96 hr	
Control	**-**	**0**	**0**	**0**	**24 hr : 5.8 mg/l** **48 hr : 5.8 mg/l** **96 hr : 5.4 mg/l**
Solvent control	**-**	**0**	**0**	**0**	
0.56	**0.46**	**0**	**0**	**0**	
1.0	**0.65**	**0**	**0**	**0**	
1.8	**0.86**	**0**	**0**	**0**	
3.2	**2.0**	**0**	**0**	**0**	
5.6	**3.0**	**0**	**0**	**0**	
10	**7.0**	**70**	**70**	**80**	

*: mean of measured concentrations at 0 and 96 hr

B. Analytical verification

The measured concentration of flutolanil technical in the analysed test medium ranged from 48% to 82% of nominal at the start and the end of the test period, respectively. The analytical results are reported in the following table.

Measured concentrations of flutolanil technical during the test

Test item nominal (mg/L)	**Mean measured (mg/L)**		**Recovery (%)**
	Sampling (hrs)		
	0 (fresh)	96 (old)	
0.56	0.46	0.47	82-84
1	0.69	0.61	61-69
1.8	0.84	0.88	47-49
3.2	1.8	2.1	56-66
5.6	2.8	3.2	50-57
10	7.2	6.7	67-72

C. Toxicity endpoints

The 24-hour, 48-hour and 96-hour LC_{50} values and the 96-h NOEC value for the flutolanil technical to rainbow trout are presented in the following table.

Toxicity endpoints for the test item flutolanil technical

Effect concentration (mg/L)	**24 h**	**48 h**	**96 h**
LC_{50}	5.8	5.8	5.4
96-h NOEC	3.0		

III. CONCLUSION

In a static 96-h acute toxicity test on rainbow trout, based on the measured test concentrations the NOEC (no observed effect concentration) of flutolanil technical was determined to be 3.0 mg/L. The median lethal concentration (96-h LC_{50}) of flutolanil technical was calculated to be 5.4 mg/L; 95% confidence limits could not be calculated due to insufficient mortality seen during the test.

Equivalent to OECD 203, 1992. The study has already been reviewed under Uniform Principles for the first approval of flutolanil under Directive 91/414/EEC. (DAR, 2006: B9.2.1). The validity criteria were met and the study deviations are considered to be minor and do not affect the outcome of the study, therefore the study is acceptable.

Study CA 8.2.1.02

Report	CA 8.2.1-02. [redacted] 1987b
Title	Acute toxicity of flutolanil technical to bluegill sunfish (*Lepomis macrochirus*).
Report no.	35377 (W-3009)
Guidelines	Directive 96/12/EC, Directive 92/69/EEC Method C.1
GLP	Yes
Previous evaluation	DAR (2006)
RMS Comment	The applicant submitted a new, more extensive summary. Considered acceptable for use in risk assessment
Endpoint	NOEC = 2.5 mg/L 96-h LC_{50}= > 5.4 mg/L (mean measured)

Executive Summary

The effects of flutolanil technical (purity: 97.8%) to the bluegill sunfish (*Lepomis macrochirus*), were determined in a 96-hour static system. The methods followed were as per the Committee on Methods for Toxicity Tests with Aquatic Organisms. In the final test, groups of ten bluegill sunfish (*Lepomis macrochirus*) were exposed to nominal concentrations of 0.56, 1.0, 1.8, 3.2, 5.6 and 10 mg/L for 96 hr. Two control groups were exposed to dilution water and dilution water containing dimethylformamide (100 µL/L). The test fish were observed after 24, 48, 72 and 96 hours for mortality and symptoms of toxicity. The concentration of flutolanil in the test water was measured at the beginning and at the end of the test.

The measured concentration of flutolanil in the analysed test medium ranged from 44% to 79% of nominal at the start and the end of the test period, respectively. As the measured concentrations deviated from nominal by more than 20%, all study results were therefore based on mean measured concentrations of 0.31, 0.79, 0.80, 1.6, 2.5 and 5.4 mg/L.

No mortalities were observed in control groups and at the test concentrations of 0.31 to 2.5 mg/L (mean measured). Treatment-related effects (loss of equilibrium, fish on the bottom of test chamber and/or quiescence) were observed in the 1.6 and 2.5 mg/L (mean measured) test concentrations up to 72-hour exposure period, and in the measured test concentrations of 5.4 mg/L throughout the 96-hour exposure period. At the highest test concentration of 5.4 mg/L (mean measured), 20% mortalities were observed after 96 hours. Therefore, the 96-hour LC_{50} was found to be greater than 5.4 mg/L and the 96-hour NOEC was determined to be 2.5 mg/L.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test material:**	Flutolanil technical
	Lot no.:	521169
	Purity:	97.8%
	Description:	White powder
2.	**Test organism:**	Bluegill sunfish (*Lepomis macrochirus*)
	Mean length:	28 ± 2.3 mm
	Mean weight:	0.54 ± 0.14 g
	Source:	[redacted]
3.	**Treatment:**	0 (control and solvent control), 0.56, 1.0, 1.6, 3.2, 5.6 and 10 mg/L
4.	**Test vessels:**	Glass vessels (total capacity of 5 gallon); Depth was 29.8 cm, equivalent to 15 L
	Test water:	Soft reconstituted water

5. Environmental conditions:

Temperature:	22 – 23 °C
pH:	6.7 – 7.3
Dissolved oxygen:	4.8 – 9.4 mg/L (55 – 108% saturation)
Water hardness:	54 – 58 mg/L $CaCO_3$
Photoperiod:	16 h light : 8 h darkness

B. STUDY DESIGN AND METHODS

1. In-life phase: Mar 02 to Mar 06, 1987

2. Test organism assignment and treatment

Fish were in good health and were acclimatised for 14 days in test medium of the same quality of test medium used in the test and were last fed 48-96 hours before the test was started. A 96 hr range finding test exposing groups of five fish to nominal flutolanil technical concentrations 1, 10 and 100 mg/L was performed. No mortality was recorded at 1 and 100 mg/L concentrations, however, 10 mg/L concentration elicited 100% mortality. Observations of precipitate in the test solutions indicated that the test material solubility was exceeded in the 10 and 100 mg/L test levels. Based on these results,

the definitive test employed nominal exposure concentrations of 0.56, 1.0, 1.8, 3.2, 5.6 and 10 mg/L. Control groups were placed in dilution water alone and dilution water containing dimethylformamide (DMF) at a concentration of 100 µL/L. The fish were randomly allocated to the vessels containing the prepared test or control media until each contained ten animals within approximately 30 minutes after the test item was added to the water. The fish were not fed during the test. The study was carried out under static exposure conditions.

3. Dose preparation

Appropriate aliquots (between 0.084 and 1.5 mL) of a working standard prepared in DMF (100 mg/mL) were transferred to test chambers (15 L) to obtain the test solutions. The solvent control chamber received 1.5 mL aliquot of DMF, which was equivalent to the highest amount used in any test solution. Precipitation occurred during the definitive test and was present throughout the 96 hours in the 1.8, 3.2, 5.6 and 10 mg/L (nominal) test concentrations.

4. Measurements and observations

Observations of the fish were made 24, 48, 72 and 96 hours after the start of the tests; additional observations were made during the first four hours. The temperature, pH and concentration of dissolved oxygen of the contents of each vessel were measured at the start of the tests and thereafter each day either immediately before or following the observations of fish behaviour. The total hardness of the dilution water control and selected test dilutions were determined at the start and end of the tests.

For the chemical analysis, water samples were taken from each test vessel at 0 and 96 hours. Analysis was performed by gas liquid chromatography (GLC).

5. Statistics

There was insufficient mortality for the statistical calculation of the median lethal concentrations (LC_{50}) or its 95% confidence limits, therefore no statistical analysis was performed.

II. RESULTS AND DISCUSSION

A. Mortality and abnormal responses of the fish

No mortalities were observed in control groups and at the test concentrations of 0.31 to 2.5 mg/L (mean measured). Treatment-related effects (loss of equilibrium, fish on the bottom of test chamber and/or quiescence) were observed in the 1.6 and 2.5 mg/L (mean measured) test concentrations up to 72-hour exposure period, and in the measured test concentrations of 5.4 mg/L throughout the 96-hour exposure period. At the highest test concentration of 5.4 mg/L (mean measured), 20% mortalities were observed after 96 hours. The discrepancy with the range finding test results may have been due to a solubility factor or due to an age or condition factor of the fish. In any case, a cause-effect relationship was not obvious during the conduct of this study.

Cumulative mortality of fish exposed to flutolanil technical

Mean measured concentration (mg/L)	**Mortality (%)**			
	24 h	**48 h**	**72 h**	**96 h**
0 (control, dilution water)	0	0	0	0
0 (solvent control)	0	0	0	0
0.31	0	0	0	0
0.79	0	0	0	0
0.80	0	0	0	0
1.6	0	0	0	0
2.5	0	0	0	0
5.4	10	20	20	20

B. Analytical verification

Analysis of the sample taken during the test showed that the measured concentrations deviated from nominal by more than 20% (41-85%). The analytical results are reported in the following table.

Measured concentrations of flutolanil during the test

Test item nominal (mg/L)	**Measured (mg/L)**				
	0 hours		**96 hours**		**Mean**
	Sample	mean as % nominal	Sample	mean as % nominal	Mean measured
0 (control, dilution water)	n.d.	-	n.d.	-	-
0 (solvent control)	n.d.	-	n.d	-	-
0.56	0.33	59%	0.29	52%	0.31
1.0	0.85	85%	0.73	73%	0.79
1.8	0.83	46%	0.77	43%	0.80

Test item nominal (mg/L)	Measured (mg/L)				
	0 hours		96 hours		Mean
	Sample	mean as % nominal	Sample	mean as % nominal	Mean measured
3.2	1.9	59%	1.3	41%	1.6
5.6	2.7	48%	2.3	41%	2.5
10	5.9	59%	5.0	50%	5.4

n.d. none detected

C. Toxicity endpoints

The 24-hour, 48-hour, 72-hour and 96-hour LC_{50} values and the 96-h NOEC value for the flutolanil technical to bluegill sunfish are presented in the following table.

Toxicity endpoints for the test item flutolanil technical

Effect concentration (mg/L)	24 h	48 h	76 h	96 h
LC_{50}	> 5.4	> 5.4	>5.4	> 5.4
96-h NOEC	2.5			

III. CONCLUSION

Not all validity criteria of OECD 203 (1992) were met. The dissolved oxygen concentration did not remain constant throughout the test. It was found to be 108% at the start of the test and decreased below 60% (55%) at the end of the test. Even though this deviates from the guideline and one of the validity criteria has not been met, it is considered to be a minor deviation and it does not affect the outcome of the study, therefore the study is acceptable.

In a static 96-h acute toxicity test on bluegill sunfish, based on the nominal test concentrations the NOEC (no observed effect concentration) of flutolanil technical was determined to be 2.5 mg/L. The median lethal concentration (96-h LC_{50}) of flutolanil technical was calculated to be greater than 5.4 mg/L (mean measured).

Study CA 8.2.1-03

Report	CA 8.2.1-03, [redacted] 1990
Title	Acute toxicity of flutolanil technical to fathead minnow (*Pimephales promelas*)
Report no.	38101 (W-3010)
Guidelines	US EPA 72-1, Directive 96/12/EC, Directive 92/69/EEC Method C.1
GLP	Yes (USA/EPA)

Previous evaluation	DAR (2006)
Comment:	The applicant submitted a new, more extensive summary. Considered acceptable for use in risk assessment
Endpoint	NOEC =1.2 mg/L 96-h LC_{50} = 4.8 mg/L (3.8 - 6.2 mg/L)

Executive Summary

The effects of flutolanil technical, to the fathead minnow (*Pimephales promelas*), were determined in a 96-hour static system. Groups of ten fish (one replicate) were exposed to nominal concentrations of 1.3, 2.2, 3.6, 6.0 and 10 mg/L for 96 hours. Two control groups were exposed to dilution water and dilution water containing dimethylformamide (DMF). The test fish were observed after 24, 48, 72 and 96 hours for mortality and symptoms of toxicity. The concentration of flutolanil technical in the test water was measured at the beginning and at the end of the test.

The measured concentration of flutolanil technical in the analysed test medium ranged from 91% to 123% of nominal at the start and the end of the test period, respectively. All study results were therefore based on measured concentrations.

No mortalities or treatment-related effects were observed in control groups and at the test concentration of 1.2 mg/L, during the test period of 96 hours. The abnormal effects of mortality, loss of equilibrium, fish on the bottom of test chamber, erratic swimming, surfacing and/or quiescence were observed in the 2.2, 3.7, 6.6 and 11 mg/L measured test concentrations during the 96-hour exposure period. Complete mortality occurred in the 11 mg/L chamber within 24 hours.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Flutolanil technical
Lot no.: 543251
Purity: 97.6%
Description: White powder, stored at room temperature

2. Test organism: Fathead minnow (*Pimephales promelas*)
Mean length: 26 mm (range: ± 2 mm)
Mean weight: 0.23 g (± 0.049g)
Source: [redacted]

3. Treatment: Control, Solvent control (DMF), 1.3, 2.2, 3.6, 6.0 and 10 mg/L

4. Test vessels: Glass aquaria (total capacity of *ca*. 15 L); Depth was ~29.9 cm
Test water: Soft reconstituted water

5. Environmental conditions:

Temperature: 22°C

pH:	7.4 – 7.9
Dissolved oxygen:	73 – 94% oxygen saturation
Water hardness:	44 mg/L $CaCO_3$ (measured)
Photoperiod:	16 h light : 8 h darkness

B. STUDY DESIGN AND METHODS

1. In-life phase: Sept 01, 1989 to Sept 05, 1989

2. Test organism assignment and treatment

Fish were in good health and were acclimatised to the dilution water and test temperature for 48-96 hours prior to testing and were last fed 48-96 hours before the test was started. Based on the results of a preliminary test, the definitive test employed nominal exposure concentrations of 1.3, 2.2, 3.6, 6.0 and 10 mg/L. Control groups were placed in dilution water alone and dilution water containing an aqueous solution of dimethylformamide (DMF) at the same concentrations as in the test medium (0.5mL/L). The fish were allocated in a random order to the vessels containing the prepared test or control media until each contained ten animals within approximately 30 minutes after the test item was added to the water. The fish were not fed during the test. The study was carried out under static exposure conditions.

3. Dose preparation

The definitive test concentrations were obtained by transferring appropriate weights of test compound directly to the test chambers. Approximately 7.5 mL of dimethylformamide (DMF) was added to each test weight prior to addition to the test chamber. Each solution was then vigorously stirred several times with a glass rod. The solvent control chamber received a 7.5 mL aliquot of DMF, which was equivalent to the highest amount used in any test solution. Precipitation occurred during the definitive test and was present throughout the 72 hours in the 10 mg/L (nominal) test concentrations.

4. Measurements and observations

Observations of the fish were made at 24, 48, 72 and 96 hours after the start of the tests. The temperature, pH and concentration of dissolved oxygen of the contents of each vessel were measured at the start of the tests and thereafter each day either immediately before or following the observations of fish behaviour. The total hardness of the dilution water control and selected test dilutions were determined.

For the chemical analysis, 50 mL samples were taken at the start of the test and after 96 hours. Analysis was performed by GLC.

5. Statistics

Statistical analysis was performed using LC_{50} software developed by Stephan, C.E. (1978). This program calculated the LC_{50} statistic and its 95 percent confidence limits using the binomial, the moving average, and the probit tests.

II. RESULTS AND DISCUSSION

A. Mortality and abnormal responses of the fish

No mortalities or treatment-related effects were observed in control groups and at the test concentration of 1.2 mg/L, during the test period of 96 hours. The abnormal effects of mortality, loss of equilibrium, fish on the bottom of test chamber, erratic swimming, surfacing and/or quiescence were observed in the 2.2, 3.7, 6.6 and 11 mg/L measured test concentrations during the 96-hour exposure period. Complete mortality occurred in the 11 mg/L chamber within 24 hours.

Cumulative mortality of fish exposed to flutolanil technical

Nominal Concentration (mg/L)	Mean measured Concentration* (mg/L)	% Mortality				LC_{50} value
		24 hr	48 hr	72 hr	96 hr	
Control	**-**	**0**	**0**	**0**	**0**	
Solvent control	**-**	**0**	**0**	**0**	**0**	**24 hr : 8.5 mg/L**
1.3	**1.2**	**0**	**0**	**0**	**0**	
2.2	**2.2**	**0**	**0**	**10**	**10**	**48 hr : 7.8 mg/L**
3.6	**3.7**	**0**	**0**	**0**	**10**	
6.0	**6.6**	**0**	**20**	**80**	**80**	**96 hr : 4.8 mg/L**
10	**11**	**100**	**100**	**100**	**100**	

*: mean of measured concentrations at 0 and 96 hr

Sub-lethal effects of flutolanil technical to fish

Nominal Concentration (mg/L)	**Mean measured Concentration* (mg/L)**	**Observations**			
		24 hr	**48 hr**	**72 hr**	**96 hr**
Control	**-**	10 N	10 N	10 N	10 N
Solvent control	**-**	10 N	10 N	10 N	10 N
1.3	**1.2**	10 N	10 N	10 N	10 N
2.2	**2.2**	1SUR/Q; 9Q/OB	10Q/OB	1SUR/Q; 8Q/OB	1N; 1SUR/ER; 6Q/OB;1LOE/Q
3.6	**3.7**	1LOE/OB; 8Q/OB; 1Q	2SUR/Q; 1ER/LOE; 7Q/OB	2SUR/Q; 1LOE/OB/Q; 7Q/OB	3SUR/LOE/Q; 6SUR/LOE/Q
6.0	**6.6**	7LOE/OB; 3SUR/LOE;	5LOE/OB/Q; 3SUR/LOE/Q;	2 LOE/SUR/Q	2 LOE/SUR/Q
10	**11**	10 D	10 D	10 D	10 D

*: mean of measured concentrations at 0 and 96 hr

Key observations: N = Normal; LOE = Loss of Equilibrium; Q = Quiescent; SUR = Surfacing; OB = On Bottom of Test Vessel; ER = Erratic Swimming; D = Dead

B. Analytical verification

The measured concentration of flutolanil in the analysed test medium represented an average of 103 ±7.5% of the nominal concentrations. The analytical results are reported in the following table.

Measured concentrations of flutolanil technical during the test

Test item nominal (mg/L)	**Mean measured (mg/L)**		**Recovery (%)**
	Sampling (hrs)		
	0 (fresh)	96 (old)	
1.3	1.2	1.1	85-92
2.2	2.3	2	91-105
3.6	3.9	3.4	94-108
6	7.4	5.8	97-123
10	11	10.1	101-110

C. Toxicity endpoints

The 24-hour, 48-hour and 96-hour LC_{50} values and the 96-h NOEC value for the flutolanil technical to fathead minnow are presented in the following table.

Toxicity endpoints for the test item flutolanil technical

Effect concentration (mg/L)	**24 h**	**48 h**	**72 h**	**96 h**
LC_{50} (95% confidence intervals)	8.5 (6.6, 11)	7.8 (3.7, 11)	5.1 (4.0, 6.5)	4.8 (3.8, 6.2)
96-h NOEC (lack of mortality and abnormal behavioural effects)	1.2			

III. CONCLUSION

In a static 96-h acute toxicity test on fathead minnow, based on the measured test concentrations the NOEC (no observed effect concentration) of flutolanil technical was determined to be 1.2 mg/L. The median lethal concentration (96-h LC_{50}) of flutolanil technical was calculated to be 4.8 mg/L with confidence limits 3.8 and 6.2 mg/L.

B.9.2.2 Long-term and chronic toxicity to fish

B.9.2.2.1 Fish early life stage toxicity test

Study CA 8.2.2.1-01

Report	CA 8.2.2.1-01, [redacted]1995
Title	Early life-stage toxicity of flutolanil to the fathead minnow (*Pimephales promelas*) under flow-through conditions
Report no.	41685 (W-3030)

Guidelines	U.S. EPA 72-4, ASTM Standard E-1241.92 (1992)
GLP	Yes (USA/EPA)
Previous evaluation	DAR (2006)
Comment	The applicant submitted a new, more extensive summary. Considered acceptable for use in risk assessment
Endpoint	NOEC = 0.233 mg a.s./L.

Report	CA 8.2.2.1-02. Palmer, D.A., 2016
Title	EAG Laboratories letter – To whom it may concern
Report no.	October 18, 2016
Guidelines	N/A
GLP	No
Previous evaluation	New study analysis
Comment	Considered acceptable for use in risk assessment

Executive Summary

A flow-through early life-stage toxicity study with the fathead minnow (*Pimephales promelas*) was conducted in order to determine the maximum acceptable toxicant concentration (MATC) limits of flutolanil technical. 30 newly fertilized eggs (< 24 hours post-fertilization) were exposed to 0.13, 0.25, 0.50, 1.0, and 2.0 mg/L (nominal) for 35 days (30 days post-hatch). One replicate treatment consisting of untreated test medium only was used as control. Additionally, a solvent control (DMF) was included.

Analysis of the test solutions for the active substance flutolanil was performed in samples taken at on days -6, 0, 1, 3, 7, 14, 21, 28, and at study termination on day 35. The determination of the content of flutolanil in the test solution ranged from 89.2 to 97.2 % of the nominal test concentrations and the mean measured concentrations of flutolanil, determined by gas chromatography, were 0.116, 0.233, 0.486, 0.933, and 1.94 mg/L. Effect concentrations were expressed as mean measured test concentrations.

Egg hatchability was not significantly affected at any test concentration when compared to the pooled control group. Survival was significantly reduced at 1.94 mg/L (measured) flutolanil when compared to the pooled control group. Compound-related morphological and behavioural effects were not observed at any test concentration. Based on the most sensitive endpoint (standard length), the MATC value for flutolanil was calculated to be 0.337 mg a.s./L, the no-observed effect concentration (NOEC) was 0.233 mg/L, and the low-observed effect concentration (LOEC) was 0.486 mg/L.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test material:**	Flutolanil Technical
	Lot no.:	01104
	Purity:	98.9%
	CAS number:	66332-96-5
	Description:	White powder, stored in a room temperature
2.	**Test organism:**	Fathead minnow (*Pimephales promelas*)
	Age:	< 24 hours post-fertilization (eggs)
	Source:	[redacted]
	Feeding:	Fry were fed *ad libitum* live rotifers (*Brachionus* sp.) and live brine shrimp (*Artemia*) nauplii soon after hatch began; growing fry were fed ad libitum at least three times a day at approximately 4-hour intervals during the week and at least twice a day at approximately the same interval on weekends and holidays.
3.	**Treatment:**	0.13, 0.25, 0.50, 1.0, and 2.0 mg/L (nominal)
4.	**Test vessels:**	16.0 x 30. 5 cm glass chambers (*ca*. 117 mL), depth ~ 24 cm
	Test water:	Deep well water screened for contaminants
5.	**Environmental conditions**	
	Temperature:	23.4 – 25.0°C
	pH:	8.14 – 8.35
	Dissolved oxygen:	6.0 – 8.0 (mg/L)
	Hardness:	149 – 150 mg/L $CaCO_3$
	Photoperiod:	16 h light : 8 h darkness (47 ± 6.8 lumens/ft^2)

B. STUDY DESIGN AND METHODS

1. In-life phase: Sept 26, 1994 to October 31, 1994

2. Test organism assignment and treatment

Newly fertilized fathead minnow (*Pimephales promelas*) eggs (approximately 2925 eggs < 24 hours post-fertilization) were obtained. A total of 30 eggs were added to each replicate (4 replicates per level). Egg hatch began on day 3 and was complete by day 6. Overall hatching success in the control, solvent control, (pooled control), and test levels 1 - 5 was 92.–, 98.4, (95.4), 89.2, 87.5, 98.2, 92.5, and 90.7 % respectively. The fry were fed *ad libitum* with live rotifers (*Brachionus* sp.) and live brine shrimp (*Artemia*) nauplii soon after hatch began and at least three times a day at approximately 4-hour intervals and two times a day on weekends and holidays. Food was withheld 24 hours before study termination.

3. Dose preparation

A 2-L proportional diluter system, with a Hamilton Model 420 syringe dispenser was used for the intermittent introduction of a solution of flutolanil, DMF solvent control and control dilution water to replicate test chambers to achieve the target nominal concentrations of flutolanil 0.13, 0.25, 0.50, 1.0 and 2.0 mg/L. Duplicate samples of 50 mL were used for analytical measurements. Samples were analysed using a sufficiently validated GC-ECD method (mean recoveries in fortified samples (0.124 - 2.48 mg/L– ranged from 96.2 to 106%, n=11 per level)

4. Measurements and observations

Developing embryos were observed daily for mortality. Dead eggs, identified by a distinct change in coloration, were recorded and removed from the exposure system each day. Eggs infected with fungus, if any, were removed and discarded. Positive counts of the number of embryos present were made at initiation and on days 2 and 3. The number of larvae in each chamber was estimated from the cumulative egg mortality and observed larval mortality. The post-hatch growth period began on day 5, when 95% of the remaining eggs in the dilution water control and vehicle blank had hatched. Newly hatched larvae were released into the growth chamber on day 8.
Observations of abnormal behavior, a ehaviourphysical change, and mortality were recorded daily by visual inspection of each growth chamber. Dead fry, if observed, were removed and discarded. Cumulative mortality estimates were based on observed fry mortality. Positive counts of surviving fry were made periodically throughout the study and also at termination.
Fry growth, as measured by standard length (mm) and blotted wet weight (g), was determined on day 30 post-hatch. All water quality parameters remained within the acceptable ranges.

5. Statistics

Data recorded on a continuous scale were analysed using analysis of variance methods (ANOVA). A one-tailed Dunnett's multiple’comparison procedure was used to assess differences between the control and each of the flutolanil test concentrations. The dilution water control and DMF vehicle blank were compared using a student t-test to determine whether or not they could be pooled. All statistical analyses were performed using SAS software. Inferences of statistical significance were based upon a $p \leq 0.05$. In order to attempt EC_{10} and EC_{20} calculation, data were re-analysed using the Spearman-Karber method for the 30-day post hatch survival endpoint and a logistics model for standard length and blotted wet weight endpoints.

II. RESULTS AND DISCUSSION

A. Biological data

The evaluated parameters were the number of hatching eggs, the survival of fry present, the presence of morphological and behavioural abnormalities and the growth.

For the egg hatchability, analysis of variance and Dunnett's test sho’ed no significant differences between the pooled controls and any flutolanil treatment. The reduction at 0.116, 0.233, and 0.486 mg/L (measured) were not considered to be biologically significant due to the lack of a significant dose-response for this parameter. The same analysis was also performed for the survival rates and indicated a significant reduction in survival only at 1.94 mg/L when compared to the pooled controls.

Due to significant mortality at the highest level of 1.94 mg/L, the treatment was excluded from the statistical analysis of the mean standard length and the mean blotted wet weight.

For the mean standard length, there was significant reduction indicated in the levels of 0.486 and 0.933 mg/L (measured). For the mean blotted wet weight, significant reduction was measured at the concentration of 0.933 mg/L.

No statistically significant dose-response related morphological and behavioural effects were observed at any test concentrations or the controls.

Total hatching and survival of eggs in each group

Mean Measured Concentration (mg/L flutolanil)	**Mean Egg hatch (%)**	**Mean 30-Day Survival (%)**	**Mean Length (mm) 30-Day post hatch**	**Mean Blotted Wet weight (g) 30-Day post hatch**
Control	92.5	94.6	23.9 ± 1.74	0.230 ± 0.0482
Solvent control	98.4	99.2	23.8 ± 2.00	0.225 ± 0.0527
(Pooled controls)	**95.4**	**97.0**	**23.9 ± 1.87**	**0.227 ± 0.0506**
0.116	89.2*	89.7*	24.3 ± 2.15	0.244 ± 0.0553
0.233	87.5*	97.2	23.7 ± 2.04	0.229 ± 0.0588
0.486	89.2*	100	23.0* ± 2.24	0.213 ± 0.0553
0.933	92.5	93.7	21.9* ± 2.40	0.191 * ± 0.0509
1.94 [b]	90.7 [a]	0.93*	17.2 (N=1)	0.096 (N=1)

* Statistically significantly reduction (P ≤ 0. 05) when compared to the pooled control group using frequency analysis coupled with the chi-square statistic and Fisher's exact te't. No significant differences were detected using analysis of variance and Dunnett's test wit' the exception of 30-day post-hatch survival at the highest dose level

[a] one embryo accidently destroyed. Total number of eggs reduced by one

[b] Deleted from growth analysis due to significant mortality

B. Analytical verification

The determination of the content of flutolanil in the test solution showed a mean recovery range of 96.2% to 106%. The analytical results are reported in the following table.

Measured concentrations of flutolanil during the test

Nominal concentration of flutolanil (mg/L)	Measured concentration of flutolanil (mg/L)										
	Day -6	Day 0	Day 1	Day 3	Day 7	Day 14	Day 21	Day 28	Day 35	Mean	% of Nominal
0.13	0.11	0.11	0.10	0.12	0.13	0.12	0.14	0.11	0.12	0.116	89%
0.25	0.21	0.20	0.21	0.26	0.25	0.25	0.27	0.23	0.23	0.233	93%
0.50	0.50	0.47	0.46	0.43	0.53	0.51	0.57	0.47	0.49	0.486	97%
1.0	1.02	0.95	0.88	0.91	0.94	0.91	1.07	0.92	0.93	0.933	93%
2.0	2.00	2.06	1.92	1.94	2.03	1.83	2.08	1.92	1.80	1.94	97%

C. Toxicity endpoint

Based on the most sensitive endpoint (standard length), the MATC value for flutolanil was calculated to be 0.337 mg a.s./L, the no-observed effect concentration (NOEC) was 0.233 mg/L, and the low-observed effect concentration (LOEC) was 0.486 mg/L.

Toxicity endpoints for the test item flutolanil technical

Endpoint	Effect concentration (mean measured)	mg a.s./L
Percent hatch	EC_{10} (95% CI)	> 1.94
	EC_{20} (95% CI)	> 1.94
	EC_{50} (95% CI)	> 1.94
	NOEC	> 1.94
30-day post hatch survival	EC_{10}	ND
	EC_{20}	ND
	EC_{50}	1.31 (1.27 – 1.34)
	NOEC	0.933
Standard length	EC_{10}	ND
	EC_{20}	ND
	EC_{50}	ND
	NOEC	0.233
	LOEC	0.486
	MATC	0.337
Blotted wet weight	EC_{10}	ND
	EC_{20}	ND
	EC_{50}	ND
	NOEC	0.486
Most sensitive endpoint: Standard length	NOEC	0.233
	LOEC	0.486
	EC_{10} (95% CI)	ND

ND: Could not be determined

III. CONCLUSION

Egg hatchability was not significantly affected at any flutolanil concentration tested. Fry survival was significantly reduced by flutolanil at a concentration of 1.94 mg/L. Growth, as measured by standard length, exhibited a significant reduction at flutolanil concentrations of 0.486 and 0.933 mg/L. Growth, as measured by blotted wet weight, exhibited a significant reduction at a flutolanil concentration of 0.933 mg/L.

Based on the most sensitive endpoint (i.e., growth) evaluated in this fathead minnow (*Pimephales promelas*) early life-stage toxicity study, the MATC for flutolanil was 0.337 mg/L. Based on the lack of survival and growth effects, the no-observed effect concentration (NOEC) was 0.233 mg/L and the lowest-observed effect concentration (LOEC) was 0.486 mg/L.

Comments by RMS

The study was conducted according to U.S. EPA 72-4 (1982) and was in general agreement with OECD 210 (2013). A difference between these guidelines is that OECD indicates that the test should start as soon as possible after fertilization and preferably before cleavage of the blastodisc commences, while the EPA guideline does not specify the age of the eggs to be used. The current test was started within 24 hours after fertilization. The report did not specify the developmental stage of the eggs, and the age of <24 hours within fertilization is probably most accurate due to overnight spawning of the brood stock, which is acceptable. The validity criteria of the OECD 210 guideline were met (hatching success in the controls > 70% (92.5 and 98.4%), post-hatch success in the controls > 75% (94.6 and 99.2%), the dissolved oxygen concentration was > 60% of the air saturation value (87-95%), the water temperature did not differ by more than ± 1.5 ºC between test chambers or between successive days at any time during the day and test concentrations were analytically confirmed. The study is considered valid and acceptable.

Egg hatchability was not statistically significantly affected using the Dunnett's test at any dose as compared to the pooled controls. 30-day post-hatch survival was statistically significantly decreased at the highest treatment level (only one of the 108 hatched eggs survived).

Growth, as measured by length and blotted wet weight were statistically significantly reduced at 0.486 mg a.s./L and above and at 0.933 mg a.s./L, respectively.
The NOEC was determined to be 0.233 mg a.s./L.

Reliability of test endpoints

According to EFSA Supporting publication 2015:EN-924, the test guideline is optimised for the derivation of ECx concentrations and thus $EC_{10,}$ EC_{20} and EC_{50} values and their confidence intervals were considered by the applicant. This resulted in no valid EC_{10} and EC_{20} values for any of the parameters.

As the reduction in wet weight at 0.933 mg a.s/L was > 10% (16%), RMS calculated the EC_{10} for wet weight to be 0.601 mg a.s/L (95% CI 0.400-0.906) using TOXRAT v3.2 (3-parameter normal CDF).

To assess the reliability of the estimated EC_{10} value, two approaches are described in EFSA Supporting publication 2015:EN-924:

- Normalised width of the confidence interval (NW = (upper limit – lower limit) / median estimate); rating of the NW ranges from excellent (<0.2) to bad (>2)
- Relationship between EC_{10} and EC_{20}/EC_{50} confidence intervals: the best case (high certainty of protection level) is achieved when EC_{10} is lower than the lower limit of the EC_{20}; the worst case (low certainty of protection level) occurs when the median EC_{10} is greater than the lower confidence limit for the EC_{50}.

Based on these rules, the reliability of the LC_{10} is considered “fair” based on its NW and cannot be determined based on the confidence intervals of the LC_{20} and LC_{50}.
However, this EC_{10} value was > the NOEC and thus the NOEC remains.

According to Regulation (EU) No 283/2013, the statistical power of the NOEC shall be assessed in case no ECx values can be derived. However, this is not possible as long as default type I and II errors have not been fixed, default variance for any response variable have not been established and typical detectable effect sizes of each parameter have not been calculated (EFSA Supporting publication 2015:EN-924). The NOEC was based on a slight but statistically significant reduction of growth as measured by length at 0.486 mg a.s./L, and set at the next lower level. This is considered a conservative approach, which is sufficiently protective. The NOEC of 0.233 mg a.s./L may be used for risk assessment.

[redacted]	1995	Early life-stage toxicity of flutolanil to the fathead minnow (*Pimephales promelas*) under flow-through conditions	Report no 41685 (W-3030)
Reliability			
General information			
Is a guideline method or modified guideline used?*		Yes	
Is the test performed under GLP conditions?*		Yes	
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?		Yes	
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?		Yes	
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?		Yes	
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?		Yes	
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?		Not applicable	

Test organism	
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	Yes
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	Yes
Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Yes
Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	Yes
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is	Yes

the response statistically significant?	
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Yes
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the	Not applicable

framework for which the study is evaluated?	
Concluding weight of evidence/proposed action	Fully acceptable
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable
Consideration/concluding score	Fully acceptable

B.9.2.2.2 Fish full life cycle test

No data submitted. Since the bioconcentration factor (BCF) of flutolanil is determined to be < 1000 (BCF = 100, see section 8.2.2.3) and LC_{50} > 0.1 mg/L, a fish life cycle test with flutolanil is not required.

B.9.2.2.3 Bioconcentration in fish

The bioconcentration of flutolanil in fish was investigated with respect to its octanol/water partition coefficient being determined to be Log P_{ow} = 3.17. The accumulation and depuration of ^{14}C-flutolanil in bluegill (*Lepomis macrochirus*) was assessed.

The bioconcentration potential of the test substance was determined in section CA 8.2.2.3-01, and the characterisation ^{14}C-flutolanil residues in bluegill (*Lepomis macrochirus*) water and tissues of was reported separately in CA 8.2.2.3-02.

Study 9.2.2.3-01

Report	CA 8.2.2.3-01. [redacted] 1991
Title	Uptake, depuration and bioconcentration of ^{14}C-flutolanil by bluegill (*Lepomis macrochirus*)
Report no.	37902 (W-3013)
Guidelines	U.S. EPA-FIFRA 40 CFR, Section 158.130 guideline 165-4
GLP	Yes
Previous evaluation	DAR (2006)
RMS Comment	The applicant submitted a new, more extensive summary. Considered acceptable for use in risk assessment
Endpoint	BCF = 100 ± 35

Executive Summary

The bioconcentration potential of ^{14}C-flutolanil (radiopurity 98%) by bluegill (*Lepomis macrochirus*) was determined in a 28 days flow through system, followed by a depuration period of 14 days. The methods followed were as per the steady-state approach. In the final test, groups of 120 bluegill sunfish were exposed to test water with ^{14}C-flutolanil at a nominal concentration of 0.05 mg/L, and to solvent control water using dimethylformamide (DMF). Fish were observed initially and twice daily during the exposure period for any mortality and adverse behaviour. Water and fish samples were periodically taken for radioanalysis at 0.17, 1, 3, 7, 14, 21 and 28 days during the uptake phase, and at 1, 3, 7, 10 and 14 days during the depuration phase. The kinetic parameters (steady-state bioconcentration factor (BCF), uptake rate constant (k_1), depuration rate constant (k_2), $T_{1/2}$ for depuration, and time to reach 90% of steady-state) were calculated using a steady-state approach with BIOFAC 2.

The average ^{14}C-flutolanil concentration in water during the uptake phase was 0.046 mg/L, which represented 92% of the nominal concentration of 0.05 mg/L. No mortality or adverse behaviour was observed in the test fish.

Uptake of ^{14}C-flutolanil reached steady-state plateau by Day 14. The tissue residues after 28 days of exposure were 0.60 mg/kg for fillet, 3.5 mg/kg for whole fish, and 8.9 mg/kg for viscera. During the depuration period, over 90% of ^{14}C-flutolanil in fish was eliminated within 1 day. The parameters calculated for whole fish were as follows: BCF = 100, k_1 = 150 mg/kg fish/mg/L water/day, k_2 = 1.5 /day, $T_{1/2}$ for depuration = 0.46 day, time to reach 90% of steady state = 1.5 day.

I. MATERIALS AND METHODS

A. MATERIALS

1. Non-radiolabelled

Test material:	Flutolanil technical
Lot no.:	TS-3776
Purity:	Not reported
Description:	White powder

2. Radiolabelled

Test material:	^{14}C-flutolanil
Lot no.:	RS-4091
Radiopurity:	98%
Description:	White crystalline solid

2.

Test organism:	Bluegill sunfish (*Lepomis macrochirus*)
Mean length:	51 ± 3.7 mm
Mean weight:	4.7 ± 1.1 g
Source:	[redacted]

3. **Treatment:** 0 (solvent control) and 0.05 mg/L

4. **Test vessels:** Glass aquaria (total capacity of 100 L)

Test water: Aerated well water

5. Environmental conditions:

Temperature:	20 – 23 °C
pH:	7.9 – 8.3
Dissolved oxygen:	7.0 – 8.5 mg/L (55 – 108% saturation)
Water hardness:	258 – 288 mg/L $CaCO_3$
Photoperiod:	16 h light : 8 h darkness

B. STUDY DESIGN AND METHODS

1. In-life phase: Jul 31 to Sep 11, 1990

2. Test organism assignment and treatment

The 240 bluegill were estimated to be less than 1 year of age at time of testing. All test fish were held in culture tanks and observed for at least 14 days prior to testing. During the holding (culture) and test periods, the fish received 3.0 g/aquaria/day of Rangens® Salmon Starter *ad libitum*. A 7 days flow through preliminary test was performed with non-radiolabelled flutolanil technical from 0.30 to 5.0 mg/L. A 7-day EC_{50} was calculated to be 3.5 mg/L. Observation of a white precipitate in the diluter mixing box suggested that the test material solubility was exceeded. Based on these results, the flow through definitive test employed a nominal exposure concentration of 0.05 mg/L to evaluate the bioconcentration potential of ^{14}C-flutolanil. 120 fish were impartially transferred from the culture tank to the control and treatment aquaria. The control aquarium consisted of dilution water that received an equivalent amount of dimethylformamide (DMF) to that delivered to the treated aquarium.

3. Dose preparation

An aliquot (0.10 ml) of ^{14}C-flutolanil diluter stock solution (605 mg/L DMF) was delivered to the toxicant mixing cell (1205 ml of dilution water) to achieve a nominal exposure concentration of 0.05 mg/L. Aerated well water was delivered to the test aquaria at an average rate of 360 ml/minute/aquarium during the 28-day exposure period. The control aquarium received 0.10 mL of DMF, which was equivalent to that delivered to the treated aquarium. On Day 28, the addition of the ^{14}C-flutolanil test material was terminated. The depurating phase was initiated by siphoning the water in each test aquarium until 8 cm (approx. 20 L) of water remained. This process was repeated and the fish were then exposed to flowing uncontaminated well water for 14 days.

4. Measurements and observations

Observations of the fish were made initially and twice daily during the exposure period for any mortality and adverse behaviour. Water and fish samples (whole fish, separate edible (fillet) and non-edible (viscera) portions) were periodically taken for radioanalysis by liquid scintillation counting (LSC) throughout the experiment at 0.17, 1, 3, 7, 14, 21 and 28 days. Three fish from each chamber were collected and pooled into control and treated samples for dissection. Three additional fish were collected and pooled into control and treated samples for whole fish analysis. Samples were frozen immediately and stored until analysis. After 28 days exposure period, a depuration phase of 14 days was initiated by siphoning each test aquarium and refilling them with uncontaminated water. Water

and fish samples were periodically taken in the same manner as during the intake phase at 1, 3, 7, 10 and 14 days. Water quality measurements of temperature, dissolved oxygen and pH were made initially and throughout the study on each sampling date.

5. Data analysis

The following kinetic parameters were calculated using a steady-state approach by modelling two compartments (water and fish) with a computer program (BIOFAC 2): the steady-state bioconcentration factor (BCF), the uptake rate constant (k_1), the depuration rate constant (k_2) $T_{1/2}$ for depuration, and time to reach 90% of steady-state.

II. RESULTS AND DISCUSSION

A. Biological data

No mortality or adverse behaviour was observed in the test fish.

The results of radioanalysis of ^{14}C-flutolanil in water, whole fish, fillet (edible), and viscera (non-edible) during 28 days of constant exposure to ^{14}C-flutolanil and 14 days of depuration in clean water were as follows:

Day (Uptake)	**Total ^{14}C Concentration as ^{14}C-flutolanil**			
	Water	**Whole fish**	**Edible portion**	**Non-edible portion**
	mg/L	mg/kg	mg/kg	mg/kg
0	0.048	-	-	-
0.17	0.038	1.1	0.26	2.3
1	0.044	3.4	0.32	5.2
3	0.047	3.6	0.34	6.7
7	0.045	6.6	0.49	10
14	0.045	6.8	0.56	12
21	0.050	3.0	0.40	5.7
28	0.050	3.5	0.60	8.9
(Depuration)	mg/L	mg/kg	mg/kg	mg/kg
1	0.0039	0.34	ND	0.43
3	ND*	0.064	ND	0.097
7	ND	0.037	ND	0.054
10	ND	ND	ND	0.038
14	ND	ND	ND	ND

* ND: Not detectable

Uptake of ^{14}C-flutolanil reached steady-state plateau by Day 14. The tissue residues after 28 days of exposure were 0.60 mg/kg for fillet, 3.5 mg/kg for whole fish, and 8.9 mg/kg for viscera. During the depuration period, over 90% of ^{14}C-flutolanil in fish was eliminated within 1 day. The bioconcentration factor in whole fish was calculated to be 100.

B. Analytical verification

The concentration of the test substance was maintained throughout the study. The average ^{14}C-flutolanil concentration in water during the uptake phase was 0.046 mg/L, which represented 92% of the nominal concentration of 0.05 mg/L.

III. CONCLUSION

The test fish were in good health and provided acceptable data to define the parameters for characterising bioconcentration potential of ^{14}C-flutolanil.

Bioaccumulation fish (whole):

BCF = 100 ± 35
Uptake rate constant k_1 = 150 ± 39 day^{-1}
Depuration rate constant k_2 = 1.5 ± 0.35 day^{-1}
$T_{1/2}$ for depuration = 0.46 ± 0.11 day
Time to reach 90% of steady state = 1.5 ± 0.36 day

Study 9.2.2.3-02

Report	CA 8.2.2.3-02. [redacted], 1991
Title	Characterization of ^{14}C-flutolanil residues in bluegill (*Lepomis macrochirus*) water and tissues.
Report no.	38946 (W-3022)
Guidelines	U.S. EPA-FIFRA 40 CFR, Section 158.130 guideline 165-4.
GLP	Yes (USA/EPA)
Previous evaluation	DAR (2006)
RMS Comment	The applicant submitted a new, more extensive summary. Considered acceptable for use in risk assessment
Conclusion	The major residues detected were unchanged parents and the metabolites M-4 (3'-hydroxy-2-trifluorotoluanilide) and M-11 (3'-(1-carboxyethoxy)-2-trifluorotoluanilide).

Executive Summary

This study describes the characterization of residues in bluegill (*Lepomis machrochirus*) following exposure to ^{14}C-flutolanil (radio-purity 98%). The parameters for characterising bioconcentration potential of ^{14}C-flutolanil were determined in the previous study (CA 8.2.2.3-01). Fish were exposed to a mean concentration of ^{14}C-flutolanil of 0.046 mg/L for 28 days. Both uptake and depuration were rapid and bioconcentration factor (BCF) in whole fish was calculated to be 100.

Water and fish samples were taken on Days 21 and 28 of the uptake phase. Samples were processed as describe in the study above (whole fish, fillet and viscera) for metabolite characterization. The

chemical nature of the radioactive residue recovered from the water and tissues was characterized and quantified by HPLC and TLC.

In test water, approximately 91 % (mean of day 21 and day 28) of the ^{14}C-residue was flutolanil. In fillet, flutolanil was the only significant residue detected. In viscera, the major residues detected were unchanged flutolanil and the metabolites M-4 (3'-hydroxy-2-trifluorotoluanilide (desisopropylflutolanil)) and M-11 (3'-(l-carboxyethoxy)-2-trifluorotoluanilide). The latter two metabolites were most likely present as labile conjugates since they were not detected until after acid hydrolysis followed by solvent partition.

I. MATERIALS AND METHODS

Water and fish samples obtained at 21 and 28 days of the intake phase described above (CA 8.2.2.3-01) were subjected to metabolite analysis by HPLC and TLC.

Nine potential metabolite standards were used for qualitative co-chromatography. Analytical and radiolabelled flutolanil standards were used for determining extractability and for qualitative co-chromatography.

II. RESULTS AND DISCUSSION

A. Characterisation of residue in water

In organic-extractable residues from water of days 21 and 28, ^{14}C-flutolanil accounted for 98.0 and 84.0% residue, respectively. The day 28 residue was found to comprise a very polar compound, accounting for 6 % of the residue.

B. Characterisation of residue in fillet

In acetonitrile-soluble residue from fillet of days 21 and 28, parent compound accounted for 71.5% and 72.8% of the initial residue, respectively. A rapidly eluting material from HPLC accounted for 10% of the day 28 residue, possibly composed of several non-polar conjugates.

C. Characterisation of residue in viscera

In viscera residue of days 21 and 28, parent compound was found to represent 21.2 and 19.6% of initial residue, respectively. After acidification of the viscera organic-extractable material of days 21 and 28, two major metabolites found to be M-4 (3'-hydroxy-2-trifluorotoluanilide, representing 20.7 and 21.2% for day 21 and 28, respectively) and M-11 (3'-(1-carboxyethoxy)-2-trifluorotoluanilide, representing 7.8 and 18.7% for day 21 and 28, respectively). In the remaining organic-extractable material, unknown metabolites and matrix associated material accounted for 0.4 to 15.2% of the initial viscera residue.

III. CONCLUSION

Analysis of ^{14}C-flutolanil in water and fillet samples from days 21 and 28 of the uptake phase indicated that the unchanged parent was the major component. In viscera samples, the major residues detected were unchanged parents and the metabolites M-4 (3'-hydroxy-2-trifluorotoluanilide) and M-11 (3'-(1-carboxyethoxy)-2-trifluorotoluanilide).

B.9.2.3 Potential for endocrine disruption

Study CA 8.2.3-01

Report	CA 8.2.3-01, [redacted], 2011
Title	Flutolanil: Fish Short-Term Reproduction Assay with the Fathead Minnow (*Pimephales promelas*)
Report no.	397A-148
Guidelines	OECD Guideline 229, U.S. EPA OPPTS No. 890.1350
GLP	Yes
Previous evaluation	New study
RMS Comment	Considered acceptable for use in risk assessment
Conclusion	The RMS is of the opinion that the results indicate a potential endocrine effect, based on effects on vitellogenin (concentration-related reduction), fecundity, secondary sexual characteristics in males and histological alterations of both male and female gonads. Further data are needed to investigate the potential endocrine activity of flutolanil. A medaka extended one generation test or other testing strategy to address potential anti-androgenicity in fish may be recommended.

Executive Summary

The objective of this short-term reproduction assay was to determine if the test substance might impact the hypothalamus-pituitary-gonadal (HPG) endocrine axis resulting in the disruption of reproduction in fish. Effects were evaluated using fathead minnows (*Pimephales promelas*) exposed to Flutolanil for 21 days under flow-through test conditions. Endpoints that were evaluated for endocrine disruption of the reproductive system included fecundity, fertility, secondary sex characteristics (including tubercle and fatpad scores), gonadosomatic index (GSI), histopathology of gonads, as well as serum vitellogenin (VTG). Other endpoints included survival, general observations of health, weight, and length.

Fathead minnows were exposed to a series of three test concentrations, a negative (dilution water) control and a solvent control (20 µL/L dimethylformamide) under flow-through conditions. Nominal test concentrations were 0.020, 0.20 and 2.0 mg a.s./L, equivalent to mean measured 0.018, 0.18, 1.2 mg a.s./L. Treatment-related findings in this assay were observed in the middle (0.18 mg a.s./L) and high (1.2 mg a.s./L) treatment groups. There were statistically significant, treatment-related effects on cumulative egg production and eggs per female reproductive day at 1.2 mg a.s./L. In males, there were statistically significant, treatment-related decreases in fatpad and tubercle scores at 1.2 mg a.s./L. In females, there were statistically significant, treatment-related increases in wet weight at 1.2 mg a.s./L and in GSI at 0.18 and 1.2 mg a.s./L, with a statistically significant, treatment-related

decrease in VTG at 0.18 and 1.2 mg a.s./L. Treatment-related effects in the gonads of male and female fish at test termination were limited to the high test concentration (1.2 mg a.s./L).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Flutolanil technical
Lot no.: 30116
Purity: 98.7%

2. Test organism: Fathead Minnow (*Pimephales promelas*) (*ca*. 6 months old at start of pre-exposure period)
Mean weight: 3.1 g (males), 1.4 g (females) ±30% of the mean weight of each sex
Mean length: 63 mm (males), 50 mm (females)
Source: [redacted]
Feed: commercial flake food (Sera Vipan) and brine shrimp nauplii (*Artemia* sp.) twice a day

3. Treatment: Control, Solvent control (DMF), 0.020, 0.20 and 2.0 mg a.s./L (continuous flow system)
Replicates: 4/treatment and control groups
 # Organisms: 2 males and 4 females/replicate
Biomass loading: 0.19 g of fish/L of test solution
Instantaneous loading: 1.3 g of fish/L (total weight of fish per litre of water in the tank)

4. Test vessels: Glass aquaria (total capacity of 12 L) filled with approx.. 10 L; Depth was 12.5 cm
Test water: moderately-hard filtered well water

5. Environmental conditions:

Temperature: 24.8 – 25.9°C
pH: 7.8 – 8.2
Dissolved oxygen: 3.0 – 8.3 mg/L equivalent to 36.7 – 101.6% oxygen saturation
Alkalinity: 168 – 176
Water hardness: 136 – 144 mg/L $CaCO_3$ (measured)
Photoperiod: 16 h light : 8 h darkness (*ca*. 956-1487 lux)

B. STUDY DESIGN AND METHODS

1. In-life phase: 15 February 2011 to 08 March 2011

2. Test organism assignment and treatment

During the 14-day pre-exposure period, breeding groups were selected and placed for health monitoring. Fish selected were similar in weight in order to minimise the variability in egg production. The exposure period was initiated after successful spawning was verified during the pre-exposure

period. A stratified random procedure was used to assign spawning groups to treatments based on data collected during the pre-exposure period. The groups were ranked from low to high fecundity and the top 20 spawning groups were selected. The top five performers were randomly assigned to one replicate exposure chamber of each treatment or control group (i.e., replicate A). The next five performers were randomly assigned to a second replicate for each treatment or control group (i.e., replicate B) and so on. This design was intended to minimize the variability in egg production between experimental groups.

3. Dose preparation

A primary stock solution was prepared by mixing a calculated amount of test substance into HPLC-grade DMF at a nominal concentration of 100 mg a.s./mL. Two secondary stock solutions were prepared in DMF at nominal concentrations of 1.0 and 10 mg a.s./mL by proportional dilution of the primary stock. The stock solutions were mixed by inversion and then held under refrigerated conditions in amber bottles. Fresh aliquots were placed in the syringe pump weekly during the test to be diluted in the flow through system at a target rate of 4 µL/min and delivered at a target rate of 200 mL/min, to achieve the nominal test concentrations of 0.02, 0.2 and 2.0 mg a.s./L.

4. Measurements and observations

Exposure of the fish to flutolanil was conducted for 21 days. During the exposure period, survival, general observations, and assessments of fecundity and fertility were recorded daily. At test termination, all fish were euthanized with buffered MS-222 (tricaine methanesulfonate) following observations and were measured for total length and wet weight. Observations of secondary sex characteristics were recorded and the external sex was determined. Blood samples then were collected for analysis of serum concentrations of vitellogenin (VTG). Immediately following blood collection, each fish was dissected and the gonadal sex was determined. The gonads were fixed, removed and weighed for calculation of the gonadosomatic index (GSI), and were preserved for gonadal histology. The carcass of each fish was preserved for later evaluation of tubercles.

5. Statistics

Statistical analyses were performed to evaluate differences between treatment and control groups for each of the following biological endpoints: Survival, Wet Weight, Total Length, Fecundity (eggs per female reproductive day and cumulative eggs produced), Fertility (fertile eggs/total eggs laid), Gonadosomatic index (GSI), Serum vitellogenin (VTG) concentration, Tubercule score, Fatpad score, Gonad developmental stage, Incidence and severity of gonad abnormalities.

Data from the negative and solvent control groups for each parameter were compared using an appropriate statistical test. Statistical tests used to evaluate treatment effects were performed at confidence level of $\alpha = 0.05$ with SAS software.

II. RESULTS AND DISCUSSION

Deviations

The recorded temperature on Day 5 in one control replicate was recorded to deviate by 2^{o}C instead of 1 ^{o}C, this deviation had no effect in the study results

A. Biological data

Survival and General Observations

There were no treatment-related effects on survival of fathead minnows during the 21-day test. The mean percent survival on Day 21 in the negative control and solvent control groups was 91.7 and 95.8%, respectively and therefore pooled control was used for comparisons with the treatment groups. Daily clinical observations of the fathead minnows through Day 21 appeared normal in the control groups and in the 0.018 and 0.18 mg a.s./L treatment groups. In the 1.2 mg a.s./L treatment group, treatment-related lethargy was first noted among 5 of the 24 fish on Day 1 of the test, decreasing to only 1 fish by Day 4. After Day 4, all fish in the high treatment group appeared normal.

Length and Weight

There was a treatment-related increase in wet weight among female fathead minnows in the high (1.2 mg a.s./L) treatment group at test termination ($p \leq 0.05$). Due to sexual dimorphism (i.e., the difference in size between males and females in the species), length and weight measurements were statistically compared by sex and results are summarised in the table below. The increased wet weight among females in the 1.2 mg a.s./L treatment group was statistically significant ($p \leq 0.05$) and treatment-related.

Reproduction

There was a treatment-related effect resulting in a lack of reproduction in the high (1.2 mg a.s./L) treatment group during the test. The cumulative number of eggs produced, the number of eggs per female reproductive day, and the percent fertility (the number of fertile eggs of the total number collected) from the 21-day test are summarized in the table below.

Summary of Survival, Fecundity, Fertility and Growth of Fathead Minnows Exposed to Flutolanil

Mean concentration (mg a.s./L)	Mean ± Standard Deviation at Termination							
	% Survival	Cumulative No. of Eggs Produced	Eggs /Female /Reproductive Day	% Fertility	Total length (mm)		Wet weight (g)	
					Male	Female	Male	Female
Control	91.7 ± 9.6	2495 ± 450	30.5 ± 6.69	98.1±1.4	63 ± 1.6	49 ± 0.2	3.5 ± 0.16	1.4 ± 0.06
Solvent control	95.8 ± 8.4	2548 ± 455	30.4 ± 5.41	99.1±0.3	63 ± 1.0	50 ± 1.7	3.6 ± 0.35	1.4 ± 0.13
Pooled control	93.7 ± 8.6	2521 ± 420	30.4 ± 5.64	98.6±1.1	63 ± 1.2	50 ± 1.3	3.6 ± 0.26	1.4 ± 0.10
0.018	100 ± 0.0	2126 ± 536	25.3 ± 6.40	98.3±0.9	62 ± 2.3	50 ± 1.5	3.4 ± 0.31	1.5 ± 0.18
0.18	100 ± 0.0	2120 ± 441	25.2 ± 5.27	98.7±0.1	64 ± 1.2	50 ± 1.5	3.7 ± 0.14	1.4 ± 0.09
1.2	100 ± 0.0	0 ± 0*†	0 ± 0*†	-[1]	63 ± 2.2	51 ± 0.9	3.4 ± 0.20	1.7±0.12*†

[1] No mean or standard deviation was calculated; no eggs were produced in any replicate in this treatment group

* Statistically significant trend using the Jonckheere-Terpstra trend test ($p \leq 0.05$)

† Statistically significant difference from the pooled control using Dunnett's test ($p \leq 0.05$)

Secondary Sex Characteristics, GSI, VTG, Histology and Histopathology

There were treatment-related decreases in fatpad scores and tubercles scores among male fathead minnows in the high (1.2 mg a.s./L) treatment group at test termination ($p \leq 0.05$). There was a treatment-related and dose-responsive increase in Gonadosomatic Index (GSI) among female fish in the two highest treatment groups (0.18 and 1.2 mg a.s./L) and a treatment-related and dose-responsive decrease in Vitellogenin (VTG) among female fish in the same treatment groups during the 21-day test.

All fish were dissected immediately following blood collection, and gonadal sex was determined using a dissecting microscope. Histomorphologic parameters assessed included relative germ cell numbers, alterations in numbers and sizes of non-germ cells (e.g., testicular interstitial cells and ovarian perifollicular cells), and increased degenerative changes. Treatment-related effects in the gonads of male and female fish at test termination were limited to the highest test concentration (1.2 mg a.s./L). Measurements are summarised in the table below.

Summary of Biological Parameters in Fathead Minnows Exposed to Flutolanil

Mean concentration (mg a.s./L)	Mean ± Standard Deviation at Termination							
	Males				Females			
	Fatpad Score	Tubercle Score	Gonado-somatic Index (GSI)	Vitello-genin (µg/mL)	Fatpad Score[1]	Tubercle Score[1]	Gonado-somatic Index (GSI)	Vitellogenin (µg/mL)
Control	3.6±0.95	26.4±1.1	1.95±0.34	0.9±1.01	1±0	0	10.4±0.98	3531±728
Solvent control	2.9±0.25	26.4±1.4	1.76±0.2	6.23±9.8	1±0	0	11.8±1.18	4022±913
Pooled control	3.3±0.76	26.4±1.2	1.85±0.28	3.47±4.7	1±0	0	11.1±1.24	3767±342
0.018	3.4±0.48	26.6±2.6	1.83±0.2	2.32±0.9	1±0	0	12±1.06	3271±522
0.18	3.3±0.29	24.4±3.5	1.73±0.13	1.36±0.8	1±0	0	13.6±1.21* †	2742±709* †
1.2	2.3±0.29[a]	18±5.35† *	1.91±0.26	1.54±3.0	1±0	0	17.1±1.8* †	929±226* †

* Statistically significant trend using the Jonckheere-Terpstra trend test ($p \leq 0.05$)

† Statistically significant difference from the pooled control using Dunnett's test ($p \leq 0.05$)

[a] Statistically significant difference from the pooled control using Wilcoxon Rank-Sum test ($p \leq 0.05$)

[1] A fatpad score of 1 = no fatpad visible; a tubercle score of 0 = no tubercles present

B. Analytical verification

Samples were collected for analysis from all stock solutions on Day 0 to confirm concentrations being delivered to the diluter system. On Day 10, additional samples were collected from the 2.0 mg a.s./L test solution in the diluter mixing chamber and from the stock solution to confirm the concentrations in the diluter system. The samples were placed in glass vials and processed immediately for analysis.

All test solutions appeared clear and colourless during the test, with the exception of the highest concentration of 2.0 mg a.s./L which appeared to have white precipitate on the bottom of the test chambers and floating in the water column in the mixing chamber and therefore these treatment samples collected on Days 0, 7, 14, 16 and 21 were analysed both with and without centrifugation.

Nominal Test concentration (mg a.s./L)	Measured concentration (mg a.s./L)			
	Pre-exposure samples			
	Day-2	Day-1	Mean	Mean Nominal (%)
Control	<0.01	<0.01	-	-
Solvent control	<0.01	<0.01	-	-
0.02	0.0186	0.0196	0.019	96%
0.2	0.195	0.203	0.199	100%
2.0	1.39	1.655	1.523	76%

Nominal Test concentration (mg a.s./L)	Measured concentration (mg a.s./L)						
	Exposure samples						
	Day 0	Day 7	Day 14	Day 16	Day 21	Mean	Mean Nominal (%)
Control	<0.01	<0.01	<0.01	<0.01	<0.01	-	-
Solvent control	<0.01	<0.01	<0.01	<0.01	<0.01	-	-
0.02	0.0144	0.0178	0.01805	0.0194	0.0195	0.018	89%
0.2	0.188	0.176	0.183	0.191	0.181	0.184	92%
2.0[a]	1.44	0.793	0.9735	1.39	1.48	1.215	61%

[a] Samples collected on Days 0,7,10,14,16,21 and analysed with centrifugation prior to analysis – samples analysed without centrifugation were not included in the calculation of mean measured

Nominal Test concentration (mg a.s./L)	**Measured concentration (mg a.s./L) Pre-exposure samples**				**Exposure samples**						
	Day-2	**Day-1**	**Mean**	**Mean Nominal (%)**	**Day 0**	**Day 7**	**Day 14**	**Day 16**	**Day 21**	**Mean**	**Mean Nomin (%)**
Control	**<0.01**	**<0.01**	**-**	**-**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**-**	**-**
Solvent control	**<0.01**	**<0.01**	**-**	**-**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**-**	**-**
0.02	**0.0186**	**0.0196**	**0.019**	**96%**	**0.0144**	**0.0178**	**0.01805**	**0.0194**	**0.0195**	**0.018**	**89%**
0.2	**0.195**	**0.203**	**0.199**	**100%**	**0.188**	**0.176**	**0.183**	**0.191**	**0.181**	**0.184**	**92%**
2.0	**1.39**	**1.655**	**1.523**	**76%**	**1.44**	**0.793**	**0.9735**	**1.39**	**1.48**	**1.215**	**61%**

C. Validity criteria

- The dissolved oxygen concentration was at least 60% of the air-saturation value throughout the majority of the exposure period. Between Days 12 and 14, dissolved oxygen concentrations declined to a low of 3.0 mg/L (37% of saturation) due to a build-up of microbial growth in the test chambers. However, replacing the test chambers with clean tanks on Days 12 and 14 and adding gentle aeration on Day 15 resulted in dissolved oxygen concentrations above 60% of saturation for the remainder of the test.

- Water did not differ by more than 1ºC between test vessels at any one time during the exposure period and was maintained within ±1ºC of the 25ºC temperature specified, except on Day 5 of the test when the minimum temperature was 23.0ºC for a short duration.
- There was more than 90% survival of control animals over the duration of the chemical exposure.
- Fish were actively spawning in all replicates prior to initiating chemical exposure in those replicates, and in control replicates during the test.

The validity criteria were met and the deviations were considered to be minor and did not have an effect on the outcome of the study results, therefore the test was considered to be acceptable.

D. Toxicity endpoints

The endpoints evaluated to determine if the test substance might impact the hypothalamus-pituitary-gonadal (HPG) endocrine axis of fish were adult survival, body length and wet weight, fecundity (cumulative egg production, eggs per female reproductive day and fertilization success), secondary sex characteristics (including fatpad and tubercle scores), GSI, VTG and gonad histopathology. The concentration where no significant effect was observed was 0.018 mg a.s./L (NOEC).

III. CONCLUSION

Breeding groups of fathead minnows (*Pimephales promelas*) were exposed to flutolanil at mean measured concentrations of 0.018, 0.18 and 1.2 mg a.s./L for 21 days. The endpoints evaluated to determine if the test substance might impact the hypothalamus-pituitary-gonadal (HPG) endocrine axis of fish were adult survival, body length and wet weight, fecundity (cumulative egg production, eggs per female reproductive day and fertilization success), secondary sex characteristics (including fatpad and tubercle scores), GSI, VTG and gonad histopathology. Treatment-related findings in this assay were observed in the middle (0.18 mg a.s./L) and high (1.2 mg a.s./L) treatment groups. There were statistically significant, treatment-related effects on cumulative egg production and eggs per female reproductive day at 1.2 mg a.s./L. In males, there were statistically significant, treatment-related decreases in fatpad and tubercle scores at 1.2 mg a.s./L. In females, there were statistically significant, treatment-related increases in wet weight at 1.2 mg a.s./L and in GSI at 0.18 and 1.2 mg a.s./L, with a statistically significant, treatment-related decrease in VTG at 0.18 and 1.2 mg a.s./L. Treatment-related effects in the gonads of male and female fish at test termination were limited to the high test concentration (1.2 mg a.s./L).

([redacted] 2011)

The fish short-term reproduction assay (FSTRA) in the fathead minnow evaluated the ability of flutolanil to determine impact on the hypothalamus-pituitary-gonadal (HPG) endocrine axis of fish. The results of this study are ambiguous. Some endpoints with sensitivity to endocrine active substances were affected, and there was a major adverse effect on fecundity. It is possible that anti-estrogenic activity could have caused the large decrease in female VTG, but the increase in female GSI appears incompatible with this scenario. It is also possible that anti-androgenic activity could explain the slight reductions in male SSC and maturation of spermatogonia, but the large decrease in female VTG and

large increase in female GSI are inconsistent with anti-androgenicity. It is more likely that the effects are due to systemic toxicity.

(Odum J., et al. 2016)

Comments by RMS

The study was conducted according to OECD 229 and there were no major deviations that would invalidate the study.

Water samples were collected on days -2, 0, 7, 14 and 21 from the controls and treatment groups and analysed with a sufficiently validated HPLC-UV method (fortification samples at 0.02, 0.2 and 2.0 mg a.s./L, analysed concurrently with the test samples had measured concentrations between 86 and 109% of nominal).

The results of the study were based on arithmetic mean measured concentrations, which is acceptable for flow-through study designs. As in the highest test concentration a white precipitate was observed, the samples were centrifuged before analysis and these results were used in the calculation of the mean measured concentration. This is acceptable.
The NOEC was 0.018 mg a.s./L

An EC_{10} was not provided by the applicant and is not necessary as the test guideline indicates that the bioassay serves as an *in vivo* reproductive screening assay to evaluate whether additional testing is warranted. It should not be used to derive endpoints to be used in the risk assessment. Extensive histopathology was performed on the gonads from 117 fish (38 male and 79 female), but the results were not included in the summary. The results are summarized below:

Analysis of the severity scores for abnormalities in the ovaries and testes revealed no significant differences ($P > 0.05$) between the negative and solvent controls. However, statistically significant differences ($P = 0.012$) between the distributions of the development stages of the ovaries in the two control groups were observed. Median developmental stage was 2.5 in the negative control group and was 3 in the solvent control group. Because the frequency and severity of some abnormalities might be correlated with development stage, it was decided to perform two sets of statistical analyses of the observations from the study, one set being a comparison of treatment groups to the negative control (NC) group, and the second set being a comparison of treatment groups to the solvent control (SC) group.

There was a significant difference between testes of the two control groups and those of males in the high treatment group In the high (2.0 mg a.s./L) treatment group, the testis germinal epithelium showed an increased presence of spermatogonia and primary spermatocytes with a decreased presence of spermatocysts containing secondary spermatocytes and spermatids. The changes in these ratios were such that in gonads from 2 of the 8 male fish, the normal ratio of the various spermatocytes to each other was moderately altered, so that the testes could not be staged using

normal criteria. These changes appeared to result from a relative inability of spermatogonia and primary spermatocytes in the testes of affected fish to mature into secondary spermatocytes and spermatids.

There was a statistically significant increase of oocyte atresia with flutolanil concentration of 2.0 mg a.s./L. Gonad size as measured directly on the tissue sections of the histology slides was statistically increased in all three treatment groups relative to negative and solvent control groups. Gonadal development stage is also largely determined by the ratio of non-vitellogenic oocytes to vitellogenic oocytes, and was also found to be statistically different in the treatment groups relative to the control groups.

In accordance with OECD 229 “Guidance for interpretation”, a positive result of vitellogenin measurements (statistically significant increase in VTG in males or a statistically significant decrease in females at least at the highest dose compared to the controls, together with absence of signs of general toxicity) should generally be interpreted as evidence of endocrine activity *in vivo*, especially if it is further supported by the demonstration of a biologically plausible dose-response.

There was a statistically significant decrease in VTG in females at the two highest doses as compared to the control groups. At the two highest test concentrations, the GSI was statistically increased in females as compared to the controls and this was also reflected in a higher female wet weight in the highest dose as compared to the controls. Females did not produce any eggs at the highest treatment. A statistically significant increase of oocyte atresia and difference in stage of ovaries was observed at the highest dose.

Equally evident were the statistically significant lower fatpad and tubercle scores in males at the highest test concentration as compared to the controls, indicating that the secondary sex characteristics of males are influenced by the substance. A statistically significant difference was observed in testis between the highest treatment group and the controls (the spermatocyst number per tubule, size of spermatocysts and the average germinal epithelial thickness was moderate to markedly decreased in 3 of the 8 fish).

Based on the above described effects, observed mainly at the highest dose but for the female VTG also in the 0.18 mg a.s./L treatment, there are signs that endocrine activity is plausible. The applicant stated that the increase in female wet weight in the highest treatment group and the increase in female GSI at both 0.18 and 1.2 mg a.s./L were incompatible with the decrease of VTG and attributed these effects to systemic toxicity. However, increases in weight or GSI are not expected for general toxicity, and the specific effects on gonadal histology seem to suggest the possibility of a more specific toxicity.

The results indicate a potential endocrine effect, based on effects on vitellogenin (concentration-related reduction), fecundity, secondary sexual characteristics in males and histological alterations of both male and female gonads. These seem to indicate a potential anti-androgenic or steroidogenic

mechanism of action, however, assays addressing these aspects in humans (or mammals) present in the mammalian data set were negative. Further data are needed to investigate the potential endocrine activity of flutolanil in fish. A data gap is set for an extended one generation test (medaka) and/or any other pertinent information relating to mechanism of action or molecular interactions relevant to the potential for endocrine disruption in fish and whether these potential interactions might be considered adverse to fish at the population level.

[redacted]	2011	Flutolanil: Fish short-term reproduction assay with the fathead minnow (*Pimephales promelas*)	Report no 397A-148
Reliability			
General information			
Is a guideline method or modified guideline used?*		Yes	
Is the test performed under GLP conditions?*		Yes	
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?		Yes	
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?		Yes	
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?		Yes	
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?		Yes	
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?		Not applicable	
Test organism			
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?		Yes	
Are the test organisms from a trustworthy source and acclimatized to test conditions?		Yes	

Have the organisms not been pre-exposed to test compound or other unintended stressors?	
Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Yes
Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	Yes
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	Yes
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	

Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Not applicable, this study is a screening study that should not be used for risk assessment, but should be used to indicate if further testing is needed.
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Not applicable, this study is a screening study that should not be used for risk assessment, but should be used to indicate if further testing is needed.
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable
Concluding weight of evidence/proposed action	Fully acceptable
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable

Consideration/concluding score	Fully acceptable

B.9.2.4 Acute toxicity to aquatic invertebrates

B.9.2.4.1 Acute toxicity to *Daphnia magna*

Study CA 8.2.4.1-01

Report	CA 8.2.4.1-01. Forbis, A.D., Young, B.M., Hicks, S.L., 1990
Title	Acute toxicity of flutolanil to *Daphnia magna*
Report no.	38718 (W-3014)
Guidelines	Directive 96/12/EC, Directive 92/69/EEC, Method C2, U.S. EPA-FIFRA, 40 CFR, Section 158, Guideline 72-2
GLP	Yes (USA/EPA)
Previous evaluation	DAR (2006)
RMS Comment	The applicant submitted a new, more extensive summary. Considered acceptable for use in risk assessment
Endpoint	48-h EC_{50} = > 6.8 mg/L

Executive Summary

The effects (acute immobilization), of flutolanil to *Daphnia magna*, were determined in a 48-hour static system. 20 daphnids less than 24 hours old (two replicates containing ten animals each) were exposed to 0.3, 0.6, 1.3, 2.5 and 5.0 mg/L (nominal) for 48 hours. One treatment consisting of untreated test medium only item was used as control and one with dimethylformamide (DMF) used as Solvent control. The number of immobilised daphnids was assessed after 4, 24 and 48 hours from the beginning of the test.

Analysis of the test solutions (fresh and old) for flutolanil was performed in samples taken at 0 and 48 hours after application. For each concentration, the mean measured value was below the range of ±20% of the nominal, therefore, all study results were based on mean measured concentrations.

5 % and 45% immobilization was observed only at 4.1 and 6.8 mg/L (measured), respectively after 48 hours while no immobilization was observed at the control, the solvent control and the lower levels of 0.75 – 2.2 mg/L (measured) treatment. Therefore, the 48-h EC_{50}-value for flutolanil was estimated to be greater than 6.8 mg/L (measured).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Flutolanil Technical
Batch no.: 81116
CAS no: 66332-96-S

	Purity:	98.9%
2.	**Test organism:**	*Daphnia magna*
	Age:	< 24 hours old - First-instar
	Source:	ABC Laboratories in-house culture
	Feeding:	During housing the animals were fed with a suspension of at least one algae species; *S. capricornutum, A. falcatus* and/or *C. reinhardtii.* Along with the algae, the daphnids were fed a supplement consisting of trout chow and yeast.
3.	**Treatment:**	Control, solvent control, 1.3, 2.2, 3.6, 6.0 and 10 mg/L (nominal)
	Test vessels:	Glass vessels (200 mL)
	Loading:	*ca*. 20mL of media per *Daphnia*
	Test water:	Hard Blended water using reverse osmosis water (Hardness: 160 mg/L as $CaCO_3$ and Alkalinity: 180 mg/L as $CaCO_3$)
4.	**Environmental conditions**	
	Temperature:	21°C
	pH:	8.3 – 8.5
	Dissolved oxygen:	7.8 – 8.1 (mg/L)
	Photoperiod:	16 h light : 8 h darkness (*ca*.540 – 750 lux)

B. STUDY DESIGN AND METHODS

1. In-life phase: Jun 19 to Jun 21, 1990

2. Test organism assignment and treatment

Daphnids aged less than 24 hours at the start of the test were exposed at five concentrations of the test item for a period of 48 hours under static conditions. 20 daphnids were used for each treated (0.3, 0.6, 1.3, 2.5 and 5.0 mg/L) and untreated (control) and DMF (solvent control) groups, divided in two replicates of ten animals each. No feeding occurred during the test.

3. Dose preparation

The highest concentration was prepared by injecting 0.2 ml of a primary standard solution (100mg/mL in DMF) in two litres of dilution water. Due to the presence of precipitate, sonication was performed for 30 minutes. A precipitate was still present and the solution was allowed to stand for *ca*. 10 minutes before the serial dilutions were performed to prepare the lower levels. There was no renewal of the test solution from the start of the test. Precipitate was present in the highest level throughout the test and noted to be present in the test vessels.

4. Measurements and observations

The number of immobilised daphnids was assessed after 4, 24 and 48 hours from the beginning of the test. Dissolved oxygen concentrations and pH values were measured in all the test groups and the control and solvent control vessels at the beginning and at the end of the test. The temperature and light intensity in the climatic chamber were recorded.

The analysis of the concentration of flutolanil was performed for each test concentration. Samples of 50 mL were taken from each test level and were analysed. The samples were collected at 0 hours from fresh test solutions and at the end of the test from the aged test solutions (end value at 48 hours, after pooling of the replicates). Analysis was performed by Gas-Liquid Chromatography.

5. Statistics

The NOEC was based on the absence of immobility and adverse effects at or below this test concentration. EC_{50} values and 95% confidence limits were calculated using the binomial method, the moving average and the probit tests if data permitted. The 48-hour dose-response slope was calculated by transferring percent immobile to probit values and then calculation via linear regression. Statistical analysis was performed using software developed by Stephan, C.E. (1978).

II. RESULTS AND DISCUSSION

A. Immobilization data

The number of immobilized daphnids and the percentage of immobilization at 4, 24 and 48 hours of exposure are presented in the following table.

Percentage of immobilisation after 4, 24 and 48 hours of exposure

Nominal Concentration (mg/L)	Mean measured Concentration* (mg/L)	No. of replicate	No. of *Daphnia* /group	% Immobility			EC_{50} value
				4 hr	24 hr	48 hr	
Control	-	2	20	0	0	0	**4 hr > 6.8 mg/L** **24 hr > 6.8 mg/L** **48 hr > 6.8 mg/L**
Solvent control	-	2	20	0	0	0	
1.3	0.75	2	20	0	0	0	
2.2	1.4	2	20	0	0	0	
3.6	2.2	2	20	0	0	0	
6.0	4.1	2	20	0	0	5	
10	6.8	2	20	0	0	45	

*: mean of measured concentrations at 0 and 48 hr

B. Analytical verification

Analysis of the test solutions (fresh and old) for the active substance flutolanil 0 and 48 hours after application. For each concentration, the mean measured value was below the range of ±20% of the nominal, therefore, all study results were based on mean measured concentrations. The analytical results are reported in the following table.

Measured concentrations of flutolanil during the test

Test item nominal (mg/L)	Mean measured (mg/L)	Recovery (%)
	Sampling (hrs)	

	0 (fresh)	48 (old)	
1.3	0.73	0.77	56-59
2.2	1.2	1.5	55-68
3.6	2.0	2.4	56-67
6.0	3.9	4.2	65-70
10	6.8	6.8	68

C. Toxicity endpoint

The 48-hour toxicity endpoints for flutolanil to *Daphnia magna* are presented in the following table taking into account the mean measured concentrations achieved.

Toxicity endpoints of the test item flutolanil

Effect concentration	**48 h**
EC_{50} (mg/L)	>6.8

III. CONCLUSION

In a 48-h semi-static exposure immobilization test on *Daphnia magna b*ased on the mean measured test concentrations, the 48-h EC_{50} value for flutolanil was estimated to be greater than 6.8 mg/L.

B.9.2.4.2 Acute toxicity to an additional aquatic invertebrate species

Since the active substance does not have insecticidal properties, an acute toxicity test with an additional aquatic invertebrate species is not required, however the study presented below assessing the acute toxicity of flutolanil to *Mysidopsis bahia,* was available for the flutolanil registration in the United States and therefore is submitted.

Study CA 8.2.4.2-01 *Mysidopsis bahia*

Report	CA 8.2.4.2-01 Forbis, A.D., 1991
Title	Acute Toxicity of Flutolanil to *Mysidopsis bahia*
Report no.	38720 (W-3015)
Guidelines	U.S. EPA-FIFRA, 40 CFR, Part 158.145 Guideline 72-3
GLP	Yes
Previous evaluation	New study
RMS Comment	Considered acceptable (only for adults, not for juveniles) for use in risk assessment
Endpoint	96h LC_{50} = 0.13 mg a.s./L (95% CI 0.087-0.16 mg a.s./L)

Executive Summary

The 96-hour static acute toxicity test of Flutolanil to *Mysidopsis bahia* was assessed using the methods outlined by the Committee on Methods for Toxicity Tests with Aquatic Organisms. The study was performed at the following nominal test concentrations: 0.018, 0.032, 0.056, 0.10, 0.18 and 0.32 mg/L. The mean measured test concentrations were 0.020, 0.027, 0.049, 0.087, 0.16 and 0.29 mg/L. All reported results were based upon the mean measured concentrations. Water quality parameters of temperature, dissolved oxygen and pH were measured on day 0, 2 and 4 of the test.

The 96-hour LC_{50} was calculated to be 0.13 mg/L with 95% confidence intervals 0.087 and 0.16 mg/L. The no-effect level observed for Flutolanil was considered to be 0.087 mg/L due to the lack of mortality or abnormal effects.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Flutolanil
Batch no.: 81116
Purity: 98.9%

2. Dilution salt water: Salinity: 21-22‰; Temperature: 21-23°C;
D.O.: 5.5-7.5; pH: 8.0-8.2

3. Test concentrations: Nominal: Control, Solvent Control (DMF), 0.018, 0.032, 0.056, 0.10, 0.18 and 0.32 mg/L
Mean measured: Control, Solvent Control, 0.020, 0.027, 0.049, 0.087, 0.16 and 0.29 mg/L
Test vessels: 400 ml glass beakers containing 300 ml of media; loosely covered

4. Test organism: *Mysidopsis bahia* (Lot no. 3490)

Age: Juveniles approximately 4 to 5 days old
Source: Aquatic Biosystems, Inc. Ft. Collins, Colorado
Feed: brine shrimp, ad libitum, daily

5. Environmental conditions:

Temperature: 21 to 23 °C
pH: 8.0 – 8.2
Dissolved oxygen: 5.5 – 7.5 mg/L (67-88% saturation)
Photoperiod: Not reported

B. STUDY DESIGN AND METHODS

1. In-life dates: 02 July 1990 – 06 July 1990

2. Animal assignment and treatment

A total of 80 organisms were selected for the tests. Mysids aged *ca*. 4-5 days at the start of the test were exposed at six concentrations of the test item for a period of 96 hours under static conditions. 10 animals were used for each treated (0.020, 0.027, 0.049, 0.087, 0.16 and 0.29 mg/L) and untreated

(control) and DMF (solvent control) groups, divided in two replicates of five animals each. During the test, the organisms were fed brine shrimp ad libitum, daily..

3. Dose preparation

Appropriate aliquots of a stock solution were added to dilution saltwater to prepare all test concentrations. The solvent control contained an amount of DMF equivalent to 0.1 ml/L. At 0 hour, all test solutions were clear and remained so throughout the study.

4. Measurements and observations

The number of animal mortality was assessed after 24, 48, 72 and 96 hours from the beginning of the test. Dissolved oxygen concentrations, salinity, temperature and pH values were measured in all the test groups and the control and solvent control vessels at the beginning, at 48 hours and at the end of the test. The dissolved oxygen (D.O.) measurements were corrected for salinity.

The analysis of the concentration of flutolanil was performed for each test concentration. Samples of 50 mL were taken from each test level and were analysed. The samples were collected at 0 hours from fresh test solutions and at the end of the test from the aged test solutions (end value at 96 hours, after pooling of the replicates). Analysis was performed by Gas-Liquid Chromatography. The GC-ECD was sufficiently validated with overall average recoveries of 105±4% over a concentration range of 0.103 to 20.6 mg/L and 106±3.4% over a concentration range of 0.0163 to 0.0612 mg/L. Recoveries from quality control samples (fortified with 0.0102 - 0.408 mg/L) run together with the analytical measurements ranged from 97 to 103% at t=0 and from 105 to 118% at t=96h.

5. Statistics

Statistical analysis of the concentration vs. effect data (mortality) was attempted by employing a computerized LC50 program developed by Stephan *et al.* (1978). This program calculated the LC50 statistic and its 95-percent confidence limits using the binomial, the moving average and the probit tests if data permitted. Three different methods of analysing the data were used since no one method of analysis is appropriate for all possible sets of data that may be obtained. The method of calculation selected for presentation in this report was that which gave the narrowest confidence limits for the LC_{50} although all three models were valid. The 96-hour dose-response slope was calculated by linear regression using the probit transformation method.

II. RESULTS AND DISCUSSION

A. Biological data

Mortality was recorded and any abnormal effects on mysids; tending to remain at the bottom of test replicates (observed in the 0.16 mg/L measured test concentration). The no-effect concentration based on the absence of mortality and abnormal effects was 0.087 mg/L. The control, solvent control and all other concentrations were observed to be clear and normal for the duration of the 96-hour bioassay.

The dissolved oxygen concentrations ranged between 5. 5 and 7. 5 mg/L. These values represented 67 and 88 percent saturation at 23 °C and 21°C, respectively (corrected for local altitudinal pressure). The pH values ranged from 8.0 to 8.2 and the salinity was measured to be at 21 o/oo.

Summary of Mortality and Behavioural Observations During the Static Toxicity Test on Flutolanil to *Mysidopsis bahia*

Measured concentration (mg flutolanil/L)	**% Mortality**			
	24 hours	**48 hours**	**72 hours**	**96 hours**
Control	0	0	0	0
Solvent control	0	0	0	0
0.020	0	0	0	0
0.027	0	0	0	0
0.049	0	0	0	0
0.087	0	0	0	0
0.160	20	70 [a]	90	90
0.290	70	80	100	100

[a] One animal observed on the bottom of the vessel

B. Analytical verification

Analysis of the test solutions (fresh and old) for the active substance flutolanil 0 and 96 hours after application. Study results were based on mean measured concentrations. The analytical results are summarised in the following table.

Measured concentrations of flutolanil during the test

Nominal	**Day 0**	**%**	**96-hour**	**%**	**Mean**	
0.018	0.019	106%	0.021	117%	0.020	111%
0.032	0.029	91%	0.024	75%	0.027	83%
0.056	0.051	91%	0.046	82%	0.049	87%
0.1	0.088	88%	0.086	86%	0.087	87%
0.18	0.16	89%	0.16	89%	0.160	89%
0.32	0.29	91%	0.29	91%	0.290	91%
3.2	3.4	106%	-		3.400	106%

*expressed in mg/mL

C. Toxicity Endpoints

The 96-hour toxicity endpoints for flutolanil to *Mysidopsis bahia* are presented in the following table taking into account the mean measured concentrations achieved.

Toxicity endpoints of the test item flutolanil

Effect concentration	**24h**	**48h**	**72h**	**96 h**
LC_{50} (mg/L)	0.23 [a]	0.16 [b]	0.13 [c]	0.13 [c]
95% confidence intervals	0.18-0.35	0.12-0.21	0.087-0.16	0.087-0.16

[a] LC_{50} calculated using the probit method.
[b] LC_{50} calculated using the moving average method.
[c] LC_{50} calculated using the binominal method.

III. CONCLUSION

The 96-hour LC_{50} was calculated to be 0.13 mg/L with 95% confidence intervals 0.087 and 0.16 mg/L. The no-effect level observed for Flutolanil was considered to be 0.087 mg/L due to the lack of mortality or abnormal effects.

Flutolanil appeared to be stable under test conditions and was categorized as highly toxic to mysid shrimp. The LC50 derived from the definitive study was consistent with the results of the two prior studies and accurately represents the toxicity of flutolanil to mysid shrimp.

Comments by RMS

The study was conducted according to US EPA-FIFRA, 40 CFR, Part 158.145, Guideline 72-3 and, in absence of an OECD Guideline for this species, it was in general agreement with OPPTS 850.1035. Deviations from the OPPTS 850.1035 were the amount of mysids/concentration (10 instead of 20), the age of the mysids (only young adults of 4-5 days old were used whereas also juveniles (< 24 h old) should be investigated) and the temperature (21 to 23 ºC instead of 23-27 ºC).

The 96h LC_{50} was estimated based on arithmetic mean measured concentrations. According to the EFSA Supporting publication 2015:EN-924, geometric mean measured concentrations should be used for static tests. In this case, the arithmetic and geometric mean measured concentrations were very similar and RMS did not re-calculate the 96h LC_{50}.

The 96h LC_{50} for adult mysids was 0.13 mg a.s./L (95% CI 0.087-0.16 mg a.s./L).

Forbis A.D.	1991	Acute toxicity of flutolanil to *Mysidopsis bahia*	Report no 38720 (W-3015)
Reliability			
General information			
Is a guideline method or modified guideline used?*	Yes		
Is the test performed under GLP conditions?*	Yes		

If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?	Yes
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?	Yes
* these criteria are of minor importance for study reliability, but may support study evaluation	
Test compound	
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?	Yes
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	Yes
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	Not applicable
Test organism	
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	Yes
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	Yes
Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Yes

Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	Yes
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	Yes
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the	Yes (only for adults)

regulatory purpose?	
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes
Are appropriate life-stages studied?	Yes, only adults; juveniles were not studied
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable
Concluding weight of evidence/proposed action	Fully acceptable (for adults)
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable (for adults)
Consideration/concluding score	Fully acceptable (for adults)

B.9.2.5 Long-term and chronic toxicity to aquatic invertebrates

B.9.2.5.1 Reproductive and developmental toxicity to *Daphnia magna*

Report	CA 8.2.5.1-01. Blakemore G.C., Burgess, D., 1991
Title	21-Day chronic static renewal toxicity of flutolanil to *Daphnia magna*
Report no.	38721 (W-3017)
Guidelines	U.S. EPA 72-4, ASTM Standard E-47.01 (1981)
GLP	Yes (USA/EPA)
Previous evaluation	DAR (2006)
RMS Comment	The applicant submitted a new, more extensive summary. Considered acceptable for use in risk assessment

Report	CA 8.2.5.1-02. Palmer, D.A., 2016
Title	EAG Laboratories letter - To whom it may concern
Report no.	October 18, 2016
Guidelines	N/A
GLP	No
Previous evaluation	New study evaluation
RMS Comment	Considered acceptable for use in risk assessment

Executive Summary

The effects (reproductive output), of flutolanil to *Daphnia magna*, were determined in a 21-day semi-static system. 24 daphnids less than 24 hours old (four replicates containing six animals each) were exposed to 0.3, 0.6, 1.3, 2.5 and 5.0 mg/l (nominal) for 21 days. Four replicates for each group were used as control and solvent control. The reproduction rate, the immobility rate and other observed effects were assessed and compared with corresponding parameters recorded in the untreated groups.

Analysis of the test solutions (fresh and old) for the active substance flutolanil was performed in samples taken at the start and end of the test, as well as at sample points during the test solution renewal. The determination of the content of flutolanil in the test solution showed a mean recovery of 83% in the fresh samples and 87% in the old samples. The measured values did not remain in the range of ±20% of the nominal, therefore, the evaluation of effect should have been based on the geometric mean of the measured concentrations.

8.3%, 0%, 0%, 0%, 4.2%, 0% and 29.2% adult immobility was observed at control, solvent control, 0.3, 0.6, 1.3, 2.5 and 5.0 mg/l (nominal), respectively after 21 days. The reproductive output (mean number of offspring/per parent animal which not inadvertently or accidentally died during the test) was 14.95±0.31, 14.98±0.38, 14.68±0.37, 14.25±0.72, 14.08±0.53, 13.98±0.77 and 5.83±0.57 for the control, solvent control, 0.3, 0.6, 1.3, 2.5 and 5.0 mg/l (nominal) treatment groups, respectively.

In a 21-day reproduction test on *Daphnia magna*, the toxicity endpoints were based on mean measured concentrations. Based on young per reproductive day, $EC_{10, 20, 50}$ (with 95% confidence limits) were determined to be 2.2 mg a.s./L (1.8 – 2.6), 2.6 mg a.s./L (2.3 – 3.0) and 3.6 mg a.s./L (3.5 – 3.7), respectively; the NOEC was determined to be 0.53 mg a.s./L, and the MATC was estimated to be 0.76 mg a.s./L. Based on the adult survival, the mean measured 21-d EC_{50} value for flutolanil was estimated to be greater than 4.0 mg a.s./L, the EC_{10} and EC_{20} values could not be determined, and the NOEC was determined to be 2.0 mg/L. Based on time to first brood, the $EC_{10, 20, 50}$ (with 95% confidence limits) were determined to be 2.8 mg a.s./L (2.6 – 3.0), 3.6 mg a.s./L (3.5 – 3.7) and > 4.0 mg a.s./L, respectively. Based on length, the mean measured NOEC was estimated to be 1.1 mg a.s./L. The most sensitive endpoint was determined to be young per reproductive day, with NOEC, LOEC and EC_{10} values estimated to be 0.53, 1.1 and 2.2 mg a.s./L, respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test material:**	Flutolanil Technical
	Batch no.:	81116
	Purity:	98.9%
2.	**Test organism:**	*Daphnia magna*
	Age:	< 24 hours old
	Source:	ABC in-house daphnid culture (originally obtained from Pennsylvania State University in 1954)
	Feeding:	The animals were fed with algae (*S. capricornutum*) or an algae suspension of (*S. capricornutum* and *A. falcatus*) at least twice daily to supply 2 x 10^8 cells/L
3.	**Treatment:**	Control, Solvent control (DMF), 0.3, 0.6, 1.3, 2.5 and 5.0 mg/L (nominal)
	# replicates:	4/treatment and controls
4.	**Test vessels:**	1L Glass vessels (400 mL)
	Loading:	*ca*. 66mL of media per *Daphnia*
	Test water:	Hard blended water using reverse osmosis water (Hardness: 156 - 178 mg/L as $CaCO_3$)

5. Environmental conditions

Temperature:	20 – 22°C
pH:	7.9 – 8.4
Dissolved oxygen:	5.7 – 8.6 (mg/L)
Photoperiod:	16 h light : 8 h darkness (*ca*. 550-610 lux)

B. STUDY DESIGN AND METHODS

1. In-life phase: Aug 03, 1990 to Aug 24, 1990

2. Test organism assignment and treatment

First-instar daphnids aged less than 24 hours at the start of the test were exposed at five concentrations of the test item for a period of 21 days under semi-static conditions. For each of the treated and untreated groups 24 daphnids were used, maintained in groups of four, six per test vessel. The daphnids were fed with concentrated algae suspension (*Selenastrum capricornutum* and *Ankistrodesmus falcatus*), at least twice per day, to supply 8.0 x 10^7 cells/0.4 L.

3. Dose preparation

A working standard of flutolanil technical was prepared at a concentration of 50 mg/mL in DMF. The solution was stored in the freezer and was used to prepare individually the test concentrations at each renewal. Solvent control solutions were prepared at a ratio of 0.2 mL of DMF to 2 L of dilution water.

The renewal of the test solutions was performed 3 times a week. The test vessels position was modified at each test solutions renewal.

4. Measurements and observations

Survival, abnormal effects and observance of first brood of the daphnids were recorded daily throughout the study. The living offspring produced in each vessel were counted and removed at each test solutions renewal; with the same frequency, also the immobility of parent animals were recorded. The total number of living offspring per parent animal was calculated for each test vessel (replicate). The other evaluated parameters were the time of production of the first brood, the measurement of the length of the parent animals (body length excluding the anal spine) at the end of the test and the intrinsic rate of population increase (measure of population growth which integrates reproductive output and age specific mortality). Dissolved oxygen concentration and pH values were measured once a week, in fresh and old media, in the controls and the test groups.

5. Statistics

Reproduction data were analysed between the control and solvent control by a t-test. Adult daphnid length data were assessed by a one-way analysis of variance (ANOVA) procedure. A Dunnett's one-tailed multiple means comparison was used to determine those exposure levels exhibiting responses significantly different than that of a control.

II. RESULTS AND DISCUSSION

Deviations

Minor guideline deviations were found but had no significant effect on the validity of the study, therefore they are considered to be acceptable:

- mean number of living offspring produced per parent animal surviving at the end of the test cannot be calculated due to insufficient data
- *Daphnia* was not individually housed according to OECD 211
- lux intensity was not adequate (OECD requirements are 1000-1500 lux)
- - Neonates were not counted daily no observations of presence of aborted eggs
- - Diet supplied should have been defined as mg C/*Daphnia*/day

A. Biological data

The evaluated parameters were the number of living offspring produced by each group of six parent animals, the immobility of parent animals, the time to production of first brood, the length of the parent animals at the end of the test. In order to attempt EC_{10} and EC_{20} calculation, data were re-analysed using the Probit method for the survival endpoint and the weighted logistic regression method for length, young per reproductive days and time to first brood endpoints.

Adult survival and length, total offspring produced in each group

Nominal Concentration (mg/l)	Mean measured Concentration* (mg/l)	Adult observation		Reproduction parameters	
		Survival (%)	Length (mm)	Young/Repro-duction days	Time to first Brood (days)
Control	-	92	4.52	14.95	6.0
Solvent control	-	100	4.53	14.98	6.0
Pooled (Control & Solvent control)		96	4.52	14.96	6.0
0.30	0.29	100	4.49	14.68	6.0
0.60	0.53	100	4.51	14.25	6.0
1.3	1.1	96	4.45	14.08**	6.0
2.5	2.0	100	4.40**	13.98**	6.5**
5.0	4.0	71**	4.03**	5.83**	10.0**

*: mean of measured concentrations at 0, 7 and 14 days for fresh test water and at 7, 14 and 21 days for old test water
**: statistically significant difference ($p < 0.05$) from pooled control

B. Analytical verification

The determination of the content of flutolanil in the test solution showed a mean recovery of 83% in the fresh samples and 87% in the old samples. The analytical results and the range of nominal achieved are reported in the following table.

Measured concentrations of flutolanil during the test

Nominal Concentration (µg/mL)	Mean Measured (µg/mL) ± Standard deviation (µg/mL)	Mean % Nominal (min-max)
0.30	0.29 ± 0.04	82-112
0.60	0.53 ± 0.07	78-106
1.3	1.1 ± 0.08	74-88
2.5	2.0 ± 0.2	68-94
5.0	4.0 ± 0.3	74-88

C. Toxicity endpoints

The 21-day chronic toxicity, reproductive and developmental endpoints for flutolanil to *Daphnia magna* are presented in the following table.

Toxicity endpoints for the test item flutolanil technical

Endpoint	21-days Effect concentration (mean measured)	mg a.s./L
Young per reproductive day (considering all parental organisms)	EC_{10} (95% CI)	2.2 (1.8 – 2.6)
	EC_{20} (95% CI)	2.6 (2.3 – 3.0)
	EC_{50} (95% CI)	3.6 (3.5 – 3.7)
	NOEC	0.53
	LOEC	1.1
	MATC	0.76
21-day survival	EC_{10}	ND
	EC_{20}	ND
	EC_{50}	> 4.0
	NOEC	2.0
	LOEC	-
Time to first brood	EC_{10}	2.8 (2.6 – 3.0)
	EC_{20}	3.6 (3.5 – 3.7)
	EC_{50}	> 4.0
	NOEC	1.1
	LOEC	-
Length	EC10	3.8 (3.6-3.9)
	EC20	> 4.0
	EC50	> 4.0
	NOEC	1.1
	LOEC	-
Most sensitive endpoint: Young per Reproductive day	NOEC	0.53
	LOEC	1.1
	EC_{10} (95% CI)	2.2 (1.8 – 2.6)

III. CONCLUSION

In a 21-day reproduction test on *Daphnia magna*, the toxicity endpoints were based on mean measured concentrations. Based on young per reproductive day, $EC_{10,\ 20,\ 50}$ (with 95% confidence limits) were determined to be 2.2 mg a.s./L (1.8 – 2.6), 2.6 mg a.s./L (2.3 – 3.0) and 3.6 mg a.s./L (3.5 – 3.7), respectively; the NOEC was determined to be 0.53 mg a.s./L, and the MATC was estimated to be 0.76 mg a.s./L. Based on the adult survival, the mean measured 21-d EC_{50} value for flutolanil was estimated to be greater than 4.0 mg a.s./L, the EC_{10} and EC_{20} values could not be determined, and the

NOEC was determined to be 2.0 mg/L. Based on time to first brood, the $EC_{10,\ 20,\ 50}$ (with 95% confidence limits) were determined to be 2.8 mg a.s./L (2.6 – 3.0), 3.6 mg a.s./L (3.5 – 3.7) and > 4.0 mg a.s./L, respectively. Based on length, the $EC_{10,\ 20,\ 50}$ (with 95% CI) were 3.8 mg a.s./L (3.6-3.9), > 4.0 mg a.s./L and > 4.0 mg a.s./L, respectively; mean measured NOEC was estimated to be 1.1 mg a.s./L. The most sensitive endpoint was determined to be young per reproductive day, with NOEC, LOEC and EC_{10} values estimated to be 0.53, 1.1 and 2.2 mg a.s./L, respectively.

Comments by RMS

The study was conducted according to U.S. EPA 72-4. ASTM Standard E-47.01 (1981) and in general agreement with the OECD 211 (2012). Deviations from OECD 211 are the lower light conditions (550 to 614 lux) and the fact that the daphnids were not held individually but in replicates of 6 animals. Validity criteria of the OECD 211: mortality of the control parent animals was < 20% at the end of the test was met; the mean number of living offspring per animal surviving at the end of the test was ≥ 60 in the controls.

Test substance concentrations were measured in solutions of days 0, 7, 14 and 21 using a sufficiently validated method GLC-ECD (quality control fortification samples had recoveries of 95 to 101% of nominal for spikes of 0.204 to 5.1 mg/L). As the media were renewed 3 times a week, no information is available on the course of the test substance during a renewal period. Based on that, no geometric mean measured concentrations can be calculated.

However, the mean measured concentrations of the new and old solutions were comparable, as shown in the table below. It is therefore considered acceptable that the effect concentrations were expressed as arithmetic mean measured concentrations of samples taken at 0, 7 and 14 days for fresh test water and at 7, 14 and 21 days for old test water.

Table B.9.2.5.1/01_1 Nominal and measured concentrations (mg a.s./L)

Nominal (mg/L)	Measured, fresh (mg/L)				Measured, old (mg/L)			
	day 0	day 7	day 14	mean	day 7	day 14	day 21	mean
0.3	0.31	0.30	0.27	0.29	0.31	0.34	0.25	0.30
0.6	0.50	0.55	0.50	0.52	0.64	0.47	0.50	0.53
1.3	0.96	1.0	1.0	0.99	1.1	1.2	1.0	1.1
2.5	1.7	2.0	2.0	1.9	2.4	2.1	1.9	2.1
5	4.1	3.8	4.0	4.0	4.4	4.2	3.7	4.1
% of nominal								
0.3	103	100	90	98	102	112	82	98
0.6	83	92	83	86	106	78	83	89
1.3	74	77	77	76	85	88	80	84
2.5	68	80	80	76	94	84	74	84
5	82	76	80	79	88	83	74	82

The applicant derived ECx values for the data of time to first brood. The statistical analysis of these data should not be performed without transformation. These ECx values are thus not acceptable. However, as there was no effect on time to first brood at 1.1 mg a.s./L, the EC_{10} is expected to be higher than the overall NOEC of the study.

Reproduction was expressed as young/reproductive days in the summary of the applicant. According the OECD 211, the reproductive output should be expressed as the total number of living offspring produced per parent animal present at the beginning of the test in case of test substance related mortality during the test, or when more than one parental daphnid is contained in each replicate. This was the case in the current study, and therefore RMS recalculated the results based on reproduction expressed as number of offspring per introduced parent, using TOXRAT v 3.2. The results are shown in the table below.

Table B.9.2.5.1/01_02 Reproduction expressed as offspring/introduced parent

Mean measured conc. (mg/L)	**Mean young/initial female**	**% reduction of reproduction**
Pooled controls	237.3	---
0.29	234.7	1.1
0.53	227.9	4.0*
1.1	224.8	5.3*
2.0	215.9	9.0*
4.0	60.5	74.5*
NOEC = 0.29 EC_{10} = 2.03 (95% CI 1.35-2.45) EC_{20} = 2.37 (95% CI 1.74-2.75) EC_{50} = 3.18 (95% CI 2.73-3.58)		

*statistically significant difference ($p<0.05$)

Reliability of endpoints

To assess the reliability of the estimated EC_x values, two approaches are described in EFSA Supporting publication 2015:EN-924:

- Normalised width of the confidence interval (NW = (upper limit – lower limit) / median estimate); rating of the NW ranges from excellent (<0.2) to bad (>2)
- Relationship between EC_{10} and EC_{20}/EC_{50} confidence intervals: the best case (high certainty of protection level) is achieved when EC_{10} is lower than the lower limit of the EC_{20}; the worst case (low certainty of protection level) occurs when the median EC_{10} is greater than the lower confidence limit for the EC_{50}.

Based on these rules, the EC_{10} value had a good reliability based on the normalized width of CI and a medium certainty on the level of protection. However, the EC_{10} was higher than the NOEC and thus the NOEC should be used in the risk assessment.

According to Regulation (EU) No 283/2013, the statistical power of the NOEC shall be assessed. However, this is not possible as long as default type I and II errors have not been fixed, default variance for any response variable have not been established and typical detectable effect sizes of each parameter have not been calculated (EFSA Supporting publication 2015:EN-924). The NOEC was based on a slight but statistically significant reduction of young per initial female at 0.53 mg a.s./L and set at the next lower level. This is considered a conservative approach, which is sufficiently protective.

The study is considered acceptable. The overall NOEC was 0.29 mg a.s./L may be used for risk assessment.

Blakemore G.C., Burgess, D.	1991	21-Day chronic static renewal toxicity of flutolanil to *Daphnia magna*	Report no. 38721 (W-3017)
Reliability			
General information			
Is a guideline method or modified guideline used?*		yes	
Is the test performed under GLP conditions?*		Yes	
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?		Yes	
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?		Yes	
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?		Yes	
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?		Yes	
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?		Not applicable	

Test organism	
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	Yes
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	Yes
Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Yes
Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	Yes
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is	Yes

the response statistically significant?	
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Yes
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the	Not applicable

framework for which the study is evaluated?	
Concluding weight of evidence/proposed action	Fully acceptable
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable
Consideration/concluding score	Fully acceptable

B.9.2.5.2 Reproductive and developmental toxicity to additional aquatic invertebrate species

Since the active substance does not have insecticidal properties, a reproductive toxicity test with an additional aquatic invertebrate species is not required.

The study presented below assessing the long-term toxicity of flutolanil to *Mysidopsis bahia,* was available for the flutolanil registration in the United States and therefore is submitted for completeness.

Report	CA 8.2.5.2-01 Kowalski, P.L., Boeri, R.L., Ward, T.J., 1995
Title	Life-cycle Toxicity of Flutolanil to Mysid, *Mysidopsis bahia*
Report no.	481-NI (W-3029)
Guidelines	U.S. EPA Pesticide Assessment Guideline Subdivision E, 72-4(c)
GLP	Yes
Previous evaluation	New study
RMS Comment	Considered acceptable for use in risk assessment

Executive Summary

The life-cycle toxicity of Flutolanil to the mysid, *Mysidopsis bahia*, was assessed under flow-through, for 28 days and under unaerated conditions with five concentrations of test substance, a dilution water control, and a solvent control at a mean temperature of 24.8°C. Nominal concentrations of the active substance were: 0 µg a.s./L (control and solvent control), 6.0, 12, 25, 50, and 100 µg a.s./L (Mean measured concentrations: none detected in control and solvent control, 4.23, 11.3, 21.7, 42.8, and 89.7 µg a.s./L respectively) and mean measured values were used to provide the calculations of the no observed effect levels (NOELs), lowest observed effect levels (LOELs), and maximum acceptable toxicant concentrations (MATCs).

Exposure of juvenile mysids to Flutolanil resulted in a lowest observed effect level (LOEL) of 21.7 µg a.s./L, a no observed effect level (NOEL) of 11.3 µg a.s./L, and a maximum acceptable toxicant concentration (MATC) of 15.7 µg a.s./L, when treatment data are compared to pooled control and solvent control data.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Flutolanil
Batch no.: 01104
Purity: 98.9%

2. Dilution saltwater: filtered natural seawater collected at Marblehead, Massachusetts
Salinity: 16‰; Temperature: 24.1-25.6°C;
D.O.: 7.4; pH: 8.0

3. Test concentrations: Nominal: Control, Solvent Control (DMF), 6.0, 12, 25, 50, and 100 µg a.s./L
Mean measured: Control, Solvent Control, 4.23, 11.3, 21.7, 42.8, and 89.7 µg a.s/L

Test vessels: From day 0 - 18 the retention chambers consisted of 9 cm diameter glass petri dishes to which a 12 cm high collar of Nitex® screen was attached by silicone adhesive. On day 18 ten pairs of mysids were transferred to individual retention chambers which consisted of glass petri dishes to which a collar of Nitex® screen was attached by silicone adhesive.

4. Test organism: *Mysidopsis bahia*
Age: Juveniles less than 24 hours old
Source: Aquatic Research organisms, Hampton, New Hampshire
Feed: live brine shrimp, *Artemia salina* (2-3 times daily during the test)

5. Environmental conditions:
Temperature: 23.9 to 25.7 °C
pH: 7.8 – 8.3
Dissolved oxygen: 6.0 to 8.2 mg/L (always at least 78%)
Photoperiod: 16 hour light : 8 hour dark cool-white fluorescent lights (25 foot candles)

B. STUDY DESIGN AND METHODS

1. In-life dates: 08 September - 06 October 1994

2. Animal assignment and treatment

Sixty mysids were indiscriminately and equally distributed to two replicate test vessels per concentration. Within each test vessel the 30 mysids were evenly subdivided into 2 retention chambers. When the sex of mysids could be determined (after 18 days of exposure) mysids within each vessel were rearranged so that a single male and female pair was placed in each of 10 chambers. Extra, unpaired mysids were sexually differentiated and placed in chambers 11 and 12.

The test was performed in loosely covered 20L glass aquaria that contained up to 8L of test solution. The test vessels were equipped with self-starting siphons to insure adequate flow of test media to the mysids (test media depth ranged from 4 - 10 cm). Test vessels were randomly arranged in a water bath during the 28-day test (a random numbers table was used to select the location of each vessel).

3. Dose preparation

An initial stock solution was prepared at a nominal concentration of 10,000 mg/L by combining 5.056 g of test substance and dimethylformamide and adjusting the final volume to 500 ml. Appropriate amounts of this stock solution were added directly to dilution water by the toxicant injector of a proportional diluter and this diluter toxicant solution was mixed by a high shear pump. The solvent concentration was 0. 01 ml/L dimethylformamide (DMF) in the solvent control. The test substance was supplied to the test vessels under flow through conditions by an intermittent flow proportional diluter.

4. Measurements and observations

The number of surviving organisms and the occurrence of effects on behaviour or appearance (loss of equilibrium, erratic swimming, loss of reflex, excitability, discoloration, or change in behaviour) were determined visually and recorded initially and at 24 hour intervals. Dead test organisms were removed when first observed. On day 18 of the exposure the mysids were categorized by sex. Female mysids were defined as those mysids with visible brood pouches. Offspring were counted and removed every 1-3 days after day 18. At the termination of the test the total length of each surviving first generation mysid was determined. Mysids were blotted on paper towel and weighed. After length and wet weight determination, the mysids were placed in an oven adjusted to approximately 60°C for 72 hours, transferred to a desiccator at room temperature for 10 days, and weighed.

Dissolved oxygen, pH, salinity and temperature were recorded daily in each replicate test vessel and temperature was recorded continuously during the study in one of the replicates. Additionally, analytical determination of the test material concentration was performed on days 0, 7, 14, 21 and 28.

5. Statistics

Results of the toxicity test were interpreted by standard statistical techniques, when warranted using TOXSTAT version 3.3. Shapiro-Wilk's test was used to determine if data was normally distributed, and Bartlett's test was used to determine if variances were homogeneous. If variances were homogeneous, a parametric one-way analysis of variance (ANOVA) and Bonferroni’s test was used to compare treatment and control means (survival, reproduction, and dry weight data). If variances were heteroscedastic a nonparametric ANOVA was used to compare control and treatment means (wet weight and total length data). Control and solvent control data were compared using a "t" test and since there were no statistical significance found, the data of both controls were pooled.

II. RESULTS AND DISCUSSION

Deviatons

An analytical or sampling error has resulted to an outlier value and this has been reported, but excluded from the final calculations.

A. Biological data

No statistically significant differences were seen in survival, reproduction, length, or weight between the control and solvent control. Control and solvent control survival at the end of the test was at least 86.7%. Offspring production averaged 3.9 young per female in the control and 4.5 young per female in the solvent control. Sub lethal effects were never noted in either control during the test. Four mysids exposed to 89.7 µg/L exhibited erratic swimming on day 6 of the exposure. These effects were not observed at any other time during the test. No other sub lethal effects were observed at any time during the test.

The most sensitive measures of toxicity determined by statistical analysis of survival, growth, and reproduction data were the survival of first generation mysids after 28 days of exposure, the production of young by first generation females, and the dry weight of surviving first generation mysids. Results are presented in the table below.

Summary of mean survival, reproduction, length, and weight data from the toxicity test with mysids, *Mysidopsis bahia*, and Flutolanil

	Mean values				
Mean Measured Concentration (µg/L)	**Percent survival at Day 28**	**Production of Young/Female by Day 28**	**Total Length (mm)**	**Weight (mg)**	
				Wet	Dry
Control	88.4	3.9	6.7	3.2	0.56
Solvent Control	86.7	4.5	6.7	3.2	0.57
4.23	83.4	4.7	6.7	3.3	0.59
11.3	73.4	3.9	6.6	2.6	0.55
21.7	51.7	1.3	6.4	2.5	0.47
42.8	26.7	0.2	6.3	2.4	0.44
89.7	6.7	0.0	5.6	1.6	0.34

* significantly different from the pooled control and solvent control at the 95% confidence level. survival data were arc sine square root transformed prior to analysis. Bonferroni•s t test was performed on survival, reproduction, and dry weight data. Kruskal-Wallis• test was performed on total length and wet weight data.

[1]Young production is calculated as the total number of young divided by the average number of surviving females

B. Analytical verification

Mean measured concentrations of test substance ranged from 71% to 94% of the nominal concentrations after correction for mean % recovery in the appropriate matrix spike samples (day 0 = 104%, day 7 = 94.4%, day 14 = 122%, day 21 = 106% , day 28 = 89.6%). The results are presented in the table below.

Measured concentrations of flutolanil during the test (µg a.s./L)

Nominal Test concentration (µg a.s./L)	Day 0	Day 7	Day 14	Day 21	Day 28	Mean	Mean of Nominal (%)
Control	nd	nd	nd	nd	nd	-	-
Solvent control	nd	nd	nd	nd	nd	-	-
6.0	3.97	5.07	3.57	4.38	4.58	4.31	72%
12	16	11.1	8.61	10.4	10.5	11.3	94%
25	28.7	21.5	16.6	21.5	20.5	21.8	87%
50	47.1	47.8	37.8	41.5	41	43.0	86%
100	569*	97.5	81.8	90.9	88.5	89.7	90%

* outlier excluded from mean calculations

nd: not determined

C. Toxicity endpoints

The 28-day chronic toxicity, reproductive and developmental endpoints for flutolanil to *Mysidopsis bahia* are presented in the following table.

Toxicity endpoints for the test item flutolanil technical

Endpoint	NOEC (µg a.s./L)	LOEC (µg a.s./L)	MATC (µg a.s./L)
Survival of 1st Generation Mysids	11.3	21.7	15.7
Number of Young per Female	11.3	21.7	15.7
Total Length of 1st Generation Mysids	89.7	>89.7	>89.7
Wet weight of 1st Generation Mysids	89.7	>89.7	>89.7
Dry weight of 1st Generation Mysids	11.3	21.7	15.7

III. CONCLUSION

Exposure of juvenile mysids to Flutolanil resulted in a lowest observed effect level (LOEL) of 21.7 µg a.s./L, a no observed effect level (NOEL) of 11.3 µg a.s./L, and a maximum acceptable toxicant concentration (MATC) of 15.7 µg a.s./L, when treatment data are compared to pooled control and solvent control data.

Comments by RMS

The study was conducted according to US EPA Guideline Subdivision E, 72-4(c) and in absence of an OECD Guideline for this species, it was in agreement with OPPTS 850.1350. No deviations were observed.

Mean measured concentrations of test substance were corrected for mean % recovery in the appropriate matrix spike samples (day 0 = 104%, day 7 = 94.4%, day 14 = 122%, day 21 = 106%, day 28 = 89.6%). This correction is not appropriate, but the corrections are relatively small, except for day 14, which however lead to lower concentrations, representing a worst-case situation.

The report stated: “the sample collected from test vessels with a nominal concentration of 100 µg/L on day 0 had a measured value of 569 µg/L. All other samples collected on day 0 resulted in measured values that were within the expected range of concentrations. Both the primary and secondary stock solutions used by the proportional diluter to formulate the test concentrations had analytical values within the expected ranges. These factors combined with the lack of early acute toxicity in the highest tested concentration lead to the conclusion that the high analytical value for this sample resulted from either sampling or analytical error. The value was therefore treated as an outlier and reported but not used in the calculation of the mean measured value for this concentration”. This is acceptable.

Therefore, the reported mean measured concentrations are accepted. The study is considered acceptable as the validity criterion was met: in the controls, the average number of young produced per female was > 3 (3.9 for control and 4.5 for solvent control).

Reliability of endpoints

L(E)Cx values were not stated in the report and were not derived by the applicant.
RMS estimated L(E)Cx values for growth (dry weight), production of young/surviving female and survival using TOXRAT v3.2 in comparison with the pooled controls.

	$L(E)C_{10}$ (95% CI) (µg a.s./L)	$L(E)C_{20}$ (95% CI) (µg a.s./L)	$L(E)C_{50}$ (95% CI) (µg a.s./L)
Survival	3.97 (2.41-5.60)	6.85 (4.72-8.96)	19.5 (15.8-23.8)
Production young/female	11.7 (10.1-12.9)	13.6 (12.2-14.7)	18.2 (17.2-19.1)
Growth (dry weight)	16.5 (6.3-25.2)	32.1 (19.2-43.0)	115 (81.2-237)

As the median LC_{10} (for survival) is lower than the NOEC (11.3 µg a.s./L), its reliability was assessed by evaluating the width of the confidence interval around the median value and the certainty on the level of protection offered by the median LC_{10} (EFSA Supporting publication 2015:EN-924). The rating

based on the normalized width was fair and the LC_{10} gave a high certainty of the level of protection. Therefore, the LC_{10} is concluded to be reliable.

The overall value to be used in the risk assessment is the LC_{10} of 3.97 µg/L (95% CI 2.41-5.60 µg/L).

Boeri R.L. *et al*	1995	Life-cycle toxicity of flutolanil to mysid, *Mysidopsis bahia*	Report no 481-NI (W-3029)
Reliability			
General information			
Is a guideline method or modified guideline used?*		Yes	
Is the test performed under GLP conditions?*		Yes	
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?		Yes	
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?		Yes	
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?		Yes	
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?		Yes	
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?		Not applicable	
Test organism			
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?		Yes	
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?		Yes	

Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Yes
Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	Yes
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	Yes
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and	Yes

relevant for the substance being assessed?	
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Yes
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable
Concluding weight of evidence/proposed action	Fully acceptable
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable
Consideration/concluding score	Fully acceptable

B.9.2.5.3 Development and emergence in *Chironomus riparius*

Report	CA 8.2.5.3-01. Desmares-Koopmans, D., 2003
Title	Sediment-water Chironomid toxicity test using water spiked with Flutolanil
Report no.	335431 (N-3025)
Guidelines	Proposal of OECD 219 (2001)
GLP	Yes
Previous evaluation	DAR (2006)
RMS Comment	The applicant submitted a new, more extensive summary. Considered acceptable for use in risk assessment

Executive Summary

The effects of prolonged exposure (28 days) to flutolanil on the development of sediment-water dwelling larvae of *Chironomus riparius* in a water-sediment system was investigated. The test was performed with six replicates per group, each containing twenty larvae per vessel and exposed to a control group, a solvent (acetone) control group and one test level of 1.0 mg/L (nominal) (Limit test). The number of emergence was assessed during the 28-day period. Analysis of the test solutions for flutolanil was performed in samples taken at 0, 7 and 28 Days and for overlaying water, sediment and pore water. The determination of the content of flutolanil in the test solution showed a mass balance of 104% at the start of the test, which decreased to 73% on Day 7 and 52% at the end of the test.

In all levels the first emergence occurred on day 14. Some chironomids died after emergence and four dead larvae were found (three in the blank-control and one in the solvent-control). These were not considered to be significant and therefore, no substance-related effects on the life cycle of midges were observed. Furthermore, no egg packets were observed during this test in any of the vessels and no behaviour difference of the larvae compared to the controls was recorded.

The overall NOEC for flutolanil was 1.0 mg/L (nominal) and the overall EC_{50} was greater than 1.0 mg/L (nominal). Since this study has been carried out as a limit test, EC_{10} and EC_{20} could not be determined.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** Flutolanil
Batch no.: 2AE0008P
Purity: 98.5%
Stability: Stable when stored in the fridge and protected from light

2. **Test organism:** Freshwater chironomid: *Chironomus riparius*
Age: first larval stage (2 to 3 days old, i.e. 2 to 3 days after hatching)
Source: NOTOX B.V. Laboratory culture

Feeding: The animals were fed with Trouvit: daily, from Day 1 to 27

3. Treatment: Control, Solvent control (acetone) 1.0 mg/L (nominal)
(Range finding test) study) where 20 larvae per concentration were exposed to 0.01, 0.1 and 1.0 mg a.s./L.

 # replicates: 6

 # organisms/replicate: 20

4. Test vessels: 600 mL Glass vessels (*ca*. 8 cm)

Test water: ISO-medium

Sediment: Artificial soil substratum as described in OECD guideline 207

5. Environmental conditions

Temperature: 19.4 – 21.3°C

pH: 5.5 – 8.1

Dissolved oxygen: ≥ 5.0 mg/L

Photoperiod: 16 h light : 8 h darkness (675-760 lux)

B. STUDY DESIGN AND METHODS

1. In-life phase: Jan 17, 2003 to 09 April, 2003

2. Test organism assignment and treatment

Twenty larvae at the first larval stage were randomly allocated to each vessel in groups of ten. The chironomids were fed daily with the appropriate volume of Trouvit to supply the appropriate volume of feed for each larvae at every stage.

3. Dose preparation

A total of twenty five test vessels were prepared by adding a layer of *ca*. 1.5 cm of formulated sediment (mean weight 81.65 ± 0.41g, n=25). Approximately 6 cm of ISO-medium (mean weight 275.44 ± 3.72g, n=25) was added to the sediment with minimal disturbance. The height ratio sediment:overlying water was 1:4. The vessels were aerated gently and left for one week under test conditions. The volume of the vessels was checked for evaporation three times a week and if necessary milli-RO water was added.

To achieve a test concentration of 1.0 mg/L in the water layer of the test systems, spiking solutions in acetone were prepared at a nominal concentration of 10 g/L (=10 mg/mL). No special treatment other than careful mixing was necessary to completely dissolve the test substance in acetone. The test substance was added to the water column, one day after adding the larvae, in a small volume of the stock solution (27 µL) using a pipette. The water was mixed gently with the aeration pipette of the vessel, without disturbing the sediment.

4. Measurements and observations

Visual assessments of behavioural differences of the larvae and emerging chironomids were recorded at least three times a week throughout the study. Emerged male and female midges were counted daily during the period of expected emergence (normally starting between day 13 to 16), per vessel. After identification the midges were removed from the vessels. During the period of expected emergence, the number of visible pupae that failed to emerge were counted every day and per vessel. During the period of expected emergence, vessels were checked daily for egg packets. After observation, dead larvae, pupae or midges were removed and recorded. No sediment was sieved at the end of the test.

Oxygen concentration, temperature and pH values were measured three times a week, in the vessels in the controls and the test group. Aeration was checked daily.

5. Statistics

Since the emergence and development rates in the treated vessels were higher than in the controls no statistical analysis was performed. Since this study has been carried out as a limit test, EC_{10} and EC_{20} could not be determined.

II. RESULTS AND DISCUSSION

Deviations

Minor deviations occurred in the test.

- Aeration was stopped 2 hours and 43 minutes before the addition of the larvae
- pH values and oxygen values varied from the protocol
- No measurements performed on Day 7 for the reserve vessel of the control as this vessel did not contain test organisms, therefore no measurements were performed
- The vessel for analysis of the blank control on day 28 was also used for observations

Regarding the test validity, and according to the study director, these deviations had no effect on the outcome of the study.

A. Biological data

In the controls and at the highest test group tested 1.0 mg/L (nominal) the first midges emerged on Day 14. Except for some midges who had died after emergence and four dead larvae (three in the blank-control and one in the solvent-control) no effects on the life cycle of midges were observed. Furthermore, no egg packets were observed during this test in any of the vessels and no behaviour difference of the larvae compared to the controls was recorded.

Cumulative number of emergence and mortality in each group

Nominal concentration	Day-28 cumulative emerged			Day-28 cumulative mortality			Mean Emergence rate per vessel*	Mean development (Days)	Mean development rate (1/Day)
	Males	Females	Total	Males	Females	Total			
Control	50	39	89	6	7	13	0.74	17.8	0.056
Solvent control	44	46	90	5	3	8	0.75	17.7	0.057
1.0 mg/L	46	47	93	5	6	11	0.78	16.4	0.061

* 20 larvae per vessel, six vessels per group

B. Analytical verification

The distribution of flutolanil was determined with HPLC. Samples for analysis of the overlying water, sediment and pore water were taken from the blank-control and 1.0 mg/L. Samples were taken at the start of the test (day 0, 15-25 minutes after dosing), on day 7 and at the end of the test (day 28).

Procedural recoveries for ISO-medium, overlying water and sediment were all between 70 and 110% and therefore considered acceptable. Procedural recoveries for pore-water at 0.981 mg/L were between 70 and 110% and therefore considered acceptable. At nominal 0.00981 mg/L however, recoveries were below 70%. These lower recoveries might be due to storage in a deepfreeze but also to a spiking error. Because recovery was not acceptable over the concentration range tested, results for pore water have to be considered indicative. However, due to the relative low contribution of the pore water to the total mass balance this had no effect on the test.

Measured concentrations of flutolanil during the test

	Measured concentration (mg/L or mg/kg)			Recovery (%)*		
	Day 0	Day 7	Day 28	Day 0	Day 7	Day 28
Overlaying water	1	0.579	0.155	102	60	16
Sediment	0.0701	0.415	1.19	2	13	36
Pore water	0.0887	0.205	0.659	0.27	0.44	0.17
Mass balance	**1.16**	**1.20**	**2.00**	**104**	**73**	**52**

* Based on the absolute amount spiked to the system (0.269 mg active ingredient).

C. Toxicity endpoints

In a 28-day prolonged exposure test on the development of sediment-water dwelling larvae of *Chironomus riparius* in a water-sediment system the toxicity endpoints determined for flutolanil are shown below:

Effect concentration	**Nominal concentration**
NOEC for emergence rate	1.0 mg/L
NOEC for development rate/time	1.0 mg/L
Overall NOEC	1.0 mg/L
28-day EC_{50} for emergence rate	> 1.0 mg/L
28-day EC_{50} for development rate	> 1.0 mg/L
Overall EC_{50}	> 1.0 mg/L

In the controls and at 1.0 mg of flutolanil per litre, the first midges emerged on day 14. Except for some midges who had died after emergence and four dead larvae (3 in the control and 1 in the solvent control) no effects on the life cycle of midges were observed. Furthermore, no egg packets were observed during this test in any of the vessels and no behaviour difference of the larvae compared to the controls was recorded.

The No Observed Effect Concentration (NOEC) for emergence of midges and for development rate/time was 1.0 mg/L and the EC_{50} was above 1.0 mg/L.

III. CONCLUSION

Based on the nominal concentration of flutolanil, the NOEC for *Chironomus riparius* was 1.0 mg/L and the EC_{50} was greater than 1.0 mg/L. Since this study has been carried out as a limit test, EC_{10} and EC_{20} could not be determined.

Comments by RMS

The study was conducted according to the draft OECD 219 and there were no major deviations to the OECD 219 that would invalidate the study.

The validity criteria were met: emergence at the end of the test was 74% and 75% in the blank control and solvent control, respectively (and thus > 70%), the majority of emergence to adults occurred from day 14 to day 24 and the experimental conditions were acceptable.
The OECD 219 states that effect concentrations are preferably expressed as measured concentrations in the overlaying water at the beginning of the test. As measured concentration was 102 % of nominal at the start of the test and the procedural recoveries were acceptable for the overlying water, the nominal value was used as effect concentrations. This is acceptable. The NOEC of 1 mg/L may be used as value in the risk assessment.

Desmares-Koopmans, D.,	2003	Sediment-water Chironomid toxicity test using water spiked with Flutolanil	Report No. 335431 (N-3025)
Reliability			
General information			
Is a guideline method or modified guideline used?*		Yes	
Is the test performed under GLP conditions?*		Yes	
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?		Yes	
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?		Yes	
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?		Yes	
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?		Yes	
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?		Not applicable	
Test organism			
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?		Yes	
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?		Yes	
Exposure conditions			
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?		Yes	

Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Yes
Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	Yes
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	Yes
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the	Yes

species?	
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Yes
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable
Concluding weight of evidence/proposed action	Fully acceptable
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable
Consideration/concluding score	Fully acceptable

B.9.2.5.4 Sediment dwelling organisms

Please refer to CA 8.2.5.3-01.

B.9.2.6 Effects on algal growth

B.9.2.6.1 Effects on growth of green algae

Report	CA 8.2.6.1-01, Migchielsen, M.H.J., 2003
Title	Fresh water algal growth inhibition test with Flutolanil
Report no.	354904 (N-3014)
Guidelines	OECD 201 (1984)
GLP	Yes
Previous evaluation	DAR (2006)
RMS Comment	The applicant submitted a new, more extensive summary. Considered acceptable for use in risk assessment

Executive Summary

Three replicate algal suspensions (*Pseudokirchneriella subcapitata*, formerly known as *Selenastrum capricornutum*) were each exposed to nominal concentrations of 0.18, 0.32, 0.56, 1.0, 1.8 and 3.2 mg flutolanil/L for 72 hours. Six replicates without test item were used as blank-control and vehicle-control (with acetone). Observations of cell growth were recorded at 24, 48 and 72 hours to determine the potential effect on growth rate (E_rC_{50}) or cell growth inhibition (E_bC_{50}) relative to the control.

Analysis of the test solutions (fresh and spent) for the determination of the content of flutolanil was performed in samples taken at 0 and 72 hours after application. The determination of the content of flutolanil in the test solutions showed a mean recovery of 104% in the fresh samples and 103% in the spent samples. For each concentration, the measured value did not vary more than 20% from the nominal, therefore the evaluation of effect for the active substance was based on the nominal concentrations.
Growth rate inhibition was observed to increase from 0.3% at the minimum test concentration to 19.7% at the maximum concentration compared to the control after 72 hours of exposure. Cell growth inhibition was observed to increase from 0.1% at the minimum test concentration to 53.2% at the maximum concentration compared to the control.

The growth rate E_rC_{50} value for flutolanil was greater than 3.2 mg/L, i.e. above the maximum solubility of flutolanil in test medium, and the cell growth inhibition E_bC_{50} value was 0.97 mg/L (nominal). The LOE_rC and NOE_rC values for growth rate were estimated to be 0.32 mg/L and 0.18 mg/L (nominal), respectively. The LOE_bC and NOE_bC values for cell growth inhibition were estimated to be 0.32 mg/L and 0.18 mg/L (nominal), respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Flutolanil technical
Batch no.: 2AE008P
Purity: 98.5%
Description: White powder

2. Test organism: *Selenastrum capricornutum*
Strain: NIVA CHL 1
Source: In-house laboratory culture
Initial density: 10,000 cells/mL

3. Treatment: 0 (control and acetone control), 0.18, 0.32, 0.56, 1.0, 1.8 and 3.2 mg/L (nominal)

4. Test vessels: Glass flasks (100 mL)
Test water: M2-medium (according to ISO-standard "Algal growth inhibition test" Nov. 1989)
Shaking: Yes

5. Environmental conditions:

Temperature: 23.8 – 24.1°C
pH: 8.1 – 9.6
Photoperiod: Continuous lighting within the range of 4440 to 8880 lux

B. STUDY DESIGN AND METHODS

1. In-life phase: Jan 06 to Jan 30, 2003

2. Test organism assignment and treatment

The test started (0 hours) by inoculation of a biomass of 10,000 algal cells per mL test medium in each flask. These cells were taken from older growing cultures maintained in climatic rooms under breeding conditions. The test was performed with three replicates per test concentration and six replicates per control (blank and acetone). The flasks were placed on shakers.

3. Dose preparation

Preparation of test solutions started with stock solutions in acetone at a factor of 10,000 higher than the final test concentrations. Volumes of 100 µL of the respective stock solutions were added per litre of test medium to apply for the final test concentrations. A short period of ultrasonic treatment was followed by 75 to 80 minutes of magnetic stirring. All solutions were filtered through a paper filter (5 µm) to remove any undissolved test substance particles. Volumes of 50 mL were used for each treated replicate. Volumes of 50 mL of test medium without test substance (blank) and with added vehicle (acetone) were used for each untreated replicate (control).

4. Measurements and observations

The cell density in each replicate were daily assessed during the test period by microscope using a counting chamber. Thereafter, cell densities were determined by spectrophotometric measurement of samples at 720 nm using a Varian Cary 50 single beam spectrophotometer with immersion probe (path length = 20 mm). Algal medium was used as blank and extra replicates as background for the treated solutions. The pH values were measured in the untreated and treated groups at the beginning

and at the end of the test. The temperature in the climatic chamber was continuously recorded in a temperature-control vessel.

The analysis of the concentration of flutolanil was performed for each test concentration. A total of 16 samples from the inhibition test were analysed. The samples were collected from the six test item concentrations - including a no algae vessel at the highest concentration, and from the acetone control at 0 hours from fresh test solutions and at the end of the test (72 hours) from the aged solutions. Analysis was performed by HPLC.

5. Statistical analysis

The determination of the EC_x values was calculated based on linear regression analysis of the percentages of growth inhibition and the percentages of growth rate reduction versus the logarithms of the corresponding exposure concentrations of the test substance. Additionally, the data obtained were also evaluated to determine the LOEC/NOEC values, using an ANOVA, Tukey test and Bonferroni t-test procedure. The input data refers to the growth data evaluated during 72 hours of exposure at different concentrations and the statistical analysis was performed using software TOXSTAT 3.5, 1996.

II. RESULTS AND DISCUSSION

A. Biological data

Mean number of cells, growth rates and area under the growth curve (AUGC) with the corresponding percent inhibition values are presented in the following tables.

Mean number of cells (10^4 cells/mL) at each observation time

Nominal concentration (mg flutolanil/L)	Mean number of cells		
	at 24 hrs	at 48 hrs	at 72 hrs
0.00 (blank control)	4.5	28.1	94.8
0.00 (solvent control)	4.7	27.5	93.1
0.18	4.8	27.9	91.9
0.32	5.3	22.9	73.1
0.56	4.8	18.1	54.2
1.00	4.7	15.2	43.7
1.80	4.7	13.5	44.5
3.20	5.5	13.7	38.0

Growth rate, AUGC and corresponding inhibition (%) after 72 hours of exposure

Nominal concentration (mg flutolanil/L)	Growth rate 0-72 hours		AUGC	
	Mean value	% inhibition mean value	Mean value	% inhibition mean value
0.00 (solvent control)	0.06294	-	1829.80	-
0.18	0.06274	0.3	1827.12	0.1
0.32	0.05961	5.3*	1494.64	18.3*
0.56	0.05545	11.9*	1140.88	37.7*
1.00	0.05247	16.6*	942.88	48.5*
1.80	0.05270	16.3*	913.28	50.1*
3.20	0.05054	19.7*	856.60	53.2*

* Significantly different from the control (ANOVA, Tukey test and Bonferroni t-test procedure)

C. Analytical verification

The flutolanil content in the test samples showed a mean recovery of 104% in the fresh solutions and a mean recovery of 103% in the spent solutions. For each concentration, the measured value was in the range 20% from the nominal, therefore the evaluation of effect was based on the test item nominal concentrations. The analytical results are reported in the following table.

Measured concentrations of flutolanil during the test

Flutolanil nominal (mg/L)	Mean measured (mg/L) - Mean recovery (%)		Mean measured for treatment group (µg/L) *- Mean recovery for treatment group (%)
	Sampling (h)		
	0 (fresh)	72 (spent)	
0.000	Not detectable	Not detectable	-
0.177	0.178 – 101	0.185 – 105	0.182 – 103
0.315	0.325 – 103	0.331 – 105	0.328 – 104
0.552	0.593 – 107	0.591– 107	0.592 – 107
0.985	1.03 – 105	1.07 – 109	1.05 – 107
1.77	1.82 – 103	1.86 – 105	1.84 – 104
3.15	3.24 – 103	2.73 – 87	2.99 – 95
3.15†	3.35 – 106	3.37 – 107	3.36 - 107

* Mean measured concentrations for treated group are arithmetic means

† Without algae

C. Toxicity endpoints

The 72-hour toxicity endpoints of flutolanil for *Selenastrum capricornuntum* are presented in the following table.

72-h toxicity endpoints of the test item flutolanil

Effect concentration	**Growth rate [95% confidence interval] (mg flutolanil/L, nominal)**	**AUGC [95% confidence interval] (mg flutolanil/L, nominal)**
EC_{10} (95% confidence intervals)	0.49 [0.38-0.64]	0.24 [0.17-0.35]
EC_{25} (95% confidence intervals)	2.30 [1.67-3.17]	0.41 [0.28-0.58]
EC_{50} (95% confidence intervals)	> 3.20	0.97 [0.66-1.42]
NOEC	0.18	0.18
LOEC	0.32	0.32

III. CONCLUSION

In a growth inhibition test on the green alga *Selenastrum capricornutum* the 72-h EC_{50} values for flutolanil were beyond the range tested, i.e. above the maximum solubility of flutolanil in test medium for growth rate (E_rC_{50}), and 0.97 mg test item/L (nominal) for AUGC (E_bC_{50}). The growth rate LOE_rC and AUGC LOE_bC values were estimated to be 0.32 mg/L. The growth rate NOE_rC and AUGC NOE_bC values were estimated to be 0.18 mg/L.

B.9.2.6.2 Effects on growth of an additional algal species

Since flutolanil is not a herbicide, no further testing on algae species was required.

B.9.2.7 Effects on aquatic macrophytes

Since flutolanil is not a herbicide; tests on aquatic macrophytes were not required and have not been included.

B.9.2.8 Further testing on aquatic organisms

The data point is addressed by information found in the open literature. Two studies included relevant information on the effects of flutolanil on aquatic species.

Report	CA 8.2.8-01, Yang Y., Qi, S., Chen, J., Liu, Y., Teng, M., Wang, C., 2016
Title	Toxic Effects of Bromothalonil and Flutolanil on Multiple Developmental Stages in Zebrafish
Report no.	Not applicable
Guidelines	OECD 212 (1998), The experiments were performed in accordance with current Chinese legislation and were approved by the Independent Animal Ethics Committee at the China Agricultural University.
GLP	No
Previous evaluation	New study
RMS Comment	Considered as supporting information to the risk assessment

Executive Summary

In this study, various developmental stages of zebrafish were used to address the potential environmental risk and aquatic toxicity of flutolanil. Zebrafish were selected as a new vertebrate model for rapidly and economically assessing the toxicity of novel compounds, pollutants and pharmaceuticals (Alestrom *et al*. 2006; Ali *et al*. 2011; Hill *et al*. 2005)[2] considering their small size, low feed cost, short test cycle, and the ability to be easily propagated. The embryo and sac-fry stages are considered to be a perfect choice for a risk assessment of chemicals in fish, because tests on these stages reflect the development of the fish from the earliest life stages and are closer to the real environments of zebrafish. Secondly, the experimental design and protocol are simpler than those for full-cycle tests.

These results demonstrated that the acute toxicity 96-h LC_{50} of flutolanil (in mg a.s./L) were 5.47 (embryo), 4.09 (72 h old larvae), 3.91 (12 h old larvae) and 2.70 mg/L (adult). Sub lethal effects induced by flutolanil on zebrafish embryos were noted, including growth inhibition, abnormal spontaneous movement, slower heart rate, complete hatching failure, and morphological deformities. In addition, flutolanil could cause notochord deformation and short body length of larvae. This study provides a foundation for future investigation into the mechanism of flutolanil toxicity in zebrafish.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** Flutolanil
Lot no.: Not reported
Purity: 98.7%
CAS number: 66332-96-5
Source: Taizhou Baili Chemical Co., Ltd.

2. **Test organism:** Zebrafish

[2] Alestrom P, Holter JL, Nourizadeh-Lillabadi R (2006) Zebrafish in functional genomics and aquatic biomedicine. Trends Biotech 24(1):15–21;
Ali S, van Mil HGJ, Richardson MK (2011) Large-scale assessment of the zebrafish embryo as a possible predictive model in toxicity

Age: Not reported (eggs)

Source: Not reported

Feeding: Not reported

3. **Treatment:** The test solutions contained a series of flutolanil concentrations as follows: 0, 4.50, 5.40, 6.48, 7.78 and 9.33 mg/L (embryos); 2.59, 3.11, 3.73, 4.48 and 5.37 mg/L (12 h old larvae); 3.00, 3.45, 3.97, 4.56 and 5.25 mg/L (72 h old larvae); and 2.00, 2.30, 2.64, 3.04 and 3.50 mg/L (adult fish). The normal embryos were exposed to flutolanil at 0, 1.50, 1.80, 2.16, 2.59 and 3.10 mg/L for the 11-day Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages test.

4. **Test vessels:** 250 mL beakers

Test water: The standard water was prepared according to Mu *et al*. (2013)[3]

5. **Environmental conditions:** Environmental conditions were not reported.

B. STUDY DESIGN AND METHODS

1. Publication received: 09 December 2015

2. Test organism assignment and treatment

Methods for zebrafish maintenance and embryo collection were in accordance with those of Mu *et al*. (2013). The standard water was prepared according to Mu *et al*. (2013). Both the lethal toxicity tests and the developmental tests followed the protocol described in Mu et al. (2013). The toxicity test of flutolanil in the embryo and sac-fry stages was performed according to ‘‘Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages’’ provided by OECD (1998) and lasted for a total of 11 days. Normal embryos were exposed to flutolanil at concentrations of 0, 1.50, 1.80, 2.16, 2.59 and 3.10 mg/L in 250 mL beakers without feeding. Each concentration was carried out in three replicates adding 10 embryos in each replicate. All test solutions were replaced daily.

3. Dose preparation

Flutolanil stock solution was prepared by dissolving the test item in acetone AR and Tween-80 for each test. All other reagents utilized were of analytical standard.

4. Measurements and observations

Every exposure solution of flutolanil was analysed, both at the beginning and at 24 h post-exposure (hpe). Petroleum ether (10 mL) was selected as the extraction reagent and the samples (50 mL) were extracted twice. After standing, the organic phase was collected and evaporated to dryness at 35°C. Then, the residue was dissolved in 2 mL methanol and a 20 µL volume was injected into a reversed phase high-performance liquid chromatography system to measure the actual dose of the drug in each

testing. PLoS One 6(6):e21076 ;

Hill AJ, Teraoka H, Heideman W (2005) Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci 86:6–19

[3] Mu XY, Pang S, Sun XZ, Gao JJ, Chen JY, Chen XF, Li XF, Wang CJ (2013) Evaluation of acute and developmental effects of difenoconazole via multiple stage zebrafish assays. Environ Pollut 175:147–157. doi:10.1016/j.envpol.2012.12.029

sample. The chromatographic conditions were as follows: PAD detector; C18 stainless steel column (250 mm x 46 mm x 5 µm); the mobile phase, 60 % aqueous methanol; flow rate, 1 mL/min; the detection wavelength, 254 nm; and the column temperature, 40°C; the retention time, about 9.34 min.

5. Statistics

SPSS 17.0 software was used for statistical analyses. One-way ANOVA was used to test the differences, followed by Dunnett's and Duncan's posthoc comparisons. A significance level of 0.05 was employed for all experiments. Data were presented as the mean ± standard error.

II. RESULTS AND DISCUSSION

A. Biological data

In this study, according to the 96 h LC_{50} value, the lethal sensitivity sequence of flutolanil was embryos < larvae (72 hpf) and larvae (12 hpf) < adults. This result was consistent with that of the embryo and sac-fry stages experiment. The cumulative mortality increased visibly from the sixth day because of the hatching larvae (**Fout! Verwijzingsbron niet gevonden.**-1).

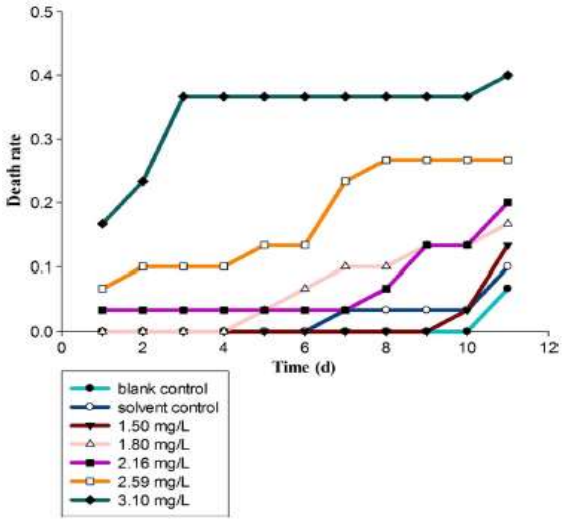


Figure 9.2.8/01-1 - Cumulative mortality of zebrafish embryo and larva exposed to flutolanil

The inhibitory effect of flutolanil on spontaneous movement was enhanced with an increase in concentration (**Fout! Verwijzingsbron niet gevonden.**.8/01-2). In the control group, the spontaneous movement of the embryos was 2 ± 1.67, while the spontaneous movement was obviously lower at 2.16 mg/L or higher concentrations.

The heartbeat of embryos exposed to flutolanil at 48 hpf was also reduced in a dose-dependent manner. The heartbeat of embryos at a 2.59 mg/L or higher dosage was significantly less than that in the control group (42.33 ± 2.58).

The hatching rate at 72 hpf was significantly reduced by 1.80 mg/L flutolanil or greater. At the highest concentration of 3.10 mg/L, the hatching rate was zero. The hatching rate at 96 hpf was significantly reduced at 2.59 mg/L or greater. The body length of the hatched larvae at 11 dpe were shown in **Fout! Verwijzingsbron niet gevonden.** and a significant decrease was found at 2.16 mg/L (386.83 ± 23.31 mm), amounting to 93.88 % of that in the controls (412.03 ± 14.13 mm). Additionally, teratogenic

effects, such as pericardial edema, yolk sac edema, sac deformity and spine deformation, were discovered in flutolanil exposure tests.

Moreover, the most sensitive toxicological indicators for flutolanil were hatching rate, followed by body length. Hatching of the embryo is a combined result of the body's biochemical mechanisms and behaviour. On the one hand, hatching embryo gland cells secrete hatching enzymes, which dissolve the chorion, while on the other hand, spontaneous movement exacerbates the destruction of the embryonic chorion (De Gaspar et al. 1999)[4].

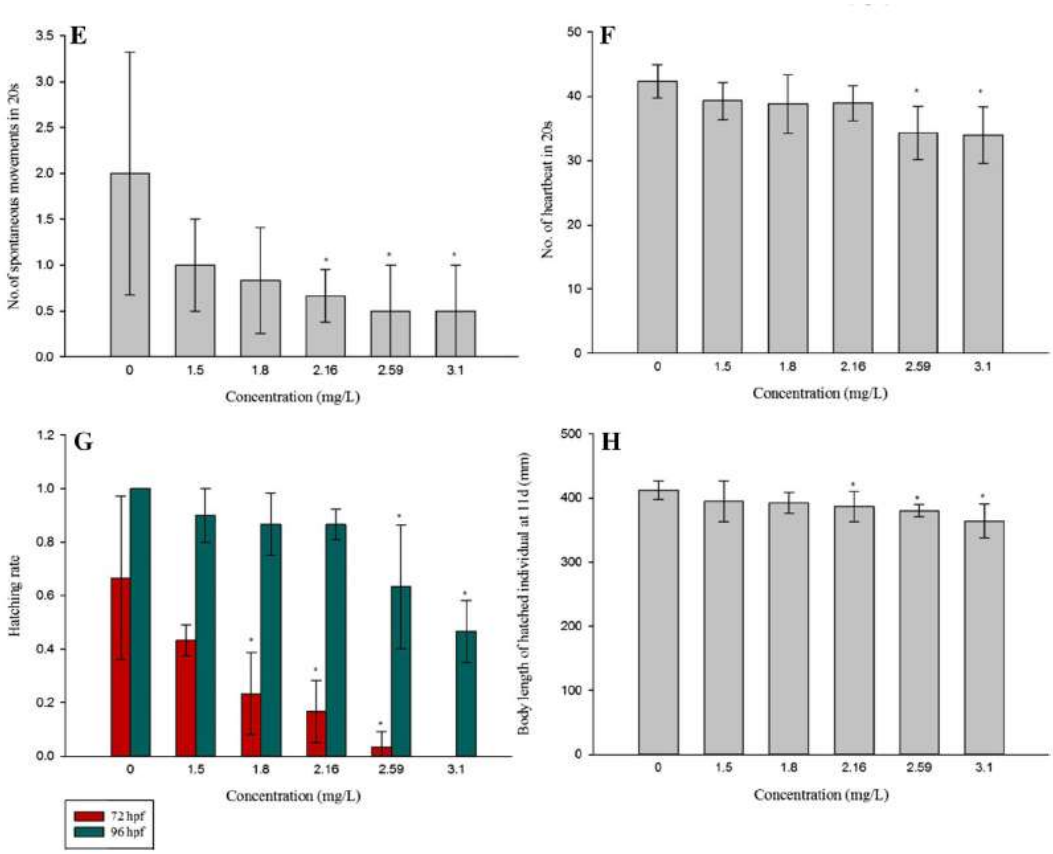


Figure 9.2.8/01-2: E–H for flutolanil: E - Spontaneous movement at 24 hpf; F - Heartbeat of embryos at 48 hpf; G - Hatching rate; H - Body length of hatched larvae after 11 days of exposure to flutolanil. Asterisks denote significant difference between treatments and control ($p < 0.05$). Error bars indicate standard deviation.

[4] De Gaspar I, Blanquez MJ, Fraile B, Paniagua R, Arenas MI (1999) The hatching gland cells of trout embryos: characterisation of Nand O-linked oligosaccharides. J Anat 194:109–118

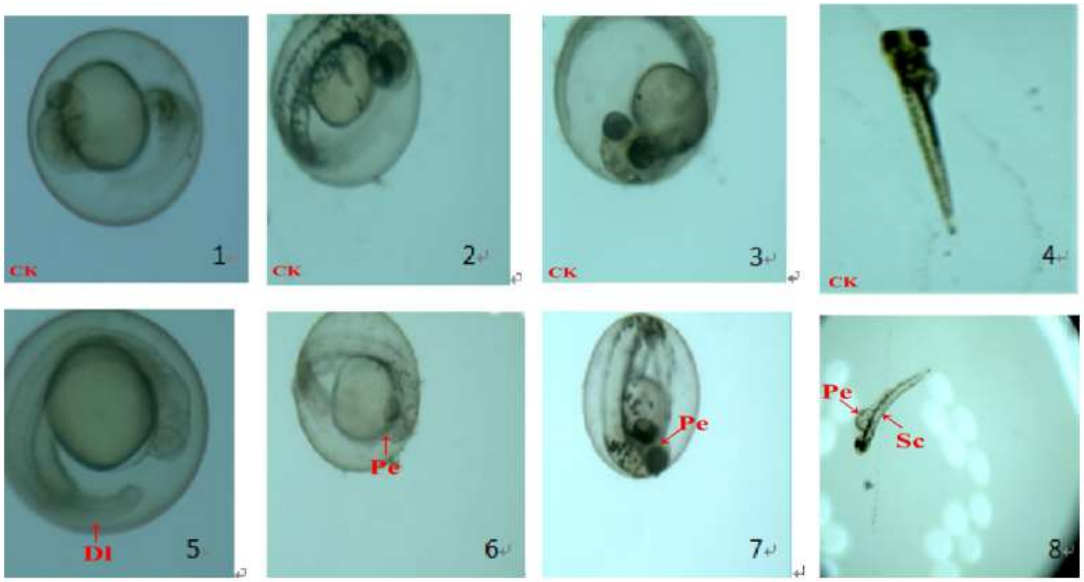


Figure 9.2.8/01-3: 1–8 for flutolanil: 1 Normal embryo at 24 hpf; 2 normal embryo at 48 h; 3 normal embryo at 72 h; 4 normal hatched larvae at 96 h; 5 development lag (Dl) at 24 hpf; 6 abnormal development of melanocytes, pericardial edema at 48 hpf; 7 pericardial edema at 72 hpf; 8 abnormal developmental tail and pericardial edema at 96 hpf.

B. Analytical verification

Analysis results determined that the actual concentration was always maintained in a range of theoretical values differing by 20 %. The analytical results are reported in the following table.

Table 9.2.8/01-1 Measured concentrations of flutolanil during the test

Test stage	Nominal concnetration of flutolanil (mg/L)	Measured concentration at 0 hpe (mg/L)	Standard deviation	Nominal (%)a	Measured concentration at 24 hpe (mg/L)	Standard deviation	Nominal (%)a	Mean Measured concentration	Nominal (%)a
Embryo	control	n.d.	-	-	n.d.		-	n.d.	-
	4.5	4.856	±0.024	7.92	4.787	±0.090	6.39	4.82	7.1%
	5.4	5.452	±0.058	0.97	5.414	±0.075	0.27	5.43	0.6%
	6.48	6.874	±0.064	6.1	6.742	±0.043	4.05	6.81	5.1%
	7.78	8.056	±0.097	3.54	7.929	±0.100	1.91	7.99	2.7%
	9.33	9.56	±0.058	2.47	9.267	±0.082	-0.67	9.41	0.9%
Larvae (12 hpf)	control	n.d.		-	n.d.		-	-	-
	2.59	2.414	±0.069	-6.8	2.277	±0.097	-12.1	2.35	-9.4%
	3.11	3.685	±0.036	18.48	3.548	±0.044	14.07	3.62	16.3%
	3.73	3.81	±0.081	2.14	3.769	±0.052	1.05	3.79	1.6%
	4.48	4.856	±0.024	8.4	4.597	±0.134	2.61	4.73	5.5%
	5.37	5.533	±0.064	3.04	5.41	±0.087	0.84	5.47	1.9%
Larvae (72 hpf)	control	n.d.	-	-	n.d.	-	-	-	-
	3	3.187	±0.013	6.23	3.404	±0.111	13.45	3.30	9.8%
	3.45	3.822	±0.111	10.79	3.563	±0.080	3.27	3.69	7.0%
	3.97	4.108	±0.077	3.46	3.844	±0.045	-3.19	3.98	0.2%
	4.56	4.967	±0.076	8.92	4.802	±0.105	5.31	4.88	7.1%
	5.25	5.703	±0.0233	8.63	5.448	±0.092	3.77	5.58	6.2%
Adult fish	control	n.d.	-	-	n.d.		-	-	-
	2	2.329	±0.078	16.47	2.277	±0.097	13.84	2.30	15.2%
	2.3	2.401	±0.091	4.41	2.352	±0.054	2.27	2.38	3.3%
	2.64	3.02	±0.070	14.44	2.895	±0.114	9.64	2.96	12.0%
	3.04	3.241	±0.085	6.61	3.167	±0.021	4.18	3.20	5.4%
	3.5	3.802	±0.0968	8.63	3.755	±0.052	7.29	3.78	8.0%

n.d. not determined

[a] % Nominal= (actual concentration-nominal concentration) / nominal concentration (%)

C. Toxicity endpoints

The 96-hour LC_{50} values for the flutolanil to zebrafish are presented in the following table.

Table 9.2.8/01-2 96 hrs LC_{50} values for flutolanil in different stages of zebrafish

Life stage	Time (hrs)	LC_{50} (mg a.s./L)	95% Confidence limits (mg a.s./L)
Adult fish	96	2.52	2.581-2.833
Embryos		5.47	5.044-5.835
Larvae (12 h)		3.91	3.625-4.241
Larvae (72 h)		4.09	3.869-4.351

III. CONCLUSION

In this test, flutolanil has appeared to inhibit the development of zebrafish significantly. The spontaneous movement, heartbeat, hatching rate of embryos and body length of larvae were significantly decreased. In addition, the abnormal phenomenon were nonspecific symptoms, which were induced by flutolanil. While in the embryonic developmental toxic tests, flutolanil was more sensitive. The acute toxicity LC_{50} of flutolanil (in mg a.s./L) were 5.47 (embryo), 4.09 (72 h old larvae), 3.91 (12 h old larvae) and 2.70 mg/L (adult).

Comments by RMS

The study summary was prepared by the applicant based on a publication in public literature with limited reporting. The study included the acute toxicity determination (96-h LC50) of different life stages of zebrafish (embryo, 12 h and 72 h old larvae and adults), and a short-term toxicity test on embryo and sac-fry stages with a total exposure duration of 11 days, performed according to the authors in agreement with OECD Guideline 212. No detailed experimental procedure was reported for the acute toxicity tests, but they were stated to be performed in accordance with a published method.

The experiments were performed in semi-static conditions, with test solutions replaced daily. The analytical concentrations in the acute toxicity test were analysed in each replicate at the beginning of exposure and 24 hours post-exposure by a reversed-phase HPLC method (validation criteria not reported in the paper) and were found to be within 20% of nominal concentrations (data reported in the supplementary information section of the paper, which was not provided by the applicant; the summary table was included in the study summary prepared by the applicant). No information was provided on the concentration measurements in the short-term toxicity test. Solvent controls were apparently not included in the study.

In the short-term toxicity test on embryo and sac-fry stages all concentrations were tested in triplicate, with 10 embryos per replicate, which is in agreement with the guideline requirements. No information was reported on the number of replicates and test fish per replicate in the acute toxicity tests;

however, they were evidently sufficiently high to allow the determination of LC50 values with 95% confidence limits.

The following parameters were analysed in a short-term toxicity test: spontaneous movement, heart rate, hatching rate, body length of the hatched larvae and teratogenic effects.

Hatching rate was determined at 72 and 96 hours post-fertilization and was found to be statistically significantly reduced at 1.80 mg/L and above in a dose-response manner at 72 hours, and at 2.59 mg/L and above at 96 hours. The body length of the hatched larvae was statistically significantly reduced at 2.16 mg/L and above in a dose-response manner following 11 days of exposure. Spontaneous movement of the embryos (determined as a number of spontaneous movements in 20 s) was statistically significantly reduced at 2.16 mg/L and above (day of evaluation not stated in the publication). Finally, the heartbeat rate (determined as a number of heartbeats in 20 seconds) evaluated at 48 hours post fertilization was statistically significantly reduced in a dose-dependent manner at 2.59 mg/L and above.
Furthermore, the following teratogenic effects were reported in the paper without stating their incidence: abnormal development of melanocytes and pericardial oedema at 48 hours post-fertilization; pericardial oedema at 72 hours post-fertilization; abnormal developmental tail and pericardial oedema at 96 hours post fertilization.

No NOEC or EC_x values were reported by the authors. Based on the significantly reduced hatching rate at 72 hours post-fertilization at 1.80 mg/L and above, the NOEC is considered to be 1.50 mg/L by RMS. As numerical raw data were not reported in the publication, the calculation of EC_x values is not possible.

The study has a number of limitations, e.g. the lack of information on the analytical validation of the test concentrations in the short-term toxicity, the lack of solvent controls, and the limited description of the experimental methods. In the acute toxicity tests, no details were reported on the number of replicates and test organisms used; however, these numbers were apparently sufficiently high to perform the statistical analysis and to calculate LC50 values with 95% confidence intervals. The analytical concentrations were measured before and after the exposure and were found to be within 20% of nominal, although no details on the validation of the analytical method are available. The study was however performed in semi-static conditions, thus the concentrations are expected to remain within 20% of the nominal. Based on this, the results of the acute toxicity tests are considered to be reliable with restrictions.

In the short-term toxicity tests, no information on the analytical verification of the test concentrations were reported. The numerical raw data were not included in the publication and no NOEC and ECx values have been derived by the study authors. Based on the statistically significant reduced hatching rate at 1.8 mg/L the RMS set the NOEC at 1.5 mg/L; however, the calculation of the ECx values is not possible based on the information provided in the publication. According to the OECD guideline 212, the short-term toxicity study is designed to be used as a screening test for either a Full Early Life

Stage test or for chronic toxicity. As a number of teratogenic effects were observed in the study, and considering the study limitations the RMS concludes that the information provided in the study is not sufficient to conclude on a sufficiently protective levels. Therefore the results of the short-term study are not suitable for risk assessment purposes and additional information is necessary.

Reliability of endpoints

Reliability of LC50:
To assess the reliability of the estimated LC_x values, two approaches are described in EFSA Supporting publication 2015:EN-924:

- Normalised width of the confidence interval (NW = (upper limit – lower limit) / median estimate); rating of the NW ranges from excellent (<0.2) to bad (>2)
- Relationship between LC_{10} and LC_{20}/LC_{50} confidence intervals: the best case (high certainty of protection level) is achieved when LC_{10} is lower than the lower limit of the LC_{20}; the worst case (low certainty of protection level) occurs when the median LC_{10} is greater than the lower confidence limit for the LC_{50}.

In the publication, LC_{10} and LC_{20} values were not provided. Based on the normalized width of the CI, the reliability of the LC_{50} values for the different life stages of fish were all excellent.

Reliability of long-term endpoints:
According to Regulation (EU) No 283/2013, the statistical power of the NOEC shall be assessed. However, this is not possible as long as default type I and II errors have not been fixed, default variance for any response variable have not been established and typical detectable effect sizes of each parameter have not been calculated (EFSA Supporting publication 2015:EN-924). In this study, a number of teratogenic effects were observed in the study, and considering the study limitations the RMS concludes that the information provided in the study is not sufficient to conclude on a sufficiently protective levels. Therefore the study is not suitable for risk assessment purposes and additional information is necessary.

The study cannot be used to derive reliable long-term endpoints. The LC_{50} values for different life stages of fish may be used for acute risk assessment.

Yang Y., Qi, S., Chen, J., Liu, Y., Teng, M., Wang, C.	2016	Toxic Effects of Bromothalonil and Flutolanil on Multiple Developmental Stages in Zebrafish	Bull Environ Contam Toxicol (2016) 97:91–97
Reliability			
General information			
Is a guideline method or modified guideline used?*		Yes; short-term toxicity test performed according to OECD guideline 2012; no information on acute toxicity test method is provided, but it appears to be comparable to OECD guideline 203	

Is the test performed under GLP conditions?*	No
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?	Yes; however, solvent controls were not included
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?	No, solvent controls not included.
* these criteria are of minor importance for study reliability, but may support study evaluation	
Test compound	
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?	Yes
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	Yes, both the purity and the source reported.
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	Not applicable
Test organism	
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	No (age, source and feeding not reported)
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	No (age, source and acclimatization not reported). No information is available on how many hours after the fertilization the exposure of embryos has started.
Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used,	No, solvent controls were not included.

is the solvent within the appropriate range and is a solvent control included?	
Is a correct spacing between exposure concentrations applied?	Yes, spacing factor 1.2 was applied in both the acute toxicity tests and the short-term toxicity test.
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	No validation of the test method reported. Also no details are reported on analytical measurements in the short-term toxicity test. The analytical concentrations during the acute test were determined at the beginning and 24 hours post-exposure and were found to be within 20% of the nominal.
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Not applicable.
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes for the short-term toxicity test. No data on the number of replicates and the number of test organisms reported for the acute toxicity test; however, the used numbers were apparently high enough to allow the calculation of LC50 values with 95% confidence intervals.
Are appropriate statistical methods used?	Yes.
Is a concentration-response curve observed? Is the response statistically significant?	Yes in the short-term toxicity test.
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	No, raw data not included in the publication.
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes.
Is the tested exposure scenario relevant for the substance?	Yes.
Is the tested exposure scenario relevant for the	Yes.

species?	
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes.
Are the organisms tested relevant for the tested compound?	Yes.
Are the reported endpoints appropriate for the regulatory purpose?	Yes, for acute exposure only. Reliable endpoints for long-term risk assessment cannot be derived.
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes.
Is the effect relevant on a population level?	Yes (decreased hatching rate in the short-term toxicity study; mortality in the acute toxicity study).
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	The EC_x values cannot be derived, as no raw numerical data were reported in the publication.
Are appropriate life-stages studied?	Yes.
Are the experimental conditions relevant for the tested species?	Yes.
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes.
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable.
Concluding weight of evidence/proposed action	Supporting information.
Type of information (Fully acceptable, supporting information, not applicable)	Supporting information.
Consideration/concluding score	Supporting information (R2/C2)

Report	CA 8.2.8-02. Matsumoto, K.-I., Hosowaka, M., Kudora, K., Endo, G., 2009
Title	Toxicity of Agricultural Chemicals in *Daphnia magna*
Report no.	Not applicable
Guidelines	OECD Guidelines for Testing of chemicals: *Daphnia* sp., Acute Immobilisation Test (OECD Guideline 202 (1984) OECD Guidelines for Testing of chemicals: *Daphnia magna* Reproduction Test (OECD Guideline 211 (1998);
GLP	No
Previous evaluation	New study
RMS Comment	Considered not acceptable for use in risk assessment

Executive Summary

Acute and sub-chronic toxicities of *Daphnia magna* were investigated using a total of 30 agricultural chemicals commonly used in Japan, including flutolanil as part of these toxicity tests.

The acute effects (acute immobilization) of flutolanil in *Daphnia magna* were determined in a 48-hour test using a static system, and the acute toxicity endpoints were determined as the concentrations yielding 50% immobility of *D. magna* after 24 hr and 48 hr exposure. During the sub-chronic assessment, *D. magna* was cultivated with flutolanil and algae until the first brood production. Lethal toxicity and the number of survival broods were determined within 13 days. Analysis and verification of the test concentrations for flutolanil were not reported.

The 24 and 48-h LC_{50}, as well as the 8 day exposure LC_{50}-value for flutolanil, were estimated to be greater than 10 mg/L (nominal). Flutolanil significantly delayed the first brood at concentrations less than half of LC_{50} (8 days) and also significantly reduced the size of the first brood at concentrations less than half of LC_{50} (8 days). Therefore, flutolanil was suggested to have parthenogenetic toxicity to *Daphnia magna*.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Flutolanil
Batch no.: Not reported
CAS no: 66332-96-5
Purity: Not reported

2. Test organism: *Daphnia magna*
Age: < 24 hours old - First-instar
Source: National Institute for Environmental Studies, Tsukuba, Japan and reared in-house (Osaka City University)
Feeding: Mixed algae species (*Chlamydomonas* sp., *Chlorella* sp., etc.)

3. Treatment: Solvent control (acetone), exact concentrations not reported, but stated to be in a range of 1-10 mg/L (nominal) withfour or five

	concentrations tested per test substance, determined using a geometric concentration ratio of 2
Test vessels:	flat bottom tubes (20 mL)
Loading:	*ca*. 3 mL of media per *Daphnia* (for the acute test)
Test water:	For acute test: activated carbon treated water, with the addition of 0.1 µg/L of vitamin B12 and 1 µg/L of selenium dioxide For sub chronic test: 0.5 mM of $CaCO_3 \cdot 2\ H_2O$, 0.3 mM of $MgSO_4 \cdot 7\ H_20$, 0.05 mM of KCI, 0.5 mM of $NaHCO_3$, 1 µg/L of selenium dioxide, and 0.1 µg/L of vitamin B_{12} were added to the treated tap water

4. Environmental conditions

Temperature:	21±1°C
pH:	Not reported
Dissolved oxygen:	Not reported
Photoperiod:	16 h light : 8 h darkness (*ca*.v550 – 720 lux)

B. STUDY DESIGN AND METHODS

1. Publication date: 06 June 2008 (received)

2. Test organism assignment and treatment

Acute toxicity test: Daphnids from the third to eighth broods, aged less than 24 hours at the start of the test, were exposed to five concentrations of the test item for a period of 48 hours under static conditions. 5 daphnids were used for each vessel and treated in groups (nominal concentrations 1.0 - 10.0 mg/L) and acetone (solvent control) group. The test vessels were placed in an incubator with stable environmental conditions, and no feeding occurred during the test. The supplied water was not changed during the test.

Sub-chronic toxicity test: Daphnids from the third to eighth broods, aged less than 24 hours at the start of the test, were exposed to four or five solutions prepared by a two-fold dilution (geometric concentration ratio of 2 with acetone).

3. Dose preparation

The test concentrations were prepared by injecting 5-50 µL of the agricultural chemical solution for flutolanil in 10 mL of the treated water in each test vessel. Treated water was added in each vessel to make a total volume of 15 mL.

4. Measurements and observations

The number of immobilised daphnids was assessed after 24 and 48 hours from the beginning of the test for the acute toxicity assays. The test vessels were placed in an incubator and therefore the environmental parameters remained constant.

5. Statistics

The NOEC was not reported for the purposes of this paper; however, in the acute test the LC_{50} (24 hr) and LC_{50} (48 hr) values were determined using the concentrations yielding 50% immobility of *D. magna*, and were calculated by recurrence straight line of concentrations and mortality rates observed after 24 hr and 48 hr exposures. In the sub-chronic toxicity test, the LC_{50} (8 days) values were calculated by recurrence straight line of the concentrations and mortality rates observed after 8 days. Concentrations resulting in significantly prolonged days of broods were determined by t-test. When broods were not observed for 13 days, the brood day period was set as 13 days. Concentrations resulting in significant reduction of broods were determined by t-test. The concentrations resulting in significantly prolonged days of broods and significant reduction of brood numbers are shown as ranges or as minimum values.

II. RESULTS AND DISCUSSION

A. Immobilization data

No immobilisation data were presented in this publication for the acute or the sub-chronic tests. However, the LC_{50} for the 24, 48 hours and the 8 days exposure were determined to be greater than the highest level tested for flutolanil, therefore it is assumed that no animals were found to be immobile in any of the test groups.

During the sub-chronic assessment, flutolanil significantly delayed the first brood at concentrations less than half of those for LC_{50} (8 days). It also significantly reduced the size of the first brood at concentrations less than half of those for LC_{50} (8 days). Therefore, flutolanil was suggested to have parthenogenetic toxicity to *Daphnia magna*.

B. Analytical verification

Analysis of the test solutions for the active substance of flutolanil after the applications were not reported.

C. Toxicity endpoint

The 24, 48-hour and 8-day toxicity endpoints for flutolanil to *Daphnia magna* are presented in the following table taking into account the nominal concentrations. The LOEC for significant reduction of broods was determined to be 5.0 mg/L (nominal concentration) at the end of the test and the LOEC concentration for significantly prolonged days of broods was 5.0 mg/L (nominal concentration).

Table 9.2.8/02-1. Toxicity endpoints of the test item flutolanil

Effect concentration	**24 hours**	**48 hours**	**8 Days**
LC_{50}	> 10 mg/L	> 10 mg/L	> 10 mg/L
NOEC	na	na	na
LOEC	na	na	na

na not applicable

III. CONCLUSION

The 24 and 48-h LC_{50}, as well as the 8-day exposure LC_{50}-value for flutolanil, were estimated to be greater than 10 mg/L (nominal). Flutolanil significantly delayed the first brood at concentrations less than half of those for LC_{50} (nominal concentration 5.0 mg/L after 8 days of exposure) and also significantly reduced the size of the first brood at concentrations less than half of those for LC_{50} (nominal concentration 5.0 mg/L after 8 days of exposure). Therefore, flutolanil was suggested to have parthenogenetic toxicity to *Daphnia magna*.

Comments by RMS

The study summary was prepared by the applicant based on the publication in a public literature with a limited reporting. The study was conducted as a part of toxicity evaluation of 30 agricultural chemicals, including flutolanil, and included an acute immobilization test and a reproduction toxicity test with *Daphnia magna*, with 4 or 5 tested exposure concentrations ranging from 1 to 10 mg/L (nominal). Exact tested concentrations were not reported, but were stated to be prepared using a geometric dilution factor of 2.

The acute toxicity tests were performed under static conditions, while in the reproduction toxicity tests daphnids were transferred to other tubes containing fresh chemical solutions and new algae on alternate days, because according to the authors the chemical and algal concentrations in the original tubes decreased with time. No information on the analytical verification of the tested concentrations was however reported in the publication.

The acute toxicity tests were performed with 5 daphnids per tested concentrations, *vs.* at least 20 daphnids per concentration, as required by the OECD guildeine 202. The reproduction toxicity test was performed with individual daphnids; no information on the number of replicates per tested concentration was provided (*vs.* at least 10 individually held daphnids per tested concentration required by the OECD guideline 211). Concurrent water and solvent controls were included and stated to be valid by the authors (neither acute nor chronic toxicity observed in the contols).

The exposure duration was 48 hours in the acute toxicity test and 13 days (*vs* 21 days, as required by the OECD guideline 211) in the reproduction toxicity test. In the acute toxicity test the EC50 values were determined after 24 and 48 hours of exposure. In the reproduction toxicity test the LC50 values and the number of survival broods were calculated after 8 days of exposure. Furthermore, the concentrations resulting in statistically significantly prolonged days of broods and significant reductions of broods were determined by a t-test (raw numerical data and the p-values not reported in the publication). The total numbers of living offspring at the end of the test were not reported. Also no NOEC or ECx values were derived.

It is stated in the publication that no immobilization of daphnids was observed following 48 hours exposure to flutalonil at the maximal tested nominal concentration of 10 mg/L. The 48-hour LC50 was thus concluded to be > 10 mg/L (nominal concentration). In the reproduction toxicity test, statistically significant reduction in the number of broods and statistically significantly prolonged brood duration

were observed at the nominal tested concentration of 5 mg/L. This value can be considered as a LOEC. No further data are reported.

Reliability of endpoints

The study has a number of limitation, e.g. the lack of analytical verification of the tested concentration, the lower numbers of tested organisms, a shorter exposure duration in a reproduction toxicity test and overall very limited reporting with a lack of raw numerical values, precluding the assessment of the validity of the reporting data. Neither NOEC nor EC_x values can be derived from the reproduction toxicity test. Although the LC50 values for 24 and 48 hours of exposure have been derived, they are reported as nominal concentrations. Considering these limitations, the study is concluded to be not reliable and not suitable for the risk assessment purposes.

Matsumoto K-I, Hosokawa M, Kuroda K, Endo G	2009	Toxicity of agricultural chemicals in *Daphnia magna*	Osaka City Med. J., 55, 89-97
Reliability			
General information			
Is a guideline method or modified guideline used?*		Yes; according to the authors the acute immobilization test was performed according to the protocol overal similar to the OECD guideline 202 and the reproduction toxicity test according to the previous (1998) version of OECD guideline 211 ; however, with a significant number of deviations.	
Is the test performed under GLP conditions?*		No	
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?		Yes	
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?		Yes	
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?		Yes	
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?		No	
If a formulation is used or if impurities are		Not applicable	

present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	
Test organism	
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	Yes
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	Yes; the chosen test organisms are considered to be acceptable. No pre-exposure to the test substance or unintended stressors apparently occurred.
Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Yes, solvent controls (acetone) were included.
Is a correct spacing between exposure concentrations applied?	Yes, spacing factor 2 was applied in both the acute toxicity tests and the reproduction toxicity test.
Is the exposure duration defined?	Yes (shorter than required by the guideline in the reproduction toxicity test).
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	No analytical measurements were apparently performed.
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Not applicable.
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate	No; the lower number of test organisms (5 vs 20) were used in the acute toxicity test. The

used for all controls and test concentrations?	reproduction toxicity test was performed with individual daphnids; the information on the number of replicates per tested concentration is lacking.
Are appropriate statistical methods used?	Yes.
Is a concentration-response curve observed? Is the response statistically significant?	No data available.
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	No, raw data not included in the publication.
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes.
Is the tested exposure scenario relevant for the substance?	Yes.
Is the tested exposure scenario relevant for the species?	Yes.
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes.
Are the organisms tested relevant for the tested compound?	Yes.
Are the reported endpoints appropriate for the regulatory purpose?	No
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	No
Is the effect relevant on a population level?	Yes (decreased number of broods and prolonged brood duration).
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	The EC_x values cannot be derived, as no raw numerical data were reported in the publication.
Are appropriate life-stages studied?	Yes.

Are the experimental conditions relevant for the tested species?	Yes.
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes for the acute toxicity study; no (shorter exposure duration) for the reproduction toxicity test.
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable.
Concluding weight of evidence/proposed action	The study is reliable with restrictions and relevant with restrictions.
Type of information (Fully acceptable, supporting information, not applicable)	Not applicable
Consideration/concluding score	Not applicable (R3/C3)

B.9.3 Effects on arthropods

B.9.3.1 Effects on bees

Table 9.3.1-1 Summary of toxicity data on bees

Species	Test substance	Time-scale (Test type)	End point	Toxicity	Data point /Author, year
Honey bee (*Apis mellifera* L.)	Flutolanil Technical	48h, Acute oral	LD_{50}	**> 208.7 µg a.s./bee**	CA 8.3.1.1.1-01 Schmitzer, S., 2001
		48h, Acute contact	LD_{50}	**> 200 µg a.s./bee**	
	Flutolanil 40 SC[1]	10 d, Chronic oral	LDD_{50} (95% CI) LDD_{20} (95% CI) LDD_{10} (95% CI)	**35.1 µg a.s./bee/day** (29.0-42.7) 18.3 µg a.s./bee/day (13.2 – 22.7) 13.0 µg a.s./bee/day (8.4 – 17.0)	CA 8.3.1.2-01 Ruhland, S., 2016, amended 2018
	Flutolanil 40 SC[1]	22 d, Larval toxicity	NOED LD/ED_{10} (95% CI) LD/ED_{20} (95% CI) LD/ED_{50} (95% CI)	**10 µg a.s./larva** 9.4 (6.5-14.0) µg a.s./larva 10.6 (7.1-15.9) µg a.s./larva 11.7 (10.6-13.0) µg a.s./larva	CA 8.3.1.3-01 Scheller, K., 2016, amended 2018
	Monarch 40 SC[1]	8 d, Semi-field	NOEC	> 11200 g in 400 L/ha	CP 10.3.1.6-01 Kling, A., 2003

Note: Endpoints in **bold** are the agreed endpoints retained for the risk assessment in line with the EFSA Conclusion (2008)

[1] Flutolanil 40 SC and Monarch 40 SC are equivalent to the representative formulation MONCUT 40 SC

CI = Confidence Intervals

B.9.3.1.1 Acute tests

Study 8.3.1.1.1-01

Report:	**CA 8.3.1.1.1-01. Schmitzer, S., 2001**
Title:	Laboratory Testing for Toxicity (Acute Contact and Oral LD_{50}) of Flutolanil tech. on Honey Bees (*Apis mellifera* L.) (Hymenoptera, Apidae)
Report no.:	9051036 (N-3015)
Published:	No
GLP:	Yes
Guidelines:	EPPO guideline No. 170, ICPBR Group recommendation (1999)
Deviations:	Minor guideline deviations are summarised below: -Feeding syrup not as OECD 213/214, no impact on validity -Starvation period shorter than 3hr in OECD 213, minor impact on validity -Contact dose 5 µL, OECD 214 states 1 µL, minor impact on validity (5 µL has the potential to "run-off" or be absorbed by the filter paper lining). -Vehicle for oral dose at 5%, OECD 213 states maximum of 1%. As solvent control group included, no impact on validity. -Oral dose was 20 mg, OECD 213 recommends 200 mg. No impact on validity as all dose was consumed (however due to smaller volume trophylaxis may be reduced- possible mortality at highest rate is not reflective)
Comment:	Equivalent to OECD 213 and 214, 1998. The study has already been reviewed under Uniform Principles for the first approval of flutolanil under Directive 91/414/EEC. (DAR, 2006: B.9.4.1) The validity criteria were met and the study deviations are considered to be minor and do not affect the outcome of the study, therefore the study is acceptable.
Endpoint	Contact LD_{50} >200 µg a.s./bee Oral LD_{50} >208.7 µg a.s./bee

Executive Summary

The objective of this study was to determine the acute oral toxicity levels of flutolanil technical to honeybee *Apis mellifera*, to compare the toxicity information with exposure resulting from application at the recommended rates for assessment to potential hazards to honey bees, to support for precautionary label statements and to indicate a further need for testing or field studies.

A definitive test was carried out with the five test concentrations of 12.5, 25, 50, 100 and 200 µg/bee (nominal) for both oral and contact tests. In the contact test 5 µL droplet of flutolanil in acetone was applied. In the oral test *ca*. 20 mg of syrup food with flutolanil as offered. After 4, 24 and 48 hours the mortality and behavioural abnormalities were evaluated. Solvent control (oral test) CO_2 and CO_2/solvent treated control (contact test) were also included, and a toxic standard (Dimethoate) of 0.2 µg per bee in both oral and contact tests.

No analysis for verification of achieved concentration was undertaken. Effects were reported based on nominal treatment rates for the contact exposure and calculated rates (by weight consumed) for the oral exposure.

During the evaluation the bees have shown normal appearance. There was a low effect on honey bee mortality in the oral toxicity test (3.3% at the measured concentrations of 13.2, 52.7 µg a.s./bee and 10% at the concentration of 207.7 µg a.s./bee). A low effect on mortality in the contact toxicity test was also demonstrated (3.3% mortality at the solvent control group and the nominal concentration 50.0 µg a.s./bee). The LD_{50} value was not determined because the maximum tested concentration of 200 µg

a.s./bee did not cause any mortality in the test organisms, during 48 hours of exposition and a low mortality was seen in the oral test at 208.7 µg a.s./bee. Therefore, the LD_{50} value of the tested substance is greater than 208.7 µg a.s./bee for the oral test and greater than 200 µg a.s./bee for the contact test. Flutolanil technical is considered to be harmless to *Apis mellifera* bees.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Flutolanil technical
Batch no.: 9950026 (Lot No.: 80718)
Purity: 99.3% (w/w) Flutolanil
Description: Solid white
Date of expiry: 02 August 2002

2. Test organism: Adult worker honey bees *Apis mellifera carnica* L.
Age: 4 - 6 week old female bees
Source: In-house, disease-free queen-right hive (collected without smoke or anaesthesia from the hive entrance)
Feeding: Ready to use syrup (Apiinvert, 30% saccharose, 31% glucose, 39% fructose)
Housing: The test chambers consisted of stainless steel cages that were 10 cm x 8.5 cm x 5.5 cm (length x width x height), inner walls lined filter paper, with removable glass sheet in the front side and perforated with 98 ventilation holes (*ca*. 0 1 mm) on the bottom. Each chamber was identified with the study number, the tested dosage, and the replicate number.
Loading: Three replicates per each concentration, 10 individuals per unit, 30 individuals per group
Anaesthetization: bees were anaesthetized with CO_2 (only in the contact test)

3. Treatment: 0 (control), 0 (solvent control), 0 (CO_2-free control, contact test), 12.5, 25, 50, 100 and 200 µg a.s./bee (nominal)
Vehicle: Acetone / Ready-to-use syrup (Oral test), Acetone with anesthetization (Contact test)
Toxic Reference: Dimethoate

4. Test vessels: 10 × 8.5 × 5.5 stainless steel cage, removable glass sheet, ventilated, lined with filter paper

5. Environmental conditions
Temperature: 25 – 26°C
Relative humidity: 52 – 72 %
Photoperiod: under constant darkness (except during observations)
Ventilation: ventilation to avoid possible accumulation of pesticide vapour

B. STUDY DESIGN AND METHODS

1. In-life phase: September 05 to September 08, 2000

2. Test organism assignment and treatment

Ten worker honey bees were assigned to each replicate (3 replicates per treatment rate). The honeybees were anaesthetized with carbon dioxide only in the contact test. For the oral test the collection was performed using glass tubes, from the flight board without anaesthetics and without the use of smoke.
A definitive test was carried out with five dosages (contact and oral toxicity test) in addition to one solvent control (contact and oral toxicity test), one CO_2 treated negative control (contact toxicity test) and one positive control with toxic standard (0.2 µg Dimethoate/bee in the contact test and oral test). The following dosages (nominal) were tested in both tests: 200, 100, 50, 25 and 12.5 µg a.s/bee. The average dosages measured in the oral test were: 208.7, 106.2, 52.7, 27.5, and 13.2 µg a.s./bee.

- **Contact Test**

Bees were anaesthetised with CO_2 and a single drop (5µL) of the test item solution was applied to the thorax of the individual bee. Bees were fed *ad libitum* with ready-to-use syrup.

- **Oral Test**

Bees were starved for 60 minutes prior to test feed. Bees were offered test item solution, mixed with *ca.* 20 mg/bee ready-to-use syrup, via a syringe. After consumption of the test solution, bees were fed *ad libitum* with ready-to-use syrup. Duration of consumption did not exceed 2 hours.

3. Dose preparation

Test solutions were made up in acetone and either applied directly (contact exposure) or in ready-to-use syrup (oral exposure). For oral dosing 20 mg of treated syrup (1:19 test item solution: ready-to-use syrup) was offered.

3. Measurements and observations

The number of dead bees in the individual test cages was recorded after 4, 24 and 48 hours. Behavioural abnormalities (vomiting, apathy, intensive cleaning) in the individual test cages were recorded after 4 hours, 24 hours and 48 hours.

4. Validity Criteria of the Study Control

Mortality resulted in 0.0 % (CO_2-treated control) and 3.3 % (CO_2/solvent-Treated control) in the contact test and 0.0 % in the oral test after 48 hours. The test is valid because the mortality observed in the untreated control groups and in the solvent control groups after 48 hours was ≤10%.

5. Statistical analysis

Results obtained from the bees treated with test item are compared to those obtained from the toxic standard and the controls. Due to the results it was not necessary to conduct statistical analysis.

II. RESULTS AND DISCUSSION

A. Biological data

During the evaluation, the bees have shown normal appearance with no abnormal symptoms. The mortality data of bees exposed to different doses of the test substance are shown in the following tables for the oral and contact tests.

Cumulative number of dead honeybees in each test group and the behavioural observations of organisms exposed to different doses of flutolanil in the oral toxicity test:

Nominal concentration (µg a.s./bee)	Mean measured concentration (µg a.s./bee)	Oral toxicity test						Mortality % in 48 hours
		4 hours		24 hours		48 hours		
		Mortality	Obs [1]	Mortality	Obs [1]	Mortality	Obs [1]	
Solvent control	-	0	0	0	0	0	0	0.0%
12.5	13.2	0	0	1	0	1	0	3.3%
25	27.5	0	0	0	0	0	0	0.0%
50	52.7	1	0	1	0	1	0	3.3%
100	106.2	0	0	0	0	0	0	0.0%
200	208.7	3	0	3	0	3	0	10.0%
Toxic Standard Dimethoate	0.2	12	11 (e,b,c)	29	1 (e)	29	1 (e)	96.7%

[1] Obs=Observations: a = food refusal/vomiting; b = moving coordination problems; c = apathy; d= intensive cleaning; e=nervous

Cumulative number of dead honeybees in each test group and the behavioural observations of organisms exposed to different doses of flutolanil in the contact toxicity test:

Nominal concentration (µg a.s./bee)	Contact toxicity test						Mortality % in 48 hours
	4 hours		24 hours		48 hours		
	Mortality	Obs [1]	Mortality	Obs [1]	Mortality	Obs [1]	
Control	0	0	0	0	0	0	0.0%
Solvent control	0	0	1	0	1	0	3.3%
12.5	0	0	0	0	0	0	0.0%
25	0	0	0	0	0	0	0.0%
50	0	0	1	0	1	0	3.3%
100	0	0	0	0	0	0	0.0%
200	0	0	0	0	0	0	0.0%
Toxic Standard Dimethoate	6	13 (e,b,c)	18	3 (e,c)	27	3 (e,b)	90.0%

[1] Obs=Observations: a = food refusal/vomiting; b = moving coordination problems; c = apathy; d= intensive cleaning; e=nervous

Due to the results of this study (no mortality> 50 % in any of the test item treatments) it was not necessary to calculate the LD_{50}. In both tests, the contact and oral test, no test item related behavioral abnormalities occurred.

B. Toxicity endpoint

The 48-day mortality endpoint for Flutolanil Technical to *Apis mellifera* are presented in the following table.

Endpoints of the test item Flutolanil technical

Endpoint	Effect concentration (µg a.s./bee)	28 hours
Contact	LD_{50}	>200
Oral	LD_{50}	>208.7

III. CONCLUSION

Flutolanil technical is considered to be harmless to bees *Apis mellifera* L. The LD_{50} was not determined and was considered to be greater than 208.7 µg a.s./bee (measured) for the oral test and greater than 200 µg a.s./bee (nominal) for the contact test (the maximum exposed concentration).

Comments RMS: The validity criteria of OECD 213/214 were met (average mortality of the controls < 10%, reference substance mortality in the correct range (24 hour LD_{50} of 0.1-0.35 µg/bee for dimethoate)). The study is considered acceptable for use in risk assessment. The toxicity values stated in the conclusion may be used in the risk assessment.

B.9.3.1.2 Chronic adult and larval tests

Study 8.3.1.2-01

Report:	**CA 8.3.1.2-01. Ruhland, S., 2016 as amended, 2018**
Title:	Chronic toxicity of Flutolanil 40 SC to the honey bee *Apis mellifera* L. under laboratory conditions
Report no.:	16 10 48 034 B (N-3078-2) Report amendment
Published:	No
GLP:	Yes
Guidelines:	Revised Proposal for a New OECD Guideline for the Testing of Chemicals: Honey bee (*Apis mellifera* L.), chronic oral toxicity test (10 day feeding test in the laboratory) (February 2016) EFSA Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees) (2014)
Deviations:	None
Comment:	The study meets the validity criteria and is considered to be acceptable.
Endpoint	10-day LDD_{50} 35.1 µg consumed a.s./bee/day

Executive summary

The purpose of this study was to assess the chronic oral toxicity of low doses of the test item to adult worker bees of *Apis mellifera* L. under laboratory conditions. In a ten-day chronic toxicity feeding test, 2-day old worker honey bees (*Apis mellifera* L. subspecies Buckfast) were exposed to a daily application of Flutolanil 40 SC diluted in the bee food (50 % w/v aqueous sucrose solution). The chronic toxicity of the test item was determined at nominal doses of 7.1, 14.3, 28.6, 57.2 and 114.3µg a.s./bee/day, corresponding to concentrations of 0.183, 0.367, 0.734, 1.468 and 2.936 g a.s./kg food. Effective doses were 8.2, 15.1, 32.4, 48.8 and 76.9 µg consumed a.s./bee/day. Additionally, honey bees were treated with Dimethoate EC 400 as toxic standard at a nominal dose of 27.3 ng a.s./bee/day (actual dose 16.2 ng a.s./bee/day). Untreated diet was served as a control.

The mean mortality and behavioural abnormalities were assessed and after a daily exposure of 28.4, 42.7 and 67.3 µg a.s./bee/day mortality was 53.3 %, 70.0 % and 83.3 %, respectively, which were statistically significantly increased compared to the control group after 10 days.

The 10-day LDD_{50} was determined to be 35.1 µg consumed a.s./bee/day, the LDD_{20} to be 18.3 µg consumed a.s./bee/day and the LDD_{10} to be 13.0 µg consumed a.s./bee/day, respectively. Also, With regard to the mortality of control AC the LC_{50} was determined to be 0.972 g a.s./kg food, the LC_{20} to be 0.423 g a.s./kg food and the LC_{10} to be 0.273 g a.s./kg food, respectively. The NOEDD was determined to be 15.1 µg consumed a.s./bee/day, and the NOEC was 0.367 g a.s./kg food, respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test material:**	Flutolanil 40 SC (synonyms: Moncut 40 SC, Rhino SC)
	Batch no.:	315156/1
	Purity:	40% (nominal), 41.8% (analysed) (w/w) Flutolanil
	Date of expiry:	29 June 2018
2.	**Toxic Reference:**	Dimethoate EC 400
	Batch no.:	FRE-001226
	Purity:	400 g/L (nominal), 420.3 g/L (analysed) Dimethoate
	Test concentrations:	Control (50 % w/v aqueous sucrose solution), Blank formulation (50 % w/v aqueous sucrose solution + blank formulation), 7.1, 14.3, 28.6, 57.2 and 114.3 µg a.s./bee/day (nominal dose) corresponding to 0.183, 0.367, 0.734, 1.468 and 2.936 g a.s./kg food and toxic standard at 27.3 ng Dimethoate/bee/day (nominal) corresponding to 0.702 mg a.s./kg food
3.	**Test organism:**	Young worker honey bees *Apis mellifera* L. subspecies Buckfast (Hymenoptera, Apoidea)
	Source:	Diseases-free and queen-right hives from BioChem agrar GmbH, Kupferstraße 6, 04827 Machern OT Gerichshain, Germany
	Age:	max. 2 days old – young worker bees
	Housing:	Aluminum cages with the dimensions: 95 mm (width) x 70 mm (height) x 60 mm with holes in the lateral walls for ventilation and two glass plates (one in front and one in the back) for observation of the bees.
	Feeding:	Provided continuously with treated or untreated 50 % (w/v) aqueous sucrose solution *ad libitum* via feeders (plastic syringes, tips removed).
4.	**Environmental conditions**	
	Temperature:	32.7 – 33.3 °C
	Relative humidity:	57.7 –62.0 %

Light: Darkness except during assessment

B. STUDY DESIGN AND METHODS

1. In-life phase: 02-31 August 2016

2. Test organism assignment and treatment

All bees used in the test derived from healthy, disease free and queen-right bee colonies. Examinations for *Varroa destructor, Nosema spp.* and foulbrood were carried out before the experimental start. The newly hatched worker bees were transferred into the test cages in groups of 10 bees/cage. For the following 24 ± 2 h (until Day 0), bees were held in the test cages at 33 ± 2 °C and 50 - 70 % RH and provided with sucrose solution for acclimatisation to the test conditions. Moribund and dead bees were rejected and replaced by healthy bees that were held in spare cages before starting the test.

Young worker bees of the species *Apis mellifera* L. were fed with 50 % w/v aqueous sucrose solution including the test item, blank formulation or the reference item. The control treatments were fed with 50 % w/v aqueous sucrose solution. The treated/untreated food was provided *ad libitum* in a plastic syringe, which had been weighed before application and was replaced daily.

3. Dose preparation

Test item solutions and blank formulation solution were prepared daily just before administration of food. The reference item stock solution was prepared once for the whole feeding period and stored in the refrigerator at about 6 °C (the reference item Dimethoate is stable over a period of 10 days when stored in the refrigerator). The reference item feeding solutions were prepared at least every 4 days and stored in the refrigerator at about 6 °C. The daily dose rates (administered solution) were based on a theoretical oral consumption of 33 µL per bee and day.

4. Measurements and observations

Observations for mortality and sub-lethal effects were performed daily before the next application. After 10 days a mortality of 6.7 % was observed in the control and a mortality of 13.3 % was observed in the blank formulation treatment. Assessment of behavior in comparison to the respective control group was performed daily during the test. The amount of feeding solution consumed was determined by weighing the feeders before and after feeding using calibrated equipment. Additional test units without bees but with filled syringes were included for evaluation of evaporation.

For verification of the exposure concentration, the highest test item solution (2.936 g a.s./kg food) and the lowest test item solution (0.183 g a.s./kg food) as well as the control solution were sampled in duplicate as specimens for analysis and retention directly after preparation on Days 0, 3 and 9. The analysis was part of the analytical phase of the study.

5. Statistics

Statistical calculations were performed with the computer program ToxRat Professional 3.2.1 (2015). For statistical calculation of the mortality results the Step-down Cochran-Armitage test was used. The

accepted significance level was $p \leq 0.05$ (one-sided greater). For calculation of the LDD_{50} and LC_{50} Probit analysis (linear maximum likelihood regression) was used.

The following endpoints were determined:

- mean daily intake per bee
- the NOEDD/NOEC (No observed effect dietary dose/concentration)
- LDD_{50} and LC_{50} (Median lethal dietary dose/concentration)

II. RESULTS AND DISCUSSION

A. Food consumption and mortality

The mean mortality after daily exposure of bees to five concentrations of flutolanil 40 SC is presented in the table below. There was 6.7% and 13.3% mean mortality in control and blank formulation groups, respectively at test end, ten days following start of exposure. In the test item group bees consuming doses of 76.9, 48.8 and 32.4 µg a.s./bee/day showed mortalities of 83.3 %, 70.0 % and 53.3 %, respectively, which are statistically significantly increased compared to the control group after 10 days. The reference dosage tested in the study was 27.3 ng a.s./bee/day (actual consumption on average per day: 16.2 ng a.s./bee), which caused a mean mortality of 86.7 % at day 10.

There were no sub-lethal effects observed at all treatment levels at the end of the test.

In the test item group the food consumption ranged between 26.2 and 44.7 mg solution per bee and day which is 67.2 % to 114.8 % of the expected amount (control: on average 40.4 mg/bee/day = 103.6 %) with a tendency of higher food uptake in the lower test item dosages. The food consumption per cage was corrected by subtracting the mean evaporation figure of each day of application. The mean daily amount of evaporated feeding solution ranged between 67.0 and 83.0 mg per day per feeding tube.

Food consumption and mortality of bees in a 10-day chronic oral toxicity test with Flutolanil 40SC

Treatments (g a.s./kg diet)	Mean consumption	Mean food consumption	Cumulative mortality	
	µg a.s./bee/day	mg/bee/day	Mean %	Mean corrected %
Control (0)	-	40.4	6.7	-
Blank formulation (0)	-	46.7	13.3	-
0.183	7.2	44.7	10.0	3.6
0.367	13.2	41.1	16.7	10.7
0.734	28.4	44.2	53.3*	50.0
1.468	42.7	33.2	70.0*	67.9
2.936	67.3	26.2	83.3*	82.1
Reference item (0.702 mg dimethoate/kg diet)	16.2	23.1	86.7	85.7

*** Statistically significant difference in pairwise comparison between treatment and control group AC (blank formulation BC) (Step-down Cochran-Armitage Test Procedure; α = 0.05; one-sided greater)**

B. Validity Criteria

The cumulative mortality for the controls was ≤15% across all replicates (actual 6.7 % in the control group and 13.3 % in the blank formulation treatment). The cumulative mortality for the reference item was ≥ 50 % (actual 86.7 % mean mortality after 10 days of exposure). The concentration of active substance in analysed sample of test item feeding solutions was within the limits of ± 20 % of the nominal concentration. All validity criteria were met.

C. Toxicity Endpoints

The LC_{50} and NOEC, based on nominal concentration, and the LDD_{50} and NOEDD, based on the mean uptake of test item per bee are presented in the following table. Calculated values of $LC_{20,10}$ and $LDD_{20,10}$ are also presented.

Chronic oral toxicity to honey bees exposed to Flutolanil 40SC – Summary of endpoints

LC_{50} [g a.s./kg food]	0.972 (0.767 – 1.258)*
LC_{20} [g a.s./kg food]	0.423 (0.287 – 0.551)*
LC_{10} [g a.s./kg food]	0.273 (0.162 – 0.379)*
LDD_{50} [µg consumed a.s./bee/day]	35.1 (29.0 – 42.7)*
LDD_{20} [µg consumed a.s./bee/day]	18.3 (13.2 – 22.7)*
LDD_{10} [µg consumed a.s./bee/day]	13.0 (8.4 – 17.0)*
NOEC#	0.367 g a.s./kg food
NOEDD#	15.1 µg consumed a.s./bee/day

* Calculated by using Probit analysis (linear maximum likelihood regression); between brackets: 95%-cl lower/upper

NOEDD / NOEC = No Observed Effect Dietary Dose/Concentration (calculated by using Step-down Cochran-Armitage Test Procedure; α = 0.05; one sided greater)

III. CONCLUSION

In a 10-day chronic toxicity feeding study with Flutolanil 40 SC the LDD_{50} was determined to be 35.1 µg consumed a.s./bee/day and the LC_{50} was determined to be 0.972 g a.s./kg food, respectively.

The NOEDD was determined to be 15.1 µg consumed a.s./bee/day, and the NOEC was determined to be 0.367 g a.s./kg food, respectively.

Comments by RMS

The study was conducted in general agreement with the latest version of the draft OECD test guideline for chronic oral toxicity in honeybees (October 2016) and all validity criteria were met. LDx values were calculated using probit analysis, which is acceptable. The calculations were confirmed by RMS. In addition to the reported results, the draft OECD TG indicates that the accumulated uptake of test chemical per bee over the test period should be reported. This parameter was reported but not included in the above summary and is therefore presented in the table below.

Table CA 8.3.1.2/01-2 Mean consumption of sugar solution, mean intake of test item and mortality in the flutolanil treatment (day 10)

Test organism	*Apis mellifera* L.	
Test item	Flutolanil	
Exposure	Oral 10 day chronic exposure via 50% aqueous sugar solution	
Concentration (g a.s./kg food)	Mean consumed dose (µg a.s./bee/day)	Cumulative uptake (µg a.s./bee)
Reference item (dimethoate)	16.2 ng a.s./bee/day	162
Water control	0	0
0.183	8.2	82.0
0.367	15.1	151
0.734	32.4	324
1.468	48.8	488
2.936	76.9	769

Reliability of estimated endpoints

To assess the reliability of the estimated LC_x and LDD_x values, two approaches are described in EFSA Supporting publication 2015:EN-924:

- Normalised width of the confidence interval (NW = (upper limit – lower limit) / median estimate); rating of the NW ranges from excellent (<0.2) to bad (>2)
- Relationship between EC_{10} and EC_{20}/EC_{50} confidence intervals: the best case (high certainty of protection level) is achieved when EC_{10} is lower than the lower limit of the EC_{20}; the worst case (low certainty of protection level) occurs when the median EC_{10} is greater than the lower confidence limit for the EC_{50}.

Based on these rules, the reliability of the LC_{10} and LDD_{10} is considered "fair" based on its NW and "high" based on the confidence intervals of the LC_{20} and LC_{50}. The LC_{10} and the LDD_{10} are lower than the NOEC and NOEDD, respectively. According to the EFSA supporting publication 2015:EN-924, in that case the risk assessment should be based on the LC_{10} and/or LDD_{10}.

The LC_{10} and/or LDD_{10} can be used for risk assessment.

Ruhland S.	2016	Chronic toxicity of Flutolanil 40 SC to the honey bee *Apis mellifera* L. under laboratory conditions	Report 16 10 48 034 B (N-3078)
Reliability			
General information			
Is a guideline method or modified guideline used?*	Yes		
Is the test performed under GLP conditions?*	Yes		
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?	Yes		
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?	Yes		
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with	Yes		

name or CAS-number? Are test results reported for the appropriate compound?	
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	Yes
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	The a.s. content is known. The effects should be considered the result of the formulated product.
Test organism	
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	Yes
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	Yes
Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Not applicable
Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	Yes
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	Yes
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	

Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Yes
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable
Concluding weight of evidence/proposed action	Study is reliable without restrictions and relevant without restrictions
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable
Consideration/concluding score	Fully acceptable

Study 8.3.1.3-01

Report:	**CA 8.3.1.3-01. Scheller, K., 2016**
Title:	Repeated exposure of Flutolanil 40 SC to honey bee (*Apis mellifera*) larvae under laboratory conditions (*in vitro*)
Study plan no.:	16 10 48 035 B (N-3079)
Published:	No
GLP:	Yes
Guidelines:	OECD DRAFT Guidance Document for testing chemicals: Honey bee (*Apis mellifera*) larval toxicity test, repeated exposure (February 2016) OECD 237 Guideline for testing chemicals: Honey bee (*Apis mellifera*) larval toxicity test, single exposure (2013)

Deviations:	None
Comment:	The study meets the validity criteria and is considered to be acceptable.
Endpoint	NOEC (22 days) 10 µg a.s./larva

Executive summary

In a chronic toxicity test, honeybee larvae (*Apis mellifera* L.) were repeatedly exposed to Flutolanil 40 SC. The toxicity of the test item was determined at doses of 2.1, 4.2, 8.4, 16.8 and 33.5 µg a.s./larva (corresponding to 6.0, 12.0, 23.9, 47.9 and 95.8 µg product/larva). The concentrations of test item in the diet were 13.2, 26.5, 53.0, 105.9 and 211.8 mg a.s./kg food (corresponding to 37.8, 75.7, 151.3, 302.6 and 605.3 mg product/kg food).

Additionally, further honeybee larvae were exposed to the reference item Dimethoate at a dose rate of 7.3 µg dimethoate/larva as positive control. A third group of larvae served as negative control, being fed with untreated diet, and untreated diet containing the blank formulation, respectively.

Assessments of larval mortality were performed after 24, 48, 72, 96, 120 hours, and of adult emergence after 15 and 22 days (respectively days 4-8, day 15, and day 22). Additionally, other observations like small body size or large quantities of remaining food after 96 and 120 hours (on day 7 and day 8) were noted.

Cumulated mortalities of honeybees treated with test item during the larval stages ranged from 8.3 % to 94.4 %. No statistically significant effects on adult emergence rates occurred up to and including 8.4 µg a.s./larva. Other findings such as smaller body size of surviving larvae and/or remaining food on day 8 occurred only in 16.7 % of larvae in the second highest test item group where larvae were treated with 16.8 µg a.s./larva.

In the chronic larval toxicity test with Flutolanil 40 SC, the ED_{50} (22 days) was determined to be 11.7 µg a.s./larva (corresponding to 33.4 µg product/larva), the ED_{20} was 10.6 µg a.s./larva and the ED_{10} was 9.4 µg a.s./larva (corresponding to 30.3 and 26.9 µg product/larva, respectively).

Accordingly, the NOED (22 days) was 8.4 µg a.s./larva (corresponding to 23.9 µg product/larva) and the corresponding NOEC (22 days) was 53.0 mg a.s./kg food (corresponding to 151.3 mg product/kg food).

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Flutolanil 40 SC (synonyms: Moncut 40 SC, Rhino SC)

Batch no.: 315156/1

Purity: 40% (nominal), 41.8% (analysed) (w/w) Flutolanil

Description: Pink liquid

Date of expiry: 29 June 2018

2. Toxic Reference: Dimethoate tech.

Batch no.: 35015A161

Purity: 98.8 % (w/w) (analysed) Dimethoate

Test concentrations: Control (untreated artificial diet - 50 % aqueous sugar solution with 50% royal jelly)

Blank formulation (untreated artificial diet, containing the Blank formulation for Flutolanil 40 SC in the amount of the highest concentration of the test item),

Test concentrations of 2.1, 4.2, 8.4, 16.8 and 33.5 µg a.s./larva (corresponding to 6.0, 12.0, 23.9, 47.9 and 95.8 µg product/larva)
Reference item (treated diet with a dose of 7.3 µg Dimethoate/larva - corresponding to: 46.4 mg a.s./kg)

Replicates: 3 replicates of each group tested including 12 larvae per replicate

3. Test organism: Honey bees *Apis mellifera* L. subspecies Buckfast (Hymenoptera, Apoidea)

Source: BioChem agrar GmbH, Germany - larvae taken from hives that were not treated with chemical substances for at least 1 month.

Age: First instar larvae (L1 during grafting) derived from healthy and queen-right colonies

Housing: 36 crystal polystyrene grafting cells (e.g. CNE Nicoplast, internal diameter 9 mm) were placed in three groups on each well plate. The plates were placed on an adjustable heating plate (e.g. stretching table), which was set to 35°C. Artificial diet was pipetted into the grafting cells, followed by placing one freshly grafted larva per cell.

Feeding: The aqueous sugar solutions as one component of the artificial diets was daily prepared with sugar solution and royal jelly. Each larva was fed separately using a sterile pipette. The food was composed of three different diets adapted to the needs of larvae at different stages of development:

Diet A (Day 1-2): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 2% weight of yeast extract, 12% weight glucose and 12% weigh fructose.

Diet B (Day 3): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 4% weight of yeast extract, 15% weight glucose and 15% weight fructose.

Diet C (Day 4-6): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 4% weight of yeast extract, 18% weight glucose and 18% weigh fructose.

4. Environmental conditions

Temperature: 34.0 - 35.0 °C

Relative humidity: D1-D8: 93-97 %, D8-D15: 75-77 %, D15-D22: 48-55 %

Photoperiod: Darkness (except during assessments)

B. STUDY DESIGN AND METHODS

1. In-life phase: 25 July 2016 – 15 August 2016

2. Test organism assignment and treatment

One-day old honeybee larvae of *Apis mellifera* L. were transferred from brood combs to polystrene grafting cells in 48-well cell culture plates 2 days before the start of the treatment. After this, in a 22 day chronic test, larvae were exposed daily between days 3 and 6 with Flutolanil 40 SC diluted in the larvae food (aqueous sugar solution mixed with royal jelly 1:1). In total, 3 treatment groups were set up: 5 doses of the test item, two controls of which one was untreated and one contained a blank formulation, and 1 dose of the reference item with 3 replicates per dose and 12 larvae per replicate.

Assessments of larval mortality were done after 24, 48, 72, 96, 120 hours, and of adult emergence after 15 and 22 days (respectively days 4-8, 15, and 22). Additionally, other observations like small body size or large quantities of remaining food after 96 and 120 hours (on days 7 and 8) were noted. In the analytical phase of the study the concentration of the active substance in the test item stock solutions (based on aqueous sugar solution) as well as in each final diet of the test item group was determined.

3. Dose preparation

The test/reference item was mixed daily (on days 3, 4, 5 and 6) into sterile filtered aqueous sugar solution to make a stock solution. The composition of the aqueous sugar solution varied slightly between day 3 and the other days, forming the basis of diet B (as applied on day 3) and diet C (as applied on day 4 - 6). Several dilutions were prepared by adding further sugar solution. Thereafter, the royal jelly was added to each stock solution at a ratio of 1:1, based on (w/w), to reach the final test concentrations.

Before feeding, the final test/reference item sugar solutions were heated up in a climate chamber, which was set to 34.5 °C. The larvae were fed with a defined quantity of the respective test item concentration.

4. Measurements and observations

Cumulated mortality was assessed during the test. At days 3 to 8, the well plates were taken out of the desiccator once a day at about the same time for feeding (until day 6) and larval mortality assessment. Furthermore, mortality was assessed on day 15. An immobile larva/pupa or one which did not react to contact stimulus was noted as dead. Adult emergence was also assessed until day 22. Lifeless pupae and bees or those, which were not fully developed, were marked as dead. In order to correct the effects observed in the treatment group by the control effect (e.g. background mortality) any calculations were performed using “mortality” instead of “adult emergence”. Other observations were morphological differences in comparison to the control and these were recorded during the mortality assessments. The presence of unconsumed food was qualitatively described on day 8.

Test conditions were also continuously monitored. Parameters such as temperature, relative humidity, illumination and ventilation were recorded to ensure the study requirements were met.

5. Statistics

For statistical calculation of the mortality results and for determination of No Observed Effect Level (NOEC/NOED) the Step-down Cochran Armitage Test was used (one-sided greater, α = 0.05). Moving Averages Computation for calculation of the median lethal dose/concentration (ED_{50}/EC_{50}), and non-linear regression analysis (3-parametric normal Cumulative distribution function, CDF, using arcsin transformed survival data) for calculation of lethal doses/concentrations (ED_{10}/EC_{10} and ED_{20}/EC_{20}), respectively, of the test item along with the 95 % confidence limits. Student’s t-test (α=0.05; one sided smaller) for pairwise comparison for mortality data of control and blank formulation. The statistical calculations were performed with the computer program ToxRat Professional.

II. RESULTS AND DISCUSSION

A. Food consumption and mortality

On day 8 of the test, 2.8 % of larvae were dead in the control, and a mortality of 8.3 % was observed in the blank formulation, respectively. In the test item groups, mortalities ranged between 0.0 and 94.4% (0.0 and 94.3 % as corrected by control; 0.0 and 93.9 % as corrected by blank formulation) on day 8. Mortality in the reference group was above 50 % across all replicates on day 8, being 97.2% (97.1 % as corrected by control; 97.0 % as corrected by blank formulation) on average. Other findings such as smaller body size of surviving larvae and/or remaining food on day 8 occurred only in 16.7 % of larvae in the second highest test item group where larvae were treated with 16.8 µg a.s./larva. Between days 8 and 22, 14.3 % pupal mortality was observed in the control, and 9.1 % in the Blank formulation. In the test item group, pupal mortalities ranged between 0.0 % and 42.9 % (0.0% and 33.3% as corrected by control; 0.0 % and 37.1 % as corrected by blank formulation).
In the final assessment on day 22, a cumulated mortality of 16.7% (day 3 to day 22) was determined for both, the control and the blank formulation. Cumulated mortalities of honeybees treated with test item during the larval stages ranged from 8.3 % to 94.4 % (0.0 % and 93.8 % as corrected by control; 0.0 % and 93.3 % as corrected by blank formulation). No statistically significant effects on adult emergence rates occurred up to and including 8.4 µg a.s./larva (corresponding to 53.0 mg a.s./kg food).

Nominal concentration [µg a.s./ larva]	**% Mean mortality of larvae Day 3- Day 8**		**% Mean Obs[1]**	**% Mean mortality of pupae Day 8-Day 22**		**% Mean mortality of pupae & larvae Day 3-Day 22**		**% Adult emergence rate**
	abs.	Corr.%		abs.	Corr.%	abs.	Corr.%	abs.
Control	2.8	-	0.0	14.3	-	16.7	-	83.3
Blank formulation	8.3	-	0.0	9.1	-	16.7	-	83.3
2.1	0.0	0.0 (0.0)	0.0	8.3	0.0 (0.0)	8.3	0.0 (0.0)	91.7
4.2	2.8	0.0 (0.0)	0.0	5.7	0.0 (0.0)	8.3	0.0 (0.0)	91.7
8.4	5.6	2.9 (0.0)	0.0	8.8	0.0 (0.0)	13.9	3.1 (0.0)	86.1
16.8	80.6	80.0 (78.8)	16.7	42.9	33.3 (37.1)	88.9	87.5 (86.7)	11.1*
33.5	94.4	94.3 (93.9)	0.0	0.0	0.0 (0.0)	94.4	93.8 (93.3)	5.6*
Reference item (7.3)	97.2	97.1 (97.0)	0.0					

Results are averages based on 3 replicates, containing 12 larvae each;

Corr.%: corrected mortality: test item and reference item corrected by control and blank formulation (mortality as corrected by blank formulation are shown in brackets) negative values are set to “0”; Calculation were performed with non-rounded values

abs.: absolute mortality as observed during the assessments

[1] Obs: Other observations (large quantities of remaining food, smaller body size of larva)

* Statistically significant difference for adult emergence in pairwise comparison between test item dosage group control (Step-down Cochran Armitage Test; α=0.05; one sided greater)

A. Analytical verification

Analytical determination of the nominal concentration of the active substance flutolanil in the Stock solutions confirmed mean recovery rates of 82 to 115 %. Analytical determination of the nominal concentration of flutolanil in the final diets in all treatment groups showed mean recovery rates of 83 to

100 %. No active ingredient has been detected in the control samples. Thus, the concentrations of the specimens of the biological part of the study were verified. Those values were within the acceptable limits as defined in the study plan.

Verification of stability of the active ingredient in samples of the final diet under honeybee larvae test conditions resulted in mean recoveries of 87 to 117 %. No active ingredient has been detected in the control samples. Because the concentrations of the active ingredient were between 80 and 120 %, the stability of the active ingredient in the test media was given for 24 h under the respective test conditions.

B. Validity Criteria

Validity criteria were met since control mortality was ≤ 15 % across all control replicates (between days 3-8) - 2.8 % for larvae across all control replicates. Adult emergence rate was ≥ 70 % for *Apis mellifera* L. across all control replicates (between day 3 and day 22) - 83.3 % for adult bees across all control replicates and finally mortality in the reference item was ≥ 50 % for larvae exposed to a total dose of 7.3 µg a.s./larva across all reference replicates (between day 3 and day 8) – Day 8/120 h: 97.2 % for larvae across all replicates exposed to a total of 7.3 µg a.s./larva between days 3-8.

C. Toxicity Endpoints

Chronic toxicity to honey bees after repeated exposure to Flutolanil 40SC – Summary of endpoints

Endpoint – Adult emergence	**Up to Day 22**
LD/ED_{50} [µg a.s./larva] [1,3] (95 %-Confidence limit / lower-upper)	**11.7** (10.6 – 13.0)
LD/ED_{20} [µg a.s./larva] [1,4] (95 %-Confidence limit / lower-upper)	10.6 (7.1 – 15.9)
LD/ED_{10} [µg a.s./larva] [1,4] (95 %-Confidence limit / lower-upper)	9.4 (6.5 – 14.0)
LC/EC_{50} [mg a.s./kg food] [1,3] (95 %- Confidence limit / lower-upper)	74.0 (66.9 – 82.0)
LC/EC_{20} [mg a.s./kg food] [1,4] (95 %- Confidence limit / lower-upper)	67.1 (44.8 – 100.4)
LC/EC_{10} [mg a.s./kg food] [1,4] (95 %- Confidence limit / lower-upper)	59.3 (40.7 – 88.4)
NOED [µg a.s./larva] [1,2]	**8.4**
NOEC [mg a.s./kg food] [1,2]	53.0

[1] Control and Blank formulation were compared for differences using Student's t-Test (α=0.05; one sided smaller). No differences were found between both treatments for D8 as well as for the final assessment at D22.
[2] Step-down Cochran Armitage Test; α=0.05; one sided greater
[3] Median effect dose/concentration of exposure was calculated using a Moving Average Computation
[4] Median effect dose/concentration of exposure was calculated using non-linear regression analysis (3-parametric normal Cumulative distribution function (CDF); calculations are performed with arcsin-transformed values

III. CONCLUSION

In the chronic larval toxicity test with Flutolanil 40 SC, the ED_{50} (22 days) was determined to be 11.7 µg a.s./larva (corresponding to 33.4 µg product/larva), the ED_{20} was 10.6 µg a.s./larva and the ED_{10} was 9.4 µg a.s./larva (corresponding to 30.3 and 26.9 µg product/larva respectively).

Accordingly, the NOED (22 days) was 8.4 µg a.s./larva (corresponding to 23.9 µg product/larva) and the corresponding NOEC (22 days) was 53.0 mg a.s./kg food (corresponding to 151.3 mg product/kg food).

Comments by RMS

The test was conducted in general agreement with the draft OECD guidance on larval toxicity (repeated exposure) of February 2014, without major deviations. The report however seems to contain erroneous test concentrations (see below).

The analytical method was sufficiently validated (recovery in feeding solution 100% and 96%, RSD 2.0% and 3.2% at fortification levels 0.77 and 33.3 mg a.s./kg, respectively (both levels n=5)). It should be noted however that nominal concentrations in the analytical phase report (256, 128, 64, 32 and 16 mg a.s./kg food) were not the same as in the biological phase report (212, 106, 53, 27 and 13 mg a.s./kg food). Furthermore, the biological phase report seems to include an erroneous description of test solution preparation and product based concentrations were wrongly expressed in active substance concentrations:

- Preparation of test solutions was indicated to have been done through serial dilution, but claimed nominal concentrations do not match with this dilution scheme. Instead, claimed nominal concentrations could only have been obtained using the claimed dilutions if all dilutions were made from the stock solution.
- The reported doses expressed as active substance and corresponding product relate to a 35% a.s. content of the product, while the product contained 41.8% a.s.

As analysis was performed for all concentrations, the analytical data may be used to define the test concentrations. Measured concentrations were all >80% of nominal for the nominal concentrations stated in the analytical phase report (i.e. 256, 128, 64, 32 and 16 mg a.s./kg food). Based on an a.s. content of 41.8%, these concentrations relate to 611, 306, 153, 76 and 38 mg product/kg food. Using these concentrations, RMS calculated larval consumption based on a total food consumption of 140 µL/larva, equivalent to 158 mg/larva. Resulting doses were 97, 48, 24, 12 and 6.0 µg product/larva, which is equivalent to 40, 20, 10, 5.1 and 2.5 µg a.s./larva (compared to 33.5, 16.8, 8.4, 4.2 and 2.1 µg a.s./larva as reported in the biological phase report).

Based on the above, calculations of effect concentrations were repeated by the RMS[5]. Results are shown in the table below.

[5] The notifier later submitted a revised final report in which the laboratory re-calculated the values in a similar manner to the RMS and came to almost the same values (NOED 9.7 µg a.s./ larva vs NOED 10 µg a.s./ larva as calculated by the RMS). As a result, the endpoints have not been updated and remain as calculated by the RMS.

	Exposure				% Adult emergence rate
	[mg a.s./kg food]	[mg product/kg food]	[µg a.s./ larva]	[µg product/larva]	
Control	0	0	0	0	83.3
Blank formulation	0	0	0	0	83.3
Flutolanil 40 SC	16	38	2.5	6.0	91.7
Flutolanil 40 SC	32	76	5.1	12	91.7
Flutolanil 40 SC	64	153	10	24	86.1
Flutolanil 40 SC	128	306	20	48	11.1*
Flutolanil 40 SC	256	611	40	97	5.6*
NOEC/D	64	153	10	24	
LC_{10}/LD_{10} (95% CI)	76 (11-95)	181 (26-227)	12 (1.7-15)	29 (4.1-36)	
LC_{20}/LD_{20} (95% CI)	82 (21-101)	197 (50-242)	13 (3.3-16)	31 (7.9-38)	
LC_{50}/LD_{50} (95% CI)	101 (57-126)	242 (136-302)	16 (9.0-20)	38 (22-48)	

Reliability of endpoints

To assess the reliability of the estimated LC_x and LD_x values, two approaches are described in EFSA Supporting publication 2015:EN-924:

- Normalised width of the confidence interval (NW = (upper limit – lower limit) / median estimate); rating of the NW ranges from excellent (<0.2) to bad (>2)
- Relationship between EC_{10} and EC_{20}/EC_{50} confidence intervals: the best case (high certainty of protection level) is achieved when EC_{10} is lower than the lower limit of the EC_{20}; the worst case (low certainty of protection level) occurs when the median EC_{10} is greater than the lower confidence limit for the EC_{50}.

Based on these rules, the reliability of the LC_{10} and LD_{10} is considered “poor” based on its NW and “low” based on the confidence intervals of the LC_{20} and LC_{50}. Further, the LC_{10} and LD_{10} are higher than the NOEC and NOEDD, respectively. According to the EFSA supporting publication 2015:EN-924, in that case the risk assessment should be based on the NOEC and/or NOEDD.

According to Regulation (EU) No 283/2013, the statistical power of the NOEC shall be assessed in case no ECx can be derived. However, this is not possible as long as default type I and II errors have not been fixed, default variance for any response variable have not been established and typical detectable effect sizes of each parameter have not been calculated (EFSA Supporting publication 2015:EN-924). Considering the level of effect on which the NOEC was based (i.e. a 13% reduction in emergence of adults at 20 µg a.s./larva and a total lack of effect at the next lower level of 10 µg a.s./bee), the NOEC is considered sufficiently protective.

The NOEC of 64 mg a.s./kg food, which is equivalent to 10 µg a.s./larva (and to 153 mg product/kg food and 24 µg product/larva), may be used for risk assessment.

Scheller K.	2016	Repeated exposure of Flutolanil 40 SC to honey	Report No 16 10 48 035 B (N-3079)

		bee (*Apis mellifera*) larvae under laboratory conditions (*in vitro*)	
Reliability			
General information			
Is a guideline method or modified guideline used?*	Yes		
Is the test performed under GLP conditions?*	Yes		
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?	Yes		
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?	Yes		
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?	Yes		
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	Yes		
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	The a.s. content is known. The effects should be considered the result of the formulated product.		
Test organism			
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	Yes		
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	Yes		
Exposure conditions			
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes		

Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Not applicable
Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	Yes; they were essential to identify the test concentrations, which were wrongly reported in the biological phase report.
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	Yes
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the	Yes

species?	
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Yes
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable
Concluding weight of evidence/proposed action	Study is reliable without restrictions and relevant without restrictions
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable
Consideration/concluding score	Fully acceptable

B.9.3.2 Effects on non-target arthropods other than bees

Study CA 8.3.2.1-01 Effects on *Aphidius rhopalosiphi*

Report:	**CP 10.5.1-01 Nienstedt, K.M., 1999a**
Title:	EXP10066A: A laboratory toxicity test with the parasitic wasp, *Aphidius rhopalosiphi* (Hymenoptera: Braconidae)

Report no.:	99-073-1013 (N-3016)
Published:	No
GLP:	Yes (OECD, Swiss Authority)
Guidelines:	Directive 96/12/EC, Based on IOBC Approved Method of Polgar (1988) and Mead-Briggs (1992) and Draft Guideline of the *Aphidius* Ring-Test-Group (Mead-Briggs, 1997)
Previous evaluation	In DAR (2006) for original approval.
Remark by RMS	Considered acceptable at the time of original inclusion. The study summary from the DAR is replaced with an updated (extended) version. Evaluation from the DAR is copied as a whole without changes. The study was checked by the RMS for the purpose of renewal and the following deviation was noted: during first 24 h of the fecundity phase survival rate of females decreased for more than 35 and 55% in the control and exposure treatments, respectively. It might be considered experimental artefact given the fact that survival rate in mortality phase during first 48h was very high in all treatments and the control, in fecundity phase mortalitity was increased in all treatments and the control, the number of produced offspring satisfy validity criteria, and the effects of the product on reproduction of other test species were not significant. Overall, this deviation does not invalidate the study.
Endpoint	LR_{50} > 4500 g a.s./ha ER_{50} > 4500 g a.s./ha

Executive Summary

The effects on mortality and reproduction (fecundity) of EXP10066A to the parasitic wasp, *Aphidius rhopalosiphi*, were determined in a two phase study. Mortality was observed following exposure to the test substance for 48 hours. The fecundity of treated wasps from the mortality phase was assessed after a 24 hour parasitisation period by observing parasitisation rates after 10 days incubation. Ten female adult wasps, less than 48 hours old, per replicate were used in a two rate study design comprising two treatment rates and a control. Wasps were exposed to the test item via treated enclosure surfaces for 48 hours. Four replicates for the control, and for each treatment rate 450 and 4500 g a.s./ha (nominal) were used. Spray solutions were made up in deionised water and applied at a nominal rate of 200 L/ha. Mortality and fecundity were assessed and compared with corresponding parameters recorded in the untreated group at the end of the test.

No analysis for verification of achieved concentration was undertaken. Effects were reported based on nominal treatment rates. EXP10066A was considered to be harmless to *Aphidius rhopalosiphi*.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** EXP10066A
Lot no.: OP980259
Purity: 464 g/L Flutolanil
Description: White opaque liquid
Date of expiry: October 12, 1998

2. **Test organism:** *Aphidius rhopalosiphi*
Age: < 48 hours (female)

Source: PK Nützlingszuchten, Welzheim, Germany

Feeding: 1:2 honey:water solution

3. **Treatment:** 0 (control), 450 and 4500 g a.s./ha (nominal)

Replication: **Exposure phase**: 4 (10 female wasps per replicate)

Fecundity phase: 14 (1 female wasp per replicate)

Vehicle: Deionised water

Toxic Reference: Perfekthion (Dimethoate)

4. **Test vessels:** **Exposure phase:** Two treated glass plates (10 × 10 cm) fitted into a stainless steel, ventilated frame. Water access, honey: water (1:2) solution provided

Fecundity phase: 5-10 barley plants (*Rhopalosiphum padi* aphid infested) enclosed by plastic cylinder (9 cm Ø × 20 cm tall) with mesh top.

5. **Environmental conditions**

Temperature: 18.5 - 21.0°C

Relative humidity: 72 - 89%

Photoperiod: 16:8 light:dark (810-1100 lux mortality, 1560-2000 lux fecundity)

B. STUDY DESIGN AND METHODS

1. In-life phase: December 08 to December 21, 1998

2. Test organism assignment and treatment

Ten adult female wasps per replicate were exposed to treated glass plates for 48 hours to assess effects on mortality. Wasps were impartially transferred by aspirator. Following the mortality phase, 14 wasps from each treatment were randomly selected and individually transferred using an aspirator for a 24 hour parasitisation period.

Mortality Phase			Fecundity Phase		
Nominal treatment (g a.s./ha)	**Number of replicates**	**Number of wasps per replicate**	**Mortality treatment rate (g a.s./ha)**	**Number of replicates**	**Number of wasps per replicate**
0 (control)	4	10	0 (control)	14	1
450	4	10	450	14	1
4500	4	10	4500	14	1
68 mg Dimethoate/ha Toxic reference	4	10	68 mg Dimethoate/ha Toxic reference	-	-

3. Dose preparation

A primary stock solution of 22.5 g a.s./L was prepared. Spray solutions were made up in deionised water and either used directly or following dilution. Spray solutions were applied to glass plates (10 ×

10 cm) by a calibrated laboratory sprayer at a nominal rate of 200 L/ha. Test units were assembled after a drying period of 1 hour.

4. Measurements and observations

Mortality and behavioural abnormalities were assessed at 2, 24 and 48 hours and after the parasitisation period. Wasps were recorded as alive and healthy, or affected, or moribund, or dead.

Fecundity (parasitisation rate) was assessed 10 days after infestation ended (24-hour period following mortality phase). The numbers of mummies (parasitized aphids) were counted per replicate.

5. Statistics

Test rate mortality was corrected for control mortality using Abbott's correction.

Mortality data was subjected to Fisher's exact test. Fecundity data was checked for normality using the Shapiro-Wilk's test. Since the data was non-normal, Kruskal-Wallis was used to evaluate significance. Statistical analysis was undertaken using Statistica for Windows (StatSoft Inc.).

The IOBC scheme was used to evaluate the classification of EXP10066A from corrected mortality (M):

Value of M	**Classification**
< 30%	Harmless
30 - 80%	Slightly harmful
80 - 99%	Moderately harmful
> 99%	Harmful

II. RESULTS AND DISCUSSION

A. Biological data

The evaluated parameters were mortality and fecundity after 48 hours' exposure. Results are presented below:

Mortality and Fecundity

Mortality Phase				**Fecundity Phase**			
Nominal treatment (g a.s./ha)	**Mortality (%)**			**No female wasps set up**	**Surviving females after 24 h**	**% mortality**	**Mean number of mummies per surviving female**
	2h	**24h**	**48h**				
0 (control)	0	0	0	14	9	35.7	12.2
450	0	0	2.5	14	6	57.1	12.2
4500	0	2.5	2.5	14	6	57.1	16.0
68 mg Dimethoate /ha Toxic reference	0	97.5	100	--	--	--	--

B. Toxicity endpoints

The 48-day mortality endpoint and subsequent fecundity endpoint for EXP10066A to *Aphidius rhopalosiphi* are presented in the following table.

Endpoints of the test item EXP10066A

Endpoint	Effect concentration (g a.s./ha)	48 hours
Mortality	LR_{50}	> 4500
Fecundity	ER_{50}	> 4500

III. CONCLUSION

Based on the mortality of *Aphidius rhopalosiphi* due to the test item applied at the maximum concentration (i.e. 4500 g a.s./ha), the test item was classified as "harmless" to *Aphidius rhopalosiphi* under worst-case laboratory conditions according to the IOBC scheme.

Since there were no statistically significant differences between the test item treatments and the control in their reproductive ability, there was no need to classify according to the IOBC scheme for this criterion.

Evaluation RMS (DAR 2006)

The study was well performed according to the test guideline and in compliance with GLP. The study is acceptable.

Study CA 8.3.2.2-01 Effects on *Typhlodromus pyri*

Report:	**CA 8.3.2.2-01 Nienstedt, K.M., 1999b**
Title:	EXP10066A: Laboratory Contact Toxicity Test with the Predacious Mite, *Typhlodromus pyri* Scheuten (Acari: Phytoseiidae)
Report no.:	99-074-1013 (N-3017)
Published:	No
GLP:	Yes (OECD, Swiss Authority)
Guidelines:	Directive 96/12/EC, Based on IOBC Approved Method of Overmeer (1988) and Overmeer and Van Zon (1982)
Previous evaluation	In DAR (2006) for original approval.
Remark by RMS	Considered acceptable at the time of original inclusion. The study summary from the DAR is replaced with an updated (extended) version. Evaluation from the DAR is copied as a whole without changes.
Endpoint	LR_{50} > 4500 g a.s./ha ER_{50} > 4500 g a.s./ha

Executive Summary

The effects on mortality and reproduction (fecundity) of EXP10066A to the predacious mite, *Typhlodromus pyri*, were determined in a laboratory study. Mortality and fecundity was observed following exposure to the test substance as fresh, dried residues. Twenty protonymph mites, 2-day synchronised, per replicate were used in a two rate study design comprising two treatment rates and a control. Mites were exposed to the test item via treated enclosure surfaces for 14 days. Five replicates for the control, and for each treatment rate 450 and 4500 g a.s./ha (nominal) were used. Spray solutions were made up in deionised water and applied at a nominal rate of 200 L/ha. Mortality and fecundity were assessed and compared with corresponding parameters recorded in the untreated group at the end of the test.

No analysis for verification of achieved concentration was undertaken. Effects were reported based on nominal treatment rates. EXP10066A was considered to be harmless to *Typhlodromus pyri*.

I. MATERIALS AND METHODS

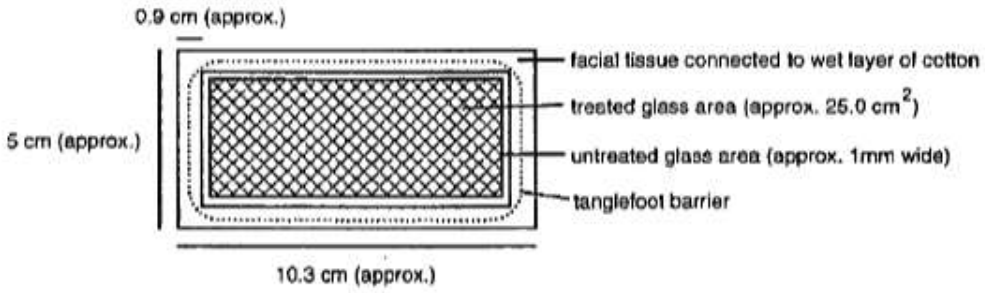
A. MATERIALS

1. **Test material:** EXP10066A
Lot no.: OP980259
Purity: 464 g/L Flutolanil
Description: White opaque liquid
Date of expiry: October 12, 1998

2. **Test organism:** *Typhlodromus pyri* Scheuten (Acari: Phytoseiidae)
Age: Protonypmph, 2-day synchronised
Source: BASF, Limburgerhof, Germany (subsequently cultured in-house)
Feeding: 1:1 wallnut:apple pollen *ad libitum*

3. **Treatment:** 0 (control), 450 and 4500 g a.s./ha (nominal)
Replication: 5 (20 protonymphs per replicate)
Vehicle: Deionised water
Toxic Reference: Ethyl parathion (parathion)

4. **Test vessels:** One treated glass plates **(**103 × 50 mm) as below:



5. **Environmental conditions**

Temperature: 24.0-25.5°C
Relative humidity: 70-79%
Photoperiod: 16:8 light:dark (1061-1500 lux)

B. STUDY DESIGN AND METHODS

1. In-life phase: January 05 to January 19, 1999

2. Test organism assignment and treatment

A total of 20 protonymph mites per replicate were exposed to treated glass surfaces for 14 days to assess effects on mortality and fecundity. For each treatment group, the control and the reference group, a total of 5 replicates were used. Wasps were impartially transferred by fine brush.

3. Dose preparation

A primary stock solution of 22.5 g a.s./L was prepared. Spray solutions were made up in deionised water and either used directly or following dilution. Spray solutions were applied to glass plates (103 × 50 mm) by a calibrated laboratory sprayer at a nominal rate of 200 L/ha. Test units were assembled after a drying period.

4. Measurements and observations

Assessments were carried out as below:

Assessment Day	Parameter Observed
3	Surviving, dead and missing mites
7	Dead and missing mites Surviving females Number of eggs Number of hatched larvae
10	
14	

Eggs and newly hatched larvae were removed at each assessment. Mortality was considered to be the sum of dead and missing mites during the first 7 days.

5. Statistics

Test rate mortality was corrected for control mortality using Abbott's correction. Mortality data was subjected to the Yates corrected Chi-squared test.

Fecundity data was checked for normality using the Shapiro-Wilk's test. Since the data was normal, ANOVA was used to evaluate significance. Statistical analysis was undertaken using Statistica for Windows (StatSoft Inc.).

II. RESULTS AND DISCUSSION

A. Biological data

The evaluated parameters were mortality and fecundity. Results are presented below.

Mortality and Fecundity

Mortality Phase			Fecundity Phase
Nominal treatment (g a.s./ha)	Mortality (%)		No eggs per female
	Day 3	Day 7	
0 (control)	3	6	8.09
450	2	4	8.81
4500	6	7	8.48
0.036% Ethyl Parathion 500 g/L /ha Toxic reference	46	52	6.50

B. Toxicity endpoint

The 48-day mortality and fecundity endpoints for EXP10066A to *Typhlodromus pyri* are presented in the following table.

Endpoints of the test item EXP10066A

Endpoint	Effect concentration (g a.s./ha)	48 hours
Mortality	LR_{50}	> 4500
Fecundity	ER_{50}	> 4500

III. CONCLUSION

EXP10066A was considered to be harmless to *Typhlodromus pyri*.

Evaluation RMS (DAR 2006)

The study was well performed and reported. The study was in compliance with GLP. According to IOBC classification flutolanil and EXP 10066A can be classified as harmless for predatory mite *T. pyri*.

B.9.4 Effects on non-target soil meso- and macrofauna

Report:	**Wüthrich, V. 1990**
Title:	Acute toxicity (LC50) study of Flutolanil Technical to earthworms, RCC
Report no.:	W-3020.
Published:	No
GLP:	Yes (OECD, Swiss Authority)
Guidelines:	Directive 96/12/EC, Directive 87/302/EEC Part C. OECD Guideline for testing of chemicals No. 207.
Previous evaluation	In DAR (2006) for original approval.
Remark by RMS	Considered acceptable at the time of original inclusion. Evaluation from the DAR is copied as a whole without changes. It is noted that acute toxicity to earthworms is not a data requirement under Regulation 1107/2009.
Conclusion	LC_{50} > 1000 mg a.s./kg dw soil

Material and methods

Adult earthworms, Eisenia foetida, were exposed to technical flutolanil (purity: 97.6 %, specification batch No.: 543251) in an artificial soil for 14 days at nominal concentrations of 0, 62.5, 125, 250, 500 and 1000 mg/kg. A reference control of chloracetamide was also used. The test was carried out at 21-22°C for 14 days under continuous illumination and with soil moisture content of 35%. Earthworms were observed for mortality and symptoms at day 7 and 14 of the exposure period. Body weights were also recorded at the beginning (day 0) and end (day 14) of the study.

Results

Number of dead worms and body weight change during the study are presented in the Table B.9.6.1.

Table B.9.6.1. Toxicity of flutolanil to Eisenia foetida.

Concentration (mg/kg)	Number of dead worms at day 14	Mean body weight (mg/worms)*		Change in body weight (%)
		Start (day 0)	End (day 14)	
0	0/40	304	233	-23.4
62.5	1/40	294	238	-19.0
125	0/40	272	248	-8.8
250	3/40	244	220	-9.8

Concentration (mg/kg)	Number of dead worms at day 14	Mean body weight (mg/worms)*		Change in body weight (%)
		Start (day 0)	End (day 14)	
500	2/40	217	183	-15.7
1000	0/40	205	172	-16.1

*: Mean value of 10 individuals.

Based on the mortality data, LC_{50} value for flutolanil was determined to be greater than 1000 mg/kg. As compared to the control group, no inhibitory effect on the body weight was observed up to the highest flutolanil concentration at 1000 mg/kg. No abnormal symptoms were detected in live worms. The 14-day LC_{50} value for toxic standard, chloracetamide was 75.19 mg/kg.

Conclusion: The 14-day LC_{50} and value for flutolanil was greater than 1000 mg/kg.

Evaluation (DAR 2006)

The study was well performed and reported and in compliance with GLP. The concentrations studied are nominal values, since analytical measurements of the substrate are not specified in the test guideline. The study is acceptable.

Report:	**CA 8.4.1-03. Wang, Y., Wu, S., Chen, L., Wu, C., Yu, R., Wang, Q. and Zhao, X., 2012**
Title:	Toxicity assessment of 45 pesticides to the epigeic earthworm *Eisenia fetida*
Report no.:	Not applicable
Published:	Yes
GLP:	No
Guidelines:	OECD guideline No. 207 (1984)
Previous evaluation	Submitted for the purpose of renewal
Remark by RMS	Supporting information. See below comments of the RMS.
Conclusion	The 7-day and 14-day LC_{50} values were estimated to be 184.9 and 150.4 mg a.s./kg, respectively.

Executive Summary

This study was conducted to investigate the comparative toxicity of 45 pesticides towards the epigeic earthworm *Eisenia fetida* in a 48-h filter paper contact toxicity test and a 14-day artificial soil toxicity test. The toxicity of flutolanil in artificial soil are presented in this summary.

A preliminary test was conducted to determine the concentration range of the test chemicals in which 0–100% mortality of the earthworm was obtained. To establish the concentration-mortality relationship, earthworms were exposed to at least five different concentrations in a geometric series and a control for each chemical. Acetone was used as a vehicle and a solvent control was included.

Ten earthworms were placed in three 500 mL glass jars pre-filled with treated soil for each concentration. The jars were loosely covered to allow for air exchange and stored at 20 ± 1 ºC with

80–85% relative humidity under 400–800 lux of constant light. Mortality was assessed at 7 and 14 days after treatment.

The 7-day and 14-day LC_{50} values of flutolanil were estimated to be 184.9 and 150.4 mg/kg, respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** Flutolanil
Lot no.: Not reported
Purity: 98%
Manufacturer: Jiangsu Taizhou Baili Chemical Co., Ltd.

2. **Test organism:** Earthworm (*Eisenia fetida*)
Age: Adult with well-developed clitella
Weight: 350-500 mg
Source: College of Animal Sciences, Zhejiang University, China

Soil: Artificial soil according to OECD 1984, mixed with decayed leaves and decomposed pig manure

Temperature: 20 ± 1ºC

Soil water content: 35% maximum water-holding capacity (measured every week)

3. **Treatment:** A preliminary test was conducted to determine the concentration range of the test chemicals in which 0–100% mortality of the earthworm was obtained. To establish the concentration-mortality relationship, earthworms were exposed to at least five different concentrations in a geometric series and a control for each chemical. Acetone was used as a vehicle and a solvent control was included. A toxic control with chloracetamide was also included

4. **Test vessels:** 500 mL glass jar (surface area, 63.6 cm^2)
Artificial soil: 10% ground sphagnum peat (< 0.5 mm), 20% kaolinite clay (> 50% kaolinite), and 70% fine sand
pH: 6.0 ± 0.5

Water content: Adjusted to 35% of the dry weight

5. **Environmental conditions**

B. STUDY DESIGN AND METHODS

1. Test organism assignment and treatment

The artificial soil test was performed according to the OECD guideline No. 207 (1984).

Before the dose-response test, earthworms were preconditioned on wet filter paper for 24 h at 20 ± 1 ºC in the dark for purging of the gut contents.

A preliminary test was conducted to determine the concentration range of the test chemicals in which 0–100% mortality of the earthworm was obtained. To establish the concentration-mortality relationship, earthworms were exposed to at least five different concentrations in a geometric series and a control for each chemical. Acetone was used as a vehicle and a solvent control was included. The mortality in the controls should not exceed 10% at the end of either test.

A total of 0.65 kg of each treated soil (equivalent to 0.5 kg dry artificial soil) was placed in three 500 mL glass jars for each concentration, then 10 adult earthworms were added to each jar. The jars were loosely covered with polypropylene lids to allow for air exchange and stored at 20 ± 1 ºC with 80–85% relative humidity under 400–800 lux of constant light. Mortality was assessed at 7 and 14 days after treatment.

2. Dose preparation

For each tested concentration, the desired amount of pesticide was dissolved in 10 mL acetone and mixed with a small quantity of fine quartz sand. The sand was mixed for least 1 h to evaporate the acetone and was then mixed thoroughly with the pre-moistened artificial soil in a household mixer. The final moisture contents of the artificial soil were adjusted to the described level by the addition of distilled water.

3. Measurements and observations

Mortality was assessed at 7 and 14 days after treatment. An earthworm was considered dead if it failed to respond to a gentle mechanical touch on the front end.

4. Statistics

A probit analysis was conducted to assess the acute toxicity of pesticides to *E. fetida* using a program developed by Chi (Chi, 1997)[6]. The significant level of mean separation ($p < 0.05$) detected was based on the lack of overlap between the 95% confidence limits of 2 LC_{50} values (Prabhaker et al., 2011)[7].

II. RESULTS AND DISCUSSION

A. Biological results

In a soil toxicity test with *Eisenia fetida*, the 7-day and 14-day LC_{50} of flutolanil were estimated to be 184.9 and 150.4 mg/kg, respectively.

[6] Chi, H., 1997. Computer Program for the Probit Analysis. National Chung Hsing University, Taichung, Taiwan.

[7] Prabhaker, N., Castle, S.J., Naranjo, S.E., Toscano, N.C., Morse, J.G., 2011. Compatibility of two systemic neonicotinoids, imidacloprid and thiamethoxam, with various natural enemies of agricultural pests. J. Econ. Entomol. 104, 773–781.

B. Toxicity endpoints

The toxicity endpoints of flutolanil to *Eisenia fetida* are presented in the following table.

7-day and 14-day LC_{50} of flutolanil to *Eisenia fetida* in a soil toxicity test

Pesticide	7-day interval		14-d interval	
	Slope (SE)	LC_{50} (95% CI) mg/kg	Slope (SE)	LC_{50} (95% CI) mg/kg
Flutolanil	8.70 (1.48)	184.9 (167.7 – 209.1)	7.02 (1.08)	150.4 (132.0 – 169.7)

III. CONCLUSION

The acute toxicity of flutolanil to the earthworm *Eisenia fetida* was determined in a soil toxicity test.

The 7-day and 14-day LC_{50} values were estimated to be 184.9 and 150.4 mg/kg, respectively.

Evaluation RMS

The study was conducted in accordance with OECD 207. The mortality in the controls was stated not to exceed 10% at the end of the test (no actual data shown).

The 7-day and 14-day LC_{50} values were estimated to be 184.9 and 150.4 mg a.s./kg, respectively.

Wang, Y., Wu, S., Chen, L., Wu, C., Yu, R., Wang, Q. and Zhao, X.	2012	Toxicity assessment of 45 pesticides to the epigeic earthworm *Eisenia fetida*	Chemosphere 88 (2012) 484–491
Reliability			
General information			
Is a guideline method or modified guideline used?*		Yes	
Is the test performed under GLP conditions?*		No	
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?		Yes, mortality in the control was stated to be <10%	
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?		Yes	
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?		Yes	
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?		Yes	
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?		Not applicable	

Test organism	
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	Yes
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	Yes
Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Not applicable
Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	Not applicable
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	The number of replicates used was lower than recommended in the guideline (i.e. 3 instead of 4), but the number of earthworms per replicate was sufficient (i.e. 10 earthworms/replicate). The confidence intervals calculated for the LC50 indicate that the number of replicates was sufficient (i.e. relatively small confidence intervals).
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	Yes, considering the normalised width of the confidence intervals (0.2 and 0.3 for 7 and 14 days, respectively), the LC50 is a good estimate, indicating that a dose response must have been observed.
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	No, no raw data were reported
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	

Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Yes, although acute toxicity to earthworms is no longer a data requirement
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes, considering the NW of the confidence interval, the calculated LC50 was reliable
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable
Concluding weight of evidence/proposed action	The study is reliable with restrictions. The study was not conducted according to GLP and the raw data were not reported. The study is relevant with restrictions as acute toxicity to earthworms is no longer a data requirement.
Type of information (Fully acceptable, supporting information, not applicable)	Supporting information
Consideration/concluding score	Supporting information

B.9.4.1 Earthworm – sub-lethal effects

Study CA 8.4.1-01 Earthworm reproduction

Report:	**CA 8.4.1-01 Lührs, U., 2000**
Title:	Effects of EXP10066A on Reproduction and Growth of Earthworms *Eisenia fetida* (Savigny 1826) in Artificial Soil
Report no.:	8411022 (N-3022)
Published:	No
GLP:	Yes (OECD)
Guidelines:	ISO 11268-2 (1998) and BBA VI 2-2 (1994)
Previous evaluation	In DAR (2006) for original approval.
Remark by RMS	Fully acceptable. The study summary from the DAR is replaced with an updated (extended) version. Re-evaluation for the purpose of renewal is presented below.
Endpoint	$NOEC_{reproduction}$ = 12.9 mg a.s./kg soil dw (10% peat)

Executive Summary

Adult mortality, growth, reproduction and feeding activity were evaluated in a dose response test during a 4-week exposure to artificial soil treated with the test item EXP10066A. Under laboratory conditions *Eisenia fetida* (40 worms per treatment group) were exposed to the following concentrations of EXP10066A which was mixed into the soil at the concentrations of 8.07 mg product/kg, 16.1 mg product/kg, 32.3 mg product/kg, 64.5 mg product/kg and 129.1 mg product/kg (corresponding to 3.2 mg a.s./kg; 6.5 mg a.s./kg; 12.9 mg a.s./kg; 25.8 mg a.s./kg and 51.7 mg a.s./kg artificial soil dry weight). Derosal SC 360 g/L (7.5 mg /kg artificial soil) was used as toxic standard and the control was treated with deionized water. The test was comprised of four replicates of 10 adult earthworms for each treatment rate, toxic standard and the control. The test organisms were exposed for 28 days to an untreated control and soil treated with EXP10066A at five nominal rates of 3.2, 6.5, 12.9, 25.8 and 51.7 mg flutolanil/kg soil dry weight.

On day 28, all adult earthworms were removed from the soil and adult mortality, biomass change and behavioural abnormalities were assessed. The soil with cocoons was incubated up to day 56 to allow hatching of the juvenile worms. On day 56, the number of juveniles per replicate was assessed. The toxic reference (32.8% w/w Carbendazim) was tested in the testing facility as a separate study.

The NOEC for *Eisenia fetida* based on mortality, growth and feeding activity was determined to be equal or greater than 51.7 mg flutolanil/kg soil dry weight.

The NOEC for *Eisenia fetida* based on reproduction was determined to be 12.9 mg flutolanil/kg soil dry weight. The LOEC was estimated at 25.8 mg flutolanil/kg soil dry weight and the EC_{50} was not calculated but is determined to be greater than 12.9 mg a.s./kg artificial soil dry weight.

In a report amendment, the EC_{10} could not be determined and EC_{20} was estimated to be 4.41 mg a.s./kg. Since 95% limits could not be determined, this endpoint was not considered to be reliable.

MATERIALS AND METHODS

A. MATERIALS

1. Test material: EXP10066A
Batch no.: OP200006
Purity: 454 g/L (analysed), 449 g a.s./L (nominal)
Description: White opaque liquid
Expiry date: 19 Apr. 2002

2. Reference material: Derosal SC 360 g/L
Batch: CO 1350029
Purity: 32.8 % (w/w) Carbendazim
Ref. concentration: 7.5 mg Derosal SC/kg (corresponding to 2.46 mg a.s./kg)

3. Vehicle: Deionised water

4. **Test substrate:** Artificial soil prepared according to OECD 207 with 10% peat
Test units: Plastic boxes (18.3 cm x 13.6 cm x 6 cm), filled with *ca*.500 g dry soil; plus *ca*.140 g water, plus *ca*. 5 g food, height of soil *ca*.5-6 cm

5. Test organism
Species: Earthworm *Eisenia fetida,* ssp. *andrei* (Savigny 1826)
Age: Adults, ca. 6 months, with clitellum, age range between test individuals not differing for > 4 weeks
Source: In-house laboratory culture bred under standardised conditions (see OECD 207) by IBACON
Weight: 350 to 561 mg differing for > 200 mg (deviation +11 mg)
Acclimation: One day in artificial soil, under test conditions
Diet: finely ground cattle manure

6. **Treatment groups:** 0 (untreated soil), 8.07, 16.1, 32.3, 64.5 and 129.1 mg product/kg soil d.w. or 3.2, 6.5, 12.9, 25.8 and 51.7 mg a.s./kg soil d.w. and toxic standard (2.46 mg a.s./kg soil dw).

7. Environmental conditions:

Temperature: 18-20°C
pH of soil: 5.8 to 5.9 at test start and 5.9 to 6.0 at test termination
Water content of soil: 26.3 % - 28.0 % (at start, corresponding to 48.8 % - 52.0 % of the total water holding capacity) 26.9 % to 30.9 % (at termination corresponding to 49 .9 % to 57.4 % of the water holding capacity)

Photoperiod: 16 hours light: 8 hours darkness (450 – 590 lux)

B. STUDY DESIGN AND METHODS

1. In-life dates: 16 May to 17 July 2000

2. Animal assignment and treatment

A reproduction and growth toxicity laboratory study with the earthworm *Eisenia fetida* was conducted for 8 weeks. The adult test organisms were exposed for 28 days to an untreated control and to the test item EXP10066A at five nominal rates of 8.07, 16.1, 32.3, 64.5 and 129.1 mg product/kg soil d.w. or 3.2, 6.5, 12.9, 25.8 and 51.7 mg flutolanil/kg soil d.w. and a toxic standard (7.5 mg Derosal SC/kg).

The test was comprised of four replicates of 10 adult earthworms for each treatment rate and for the control. Washed, dried on filter paper, weighed individually; after application the worms were placed on the surface of the artificial soil.

3. Dose preparation

A stock solution of EXP10066A was prepared by emulsifying 1.25 g of EXP10066A in 250 mL deionized water. To reach a homogeneous emulsion a magnetic stirrer was used. Appropriate amounts of solution were added to the artificial soil and were mixed (with a laboratory mixer). In the course of applying the test item the soil was ventilated and moistened.

For the preparation of the toxic standard spraying dilution 50 mg Derosal SC were suspended as described above. Deionised water was used as control.

4. Measurements and observations

Adult mortality and biomass change was assessed after 28 days of exposure to the test item, and reproduction (juveniles per vessel) was assessed after 56 days of exposure for test rates and control.

5. Statistics

Body weight changes and reproduction data were tested for normal distribution and homogeneity of variance using Kolmogoroff-Smirnov-test and Cochran's-test. Because data of body weight changes and reproduction were normally distributed Dunnett-test (multiple comparison, one sided), $\alpha = 0.05$ was used. The software used to perform the statistical analysis was EASY ASSAY, Multiple Testing, © SPiRiT, Version 4.0. In a report amendment, the determination of the EC value was determined by Logit Analysis. The software used to perform this evaluation was ToxRat Professional, Version 2.10.05, ToxRat® Solutions GmbH.

II. RESULTS AND DISCUSSION

A. Biological data

During the four weeks of exposure, none of the adult worms died in any of the treatment groups of 3.2, 6.5, 12.9, 25.8 and 51.7 mg a.s./kg soil dry weight. Mean control mortality was also 0.0 %. Mean number of juveniles produced in the control group was 246 for the four replicates (>30 worms per container), coefficient of variance 20.5%. The biomass of the controls increased by 45.5% (did not exceed 20%).

Biomass development was not statistically significantly different from the control in any of the test item treatment groups.

Reproduction of the test organisms was not statistically significantly reduced in the concentrations of 3.2, 6.5 and 12.9 mg a.s./kg soil d.w., however the reduction in reproduction was significant at the test levels of 25.8 and 51.7 mg a.s./kg soil dry weight.

In all treatment groups food was consumed. The results show that the turnover of biomass of those earthworms exposed to the five different rates of the test item was comparable to the control whereas the food intake in the toxic standard group appeared to be reduced. It can be assessed that applications of 3.2 mg a.s./kg to 51.7 mg a.s./kg of EXP10066A do not lead to a reduction of food consumption of earthworms *E. fetida*.

Additionally, no behavioural abnormalities were observed. All validation criteria were met and results of the rate response testing are given in the table below.

Summary of effects of EXP10066A on mortality, body weight change and reproduction of earthworms

Measurement	Test concentration (mg a.s./kg soil d.w.)						
	Control	3.2	6.5	12.9	25.8	51.7	Toxic standard
Mean mortality (%) after a 4-week exposure	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mean increase in weight (%) of adults	45.5	44.1	39.6	49.7	44.6	38.3	24.5 *
Mean number of juveniles/replicate	246±50	186±30	201±53	197±18	162±38*	155±23*	4±2 *
% reduction in reproduction	n.a.	24.4	18.3	19.9	34.1*	37.0*	98.4*
Mean food added (g) (weeks 1-4)	23	22	22.5	23	23	21	17.5

n.a. Not applicable
* significant compared to the control, Dunnett-test, α = 0.05

The treatment with Derosal SC 360 g/L at a concentration of 7.5 mg/kg soil dry weight resulted in a statistically significant reduction in reproduction and a statistically significant mean body weight change of *E. fetida* (t-test, $p \leq 0.05$) confirming the sensitivity of the test system.

B. Toxicity endpoints

Summary of endpoints for flutolanil on reproduction of *Eisenia fetida*

	Endpoints (mg flutolanil/kg soil dry weight)
NOEC	12.9
LOEC	25.8
EC_{10}	ND
EC_{20} (95% CI)	4.41 (ND)
EC_{50}	> 12.9

ND: could not be determined

III. CONCLUSION

In a dose response test during a 28-day exposure of earthworms to artificial soil treated with the test item EXP10066A adult mortality, biomass change, feeding activity and reproductive effects were evaluated. The results of this study showed no effects on mortality, growth and feeding activity of the earthworms *E. fetida* exposed to EXP10066A in concentrations of 3.2 to 51.7 mg a.s./kg. The reproduction of the worms was not affected in the treatment groups of 3.2 to 12.9 mg a.s./kg, but significant reduction of reproduction was observed at the higher concentrations of 25.8 mg a.s./kg and 51.7 mg a.s./kg. Therefore, the NOEC was determined to be 12.9 mg a.s./kg soil dry weight. The LOEC was estimated at 25.8 mg a.s./kg soil dry weight and the EC_{50} was not calculated and was determined to be greater than 12.9 mg/kg soil dry weight.

No mortality was observed in the control group and in any of the test item treatment groups. Biomass development was not statistically significantly different from the control in any of the test concentrations of EXP10066A and reproduction was affected up to the concentrations of 25.8 mg flutolanil/kg soil dry weight.

In a report amendment, the EC_{10} could not be determined and EC_{20} was estimated to be 4.41 mg a.s./kg. Since 95% limits could not be determined, this endpoint was not considered to be reliable.

Evaluation RMS

The test was conducted according to ISO 11268-2 (1998) and BBA VI 2-2 (1994) guidelines and in general agreement with OECD 222 with the exception that only four replicates were used in the control instead of eight. Furthermore, according to the draft update of OECD 222 (June 2015), five treatment groups should be presented rather than four. The validity criteria were met for the control (adult mortality in the first 4 weeks was 0%, each replicate produced > 30 juveniles and CV of reproduction is 20% (< 30%)). Statistically significant effects were observed for the reference substance at 2.46 mg carbendazim/kg soil dw (1-5 mg a.s./kg). The study was considered acceptable.

It is agreed with the applicant that the data do not lead to the estimation of reliable ECx values due to the lack of a clear dose-response.

Reliability of endpoints

According to Regulation (EU) No 283/2013, the statistical power of the NOEC shall be assessed in case no ECx values can be derived. However, this is not possible as long as default type I and II errors have not been fixed, default variance for any response variable have not been established and typical detectable effect sizes of each parameter have not been calculated (EFSA Supporting publication 2015:EN-924). Reproduction was reduced at all concentrations, but this was only statistically significant at the highest two concentrations. Considering the lack of a clear dose response at the lowest three concentrations, it is considered acceptable to set the NOEC at the highest concentration without a statistically significant effect.

The NOEC of 12.9 mg a.s./kg soil dw based on 10% peat may be used for risk assessment.

Lührs, U.	2000	Effects of EXP10066A on Reproduction and Growth of Earthworms *Eisenia fetida* (Savigny 1826) in Artificial Soil	Report No. 8411022 (N-3022)

Reliability	
General information	
Is a guideline method or modified guideline used?*	Yes
Is the test performed under GLP conditions?*	Yes
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?	Yes
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?	Yes. Control has less replicates than OECD 222.
* these criteria are of minor importance for study reliability, but may support study evaluation	
Test compound	
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?	Yes
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	Yes
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	The a.s. content is known. The effects should be considered the result of the formulated product.
Test organism	
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	Yes
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	Yes
Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Not applicable
Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance	Not applicable

over the duration of the study?	
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes for treatments, no for control As the CV of reproduction in the control is 20% (< 30% according to OECD 222), the test is considered acceptable.
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	Yes
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Yes
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable

Concluding weight of evidence/proposed action	Study is reliable without restrictions. Study is relevant without restrictions.
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable
Consideration/concluding score	Fully acceptable

Study CA 8.4.1-02 Earthworm reproduction

Report:	**CA 8.4.1-02 Lührs U., 2001**
Title:	Effects of EXP10066A on Reproduction and Growth of Earthworms *Eisenia fetida* (Savigny 1826) in Artificial Soil with reduced organic matter content
Report no.:	8414022 (N-3023)
Published:	No
GLP:	Yes (OECD)
Guidelines:	ISO 11268-2 (1998) and BBA VI 2-2 (1994)
Previous evaluation	In DAR (2006) for original approval.
Remark by RMS	Fully acceptable. The study summary from the DAR is replaced with an updated (extended) version. Re-evaluation for the purpose of renewal is presented below.
Endpoint	NOEC = 25 mg a.s./kg soil dw (5% peat)

Executive Summary

Adult mortality, growth, reproduction and feeding activity were evaluated in a dose response test during a 4-week exposure to artificial soil treated with reduced organic matter content and the test item EXP10066A. Under laboratory conditions *Eisenia fetida* (40 worms per treatment group) were exposed to the following concentrations of EXP10066A which was mixed into the soil at the concentrations of 32.41, 47.37, 62.33, 94.75 mg product/kg soil dry weight (corresponding to 13, 19, 25 and 38 mg flutolanil/kg soil dry weight). Derosal SC 360 g/L (7 mg /kg artificial soil) was used as toxic standard and the control was treated with deionized water. The test was comprised of four replicates of 10 adult earthworms for each treatment rate, toxic standard and the control. The test organisms were exposed for 28 days to an untreated control and soil treated with EXP10066A at four nominal rates of 13, 19, 25 and 38 mg flutolanil/kg soil dry weight.

On day 28, all adult earthworms were removed from the soil and adult mortality, biomass change and behavioural abnormalities were assessed. The soil with cocoons was incubated up to day 56 to allow hatching of the juvenile worms. On day 56, the number of juveniles per replicate was assessed. The toxic reference (31.1% w/w Carbendazim) was tested in the testing facility as a separate study.

The NOEC for *Eisenia fetida* based on mortality, growth, feeding activity and reproduction was determined to be equal or greater than 38 mg flutolanil/kg soil dry weight. The LOEC was estimated to be greater than 38 mg flutolanil/kg soil dry weight and the EC_{50} could not be calculated.

In a report amendment, the EC_{10} and EC_{20} were estimated to be 33.60 mg a.s./kg and 13.96 mg a.s./kg, respectively. Since 95% limits could not be determined, these endpoints were not considered to be reliable.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: EXP10066A
Batch no.: OP200006
Purity: 454 g/kg (analysed), 449 g a.s./L (nominal)
Description: White opaque liquid
Expiry date: 19 Apr. 2002

2. Reference material: Derosal SC 360 g/L
Batch: ABCA0410
Purity: 31.1 % (w/w) Carbendazim
Ref. concentration: 7 mg Derosal SC/kg (corresponding to 2.18 mg a.s./kg)

3. Vehicle: Deionised water

4. Test substrate: Artificial soil prepared according to OECD 207 with 5% peat (organic matter content)
Test units: Plastic boxes (18.3 cm x 13.6 cm x 6 cm), filled with *ca*.500 g dry soil; plus *ca*.110 g water, plus *ca*.5 g food, height of soil *ca*.3-4 cm

5. Test organism
Species: Earthworm *Eisenia fetida,* ssp. *andrei* (Savigny 1826)
Age: Adults, *ca*. 8 months, with clitellum, age range between test individuals not differing for > 4 weeks
Source: In-house laboratory culture bred under standardised conditions (in a breeding medium of cattle manure, peat, sand and straw, fed with cattle manure, stored at room temperature) by IBACON
Weight: 401 to 600 mg; the body weight range did not differ by more than 200 mg within this range
Acclimation: One day in artificial soil, under test conditions
Diet: finely ground cattle manure

6. Treatment groups: 0 (untreated soil), 32.41, 47.37, 62.33 and 94.75 mg product/kg soil d.w. or 13, 19, 25 and 38 mg flutolanil/kg soil d.w. and toxic standard

7. Environmental conditions:
Temperature: 19-22°C
pH of soil: 6.1 to 6.3 at test start and 6.3 to 6.4 at test termination
Water content of soil: 22.2 % - 22.6 % (at start) 22.8 % to 29.1 % (at termination)
Photoperiod: 16 hours light: 8 hours darkness (439 – 774 lux)

B. STUDY DESIGN AND METHODS

1. In-life dates: December 14 to February 12, 2001

2. Animal assignment and treatment

A reproduction and growth toxicity laboratory study with the earthworm *Eisenia fetida* was conducted for 8 weeks. The adult test organisms were exposed for 28 days to an untreated control and to the test item EXP10066A at four nominal rates of 32.41, 47.37, 62.33 and 94.75 mg product/kg soil d.w. or 13, 19, 25 and 38 mg flutolanil/kg soil d.w. and a toxic standard (7 mg Derosal SC/kg).

The test was comprised of four replicates of 10 adult earthworms for each treatment rate and for the control. Washed, dried on filter paper, weighed individually; after application the worms were placed on the surface of the artificial soil.

3. Dose preparation

A stock solution was prepared by adding 0.5 g of EXP10066A in 1L of deionized water. Appropriate amounts of solution were added to 2050 g of artificial soil to prepare the target nominal concentrations and were mixed for *ca*. 5 min (with a laboratory mixer). In the course of applying the test item the soil was ventilated and moistened. The control was treated with deionized water. 100 g of the toxic standard suspension containing 0.144 mg/mL were also added to 2050 g dry artificial soil to prepare the toxic standard group.

4. Measurements and observations

Adult mortality and biomass change was assessed after 28 days of exposure to the test item, and reproduction (juveniles per vessel) was assessed after 56 days of exposure for test rates and control. During the 4 weeks of exposure one adult worm died in the test item treated concentration of 13 mg a.s./kg. In the control no worm died. The body weights in the test item treated group changed by -0.3% to 6.0% compared to 5.3% in the control. The reproduction ranged from 226 to 304 juvenile worms in the groups treated with test item. The quantity of food consumed was 25.0 g in the control and 24.0 g in the test item treated groups.

5. Statistics

Data of mortality were analysed for significance by using Fisher-exact-test (two-sided, $\alpha = 0.05$). Data of body weight changes and reproduction were tested for normal distribution and homogeneity of variance using Kolmogoroff-Smirnov-Test and Cochran-Test. Because body weight changes and reproduction data of the worms exposed to the test item were normally distributed and homogeneous Dunnett-test (multiple comparison, two-sided), $\alpha = 0.05$ was used.

Because data of body weight changes and reproduction of the worms exposed to the toxic standard were normally distributed and homogeneous Student-t-test for homogeneous variances (pairwise comparison, two-sided), $\alpha = 0.05$ was used. The software used to perform the statistical analysis was SYST AT 9 for Windows and EASY ASSAY, Multiple Testing, © SPiRiT, Version 4.0.

The determination of the EC value was determined by Logit Analysis. The software used to perform this evaluation was ToxRat Professional, Version 2.10.05, ToxRat® Solutions GmbH.

II. RESULTS AND DISCUSSION

A. Biological data

During the four weeks of exposure, none of the adult worms died in any of the treatment groups of 19, 25 or 38 mg a.s./kg soil dry weight. Mortality of 2.5% was observed at the concentration of 13 mg a.s./kg, where only one adult worm was found dead and this was not considered to be treatment related. Mean control mortality was also 0.0 %. Mean number of juveniles produced in the control group was 311 for the four replicates (>30 worms per container), coefficient of variance 15.2%. The biomass of the controls increased by 5.3% (did not exceed a biomass loss of 20%).

Biomass development was not statistically significantly different from the control in any of the test item treatment groups. Reproduction of the test organisms was not statistically significantly reduced in any of the concentrations tested at 13, 19, 25 and 38 mg a.s./kg soil dry weight.

In all treatment groups, food was consumed. The results show that the turnover of biomass of those earthworms exposed to the five different rates of the test item was comparable to the control whereas the food intake in the toxic standard group appeared to be reduced. It can be assessed that applications of 13 mg a.s./kg up to 38 mg a.s./kg of EXP10066A do not lead to a reduction of food consumption of earthworms *E. fetida*.

Additionally, no behavioural abnormalities were observed. All validation criteria were met. Results of the rate response testing are given in the table below.

Summary of effects of EXP10066A on mortality, body weight change and reproduction of earthworms

Measurement	Test concentration (mg flutolanil/kg soil d.w.)					
	Control	**13**	**19**	**25**	**38**	**Toxic standard**
Mean mortality (%) after a 4-week exposure	0.0	2.5	0.0	0.0	0.0	0.0
Mean increase in weight (%) of adults	5.3	-0.2	0.0	6.0	-0.3	-8.0*
Mean number of juveniles/replicate	311±47	270±86	254±82	304±44	226±24	56±13*
% reduction in reproduction	n.a.	13.2	18.3	2.25	27.3	82.0*
Mean food added (g) (weeks 1-4)	25	24	24	24	24	19

n.a. Not applicable
* significant compared to the control, Student t-test, α = 0.05

The treatment with Derosal SC 360 g/L at a concentration of 7 mg/kg soil dry weight resulted in a statistically significant reduction in reproduction and a statistically significant mean body weight change of *E. fetida* (t-test, $p \leq 0.05$) confirming the sensitivity of the test system.

B. Toxicity endpoints

Summary of endpoints for flutolanil on reproduction of *Eisenia fetida*

	Endpoints (mg flutolanil/kg soil dry weight)
NOEC	38
LOEC	> 38
EC_{10} (95% CI)	13.96 (ND)
EC_{20} (95% CI)	33.60 (ND)
EC_{50}	> 38

ND: Could not be determined

III. CONCLUSION

In a dose response test during a 28-day exposure of earthworms to artificial soil treated with the test item EXP10066A adult mortality, biomass change, feeding activity and reproductive effects were evaluated. The results of this study showed no effects on lethal, sub-lethal or reproduction of the earthworms *E. fetida* exposed to EXP10066A in concentrations of 13 mg a.s./kg up to 38 mg a.s./kg. Therefore, the NOEC was determined to be 38 mg a.s./kg soil dry weight (the highest nominal concentration tested). The LOEC was estimated to be greater than 38 mg a.s./kg soil dry weight and the EC_{50} could not be calculated and was estimated to be greater than 38 mg a.s./kg soil dry weight.

No mortality was observed in the control group and in any of the test item treatment groups. Biomass development was not statistically significantly different from the control in any of the test concentrations of EXP10066A and reproduction was unaffected up to the concentrations of 38 mg flutolanil/kg soil dry weight.

In a report amendment, the EC_{10} and EC_{20} were estimated to be 13.96 mg a.s./kg and 33.60 mg a.s./kg, respectively. Since 95% limits could not be determined, these endpoints were not considered to be reliable.

Evaluation RMS

The test was conducted according to ISO 11268-2 (1998) and BBA VI 2-2 (1994) guidelines and in general agreement with OECD 222 with the exception that only four replicates were used in the control instead of eight and the artificial soil contained only 5% peat instead of 10%. Furthemore, according to the draft update of OECD 222 (June 2015), five test concentrations should be used rather than four. The validity criteria were met for the control (adult mortality in the first 4 weeks was 0%), each replicate produced > 30 juveniles and CV of reproduction is 15% (< 30%)). Statistically significant effects were observed for the reference substance at 2.18 mg carbendazim/kg soil dw (1-5 mg a.s./kg soil dw). The study was considered acceptable.
It is agreed with the applicant that the data do not lead to the estimation of reliable ECx values due to the lack of a clear dose-response.
According to Regulation (EU) No 283/2013, the statistical power of the NOEC shall be assessed in case no ECx values can be derived. However, this is not possible as long as default type I and II errors have not been fixed, default variance for any response variable have not been established and typical detectable effect sizes of each parameter have not been calculated (EFSA Supporting publication 2015:EN-924). Reproduction was reduced at all concentrations, but without statistical significance. Variation was high in all treatments, and there was no clear dose response. Without further analysis of the varation and the detectable effect sizes for this test, it is not possible to say what level of effect on reproduction could be detected. Based on an almost 30% reduction of reproduction at the highest test concentration of 38 mg a.s./kg soil dw compared to a 2% reduction at the next lower level of 25 mg a.s./kg soil dw, the NOEC is set at 25 mg a.s./kg soil dw. This is considered conservative and sufficiently protective.

The NOEC of 25 mg a.s./kg soil dw based on 5% peat may be used for risk assessment.

Lührs, U.	2001	Effects of EXP10066A on Reproduction and Growth of Earthworms *Eisenia fetida* (Savigny 1826) in Artificial Soil with reduced organic matter content	Report No. 8414022 (N-3023)

Reliability	
General information	
Is a guideline method or modified guideline used?*	Yes
Is the test performed under GLP conditions?*	Yes
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?	Yes
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?	Yes, but control has less replicates than OECD 222
* these criteria are of minor importance for study reliability, but may support study evaluation	
Test compound	
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?	Yes
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	Yes
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	The a.s. content is known. The effects should be considered the result of the formulated product.
Test organism	
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	Yes
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	Yes
Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Not applicable

Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	Not applicable
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes for treatments, no for control As the CV of reproduction in the control is 15% (< 30% according to OECD 222), the test is considered acceptable.
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	No clear dose-response. Effect of 27% but no statistical significance.
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Yes
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	No, but as the effect at the highest dose is 27%, this is considered relevant. The NOEC was set at the next lower dose with an effect of 2%.
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and	Yes

appropriate for the studied endpoints and species?	
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable
Concluding weight of evidence/proposed action	The study is reliable without restrictions . The study is relevant without restrictions.
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable
Consideration/concluding score	Fully acceptable

B.9.4.2 Effects on non-target soil meso- and macrofauna (other than earthworms)

Study CA 8.4.2-01 Collembola reproduction

Report:	**CA 8.4.2-01 Meister, A., Lührs, U., 2002**
Title:	Effects of EXP100066A on Reproduction of the Collembola *Folsomia candida* in Artificial Soil
Report no.:	10694016 (N-3010)
Published:	No
GLP:	Yes
Guidelines:	ISO 11267 (1999)
Previous evaluation	In DAR (2006) for original approval.
Remark by RMS	Fully acceptable. The study summary from the DAR is replaced with an updated (extended) version. Re-evaluation for the purpose of renewal is presented below.
Endpoint	No LCx values could be determined. $NOEC_{survival}$ < 10.4 mg a.s./kg soil dw (10% peat) $NOEC_{reproduction}$ = 37.6 mg a.s./kg soil dw (10% peat)

Executive Summary

The effects (survival and reproductive output), of EXP10066A to the hexapod, *Folsomia Candida*, were determined in an artificial soil for 28 days. Ten collembola per replicate were used in a dose-response study design comprising five treatment rates and a control. Five replicates for the control, and for each treatment rate of 0 (control), 10.4, 19.8, 37.6, 71.4 and 135.7 mg a.s./kg dry soil (nominal) were used. The reproduction rate and survival rate were assessed and compared with corresponding parameters recorded in the untreated group at the end of the test.

No analysis for verification of achieved concentration was undertaken. Effects were reported based on nominal treatment rates.

Significant effects on adult survival was observed in all treatment groups. The NOEC for mortality was therefore determined to be 37.6 mg a.s./kg and the corresponding LOEC to be 71.4 mg a.s./kg dry soil. The EC_{50} for reproduction was determined to be 110.2 mg a.s./kg dry soil.

In a report amendment, the $ER_{10, 20, 50}$ values were determined by Probit analysis and were estimated to be 44.35, 61.34 and 114.1 mg a.s./kg dry soil, respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: EXP10066A
Batch (lot) no.: OP210100
Purity: Flutolanil 458 g/L (CofA)
Description: White opaque liquid

2. Test organism: *Folsomia Candida* (Willem)
Age: 10-12 days
Source: In-house culture
Feeding: Granulated dried yeast

3. Treatment: 0 (control), 10.4, 19.8, 37.6, 71.4 and 135.7 mg a.s./kg dry soil (nominal)
Vehicle: Deionised water
Toxic Reference: Phenmedipham 166 g/L

4. Test vessels: 100 mL glass container (Ø 5cm) closed tightly
Test substrate: OECD 207 (10% peat)
Water Holding Capacity: 65.14% of the dry weight

5. Environmental conditions

Temperature: 18-23°C
pH: 5.6-6.0
Moisture content: 27-41%
Photoperiod: 16 h light : 8 h darkness (400-750 lux)

B. STUDY DESIGN AND METHODS

1. In-life phase: February 04 to March 05, 2002

2. Test organism assignment and treatment

Individual collembola (10-12 days old) were collected with an exhauster, contained in a tube in groups of ten, and then transferred to the treated surface of 30 g ± 5% wet weight artificial soil per replicate. The collembola were fed with granulated dried yeast (~2 mg) at the start of the test and *ad libitum* weekly. Collembola were exposed to the test system for a period of 28 days.

An additional, abiotic, replicate for the determination of pH and water content was set up for each group.

3. Dose preparation

A primary stock mix (1.334 mg/g) was prepared in deionised water. Aliquots of this dilution was mixed for at least 1 minute into 200 g dry artificial soil using a laboratory hand mixer. The control group was similarly prepared with deionised only, and no test item.

4. Measurements and observations

After 28 days incubation, the contents of each replicate was suspended in water tinted with blue ink and stirred with a fine brush. The collembolan (living adult and juveniles) drifted to the surface and were counted twice by binocular microscope.

Vessels were aerated by opening for a short period twice per week. Vessels were checked for water loss (by weight) on Day 14. No compensation for loss was required.

Values of pH and moisture content were determined at the start and end of the test from an abiotic satellite vessel per treatment group.

5. Statistics

The determination of the EC_x values was calculated by moving average analysis with averages over 2 values using the software EASY ASSAY version 3.0 ©. Mortality data was assessed for significance, at the 5% level, by using Fisher's Exact Test (one-sided). Reproduction data were tested for normal distribution and homogeneity of variance using Kolmogoroff-Smirnov and Cochran test. Further comparison was undertaken using Dunnett-test and Student t-test at the 5% level. Statistical analysis was undertaken with SYSTAT © and TOXRAT Pro 1.08 ©.

In a report amendment, the determination of the $ER_{10, 20, 50}$ were performed by Probit Analysis. The software used to perform this evaluation was ToxRat Professional, Version 2.10.05, ToxRat® Solutions GmbH.

II. RESULTS AND DISCUSSION

A. Biological data

The evaluated parameters were adult survival and the number of living offspring produced by each replicate.

Mean Adult Mortality and Juvenile Production with % inhibition

Nominal concentration (mg a.s./kg dry soil)	Replication	Adult Mortality (%) ± s.d. (%)	Juvenile Production ± s.d.	% of control Juvenile Production
0 (control)	5	10 ± 0	568.1 ± 112.4	--
10.4	5	34 ± 9*	638.7 ± 77.7	112.4
19.8	5	30 ± 10*	621.3 ± 117.2	109.4
37.6	5	32 ± 11*	627.2 ± 62.4	110.4
71.4	5	36 ± 13*	395.2 ± 87.8	69.6*
135.7	5	30 ± 7*	240.4 ± 49.3	42.3*
Toxic std.	5	34 ± 11*	225.5 ± 29.7	39.7*

* $p < 0.05$

s.d. standard deviation

B. Toxicity endpoint

The 28-day mortality and reproductive endpoints for EXP10066A to *Folsomia candida* are presented in the following table.

Endpoints of the test item EXP10066A

Endpoint	Effect concentration (mg a.s./kg dry soil)	28 days
Mortality	NOEC	< 10.4
	LOEC	10.4
	LC_{50}	>10.4
Reproduction	NOEC	37.6
	LOEC	71.4
	EC_{10} (95% CI)	44.35 (30.72 – 54.75)
	EC_{20} (95% CI)	61.34 (48.06 – 71.39)
	EC_{50} (95% CI)	114.1 (101.2 – 132.6)

III. CONCLUSION

Significant effects on adult survival was observed in all treatment groups. The NOEC for mortality was therefore determined to be less than 10.4 mg a.s./kg dry soil and the corresponding ER_{50} for mortality was determined to be greater than 10.4 mg a.s./kg dry soil. The NOEC for reproduction was 37.6 mg a.s./kg and the corresponding ER_{50} for reproduction was determined to be 110.2 mg a.s./kg dry soil.

In a report amendment, the $ER_{10, 20, 50}$ were determined by Probit analysis and were estimated to be 44.35, 61.34 and 114.1 mg a.s./kg dry soil, respectively.

Evaluation RMS

The study was conducted according with ISO 11267 (1999) and was in general agreement with OECD 232 (2016). The deviation from the amount of replicates in the control (5 versus 8) did not invalidate the study.

The validity criteria for the controls were met: mean adult mortality was 10% (< 20%) at the end of the test; the mean number of juveniles per vessel was 568 (> 100) at the end of the test; the coefficient of variation calculated for the number of juveniles was ca. 20% (< 30%) at the end of the test.

Mortality was statistically significantly affected at all tested concentrations but without a dose response. No LC_x values could be determined from these data. The NOEC for mortality was determined to be < 10.4 mg a.s./kg soil dw.

The applicant derived EC_x values for reproduction, but as these are higher than the NOEC of the study, the reliabilities were not evaluated by RMS.

A NOEC could not be derived as mortality was statistically significantly increased by ~30% at all tested concentrations (i.e. NOEC < 10.4 mg a.s./kg soil dw).

Meister, A., Lührs, U.	2002	Effects of EXP100066A on Reproduction of the Collembola *Folsomia candida* in Artificial Soil	Report 10694016 (N-3010)
Reliability			
General information			
Is a guideline method or modified guideline used?*	Yes		
Is the test performed under GLP conditions?*	Yes		
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?	Yes		
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?	Yes		
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?	Yes		
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	Yes		
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	The a.s. content is known. The effects should be considered the result of the formulated product.		
Test organism			
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	Yes		
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	Yes		

Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Not applicable
Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	Not applicable
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes, although the number of replicates used for the control was lower than recommended by OECD 232 (i.e 5 instead of 8). This does not compromise the reliability of the test, as mortality was demonstrated to be statistically significantly induced at all concentrations.
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	The mortality was statistically significant in all treatments without a dose-response.
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes

Are the reported endpoints appropriate for the regulatory purpose?	A NOEC could not be derived as survival was significantly reduced at all concentrations tested.
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable
Concluding weight of evidence/proposed action	Reliable and relevant without restrictions
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable
Consideration/concluding score	Fully acceptable

Study CA 8.4.2.1-01 *Hypoaspis* reproduction

Report:	**CA 8.4.2.1-01 Ganßmann M., 2015**
Title:	MONCUT 40SC (EU): Effects on reproduction of the predatory mite *Hypoaspis aculeifer* in artificial soil with 5% peat
Report no.:	105911089
Published:	No
GLP:	Yes
Guidelines:	OECD 226 (2008)
Previous evaluation	Submitted for the purpose of renewal
Remark by RMS	Fully acceptable. See below comments of the RMS.
Endpoint	NOEC = 407 mg a.s./kg soil dw (5% peat)

Executive Summary

The purpose of this study was to determine the effects of flutolanil on survival and reproductive output of predatory mites, *Hypoaspis aculeifer*, in a defined artificial soil over a 14-day exposure. Soil was treated with Moncut 40SC (40% w/w flutolanil) at five concentrations: 62.5, 125, 250, 500 and 1000 mg test item/kg artificial soil (dry weight) and a control group. There were 4 replicates per treatment group and 8 per control group (each containing 10 female mites). A reference item (dimethoate 400g/L nominal) was also tested at a range of concentrations to evaluate the sensitivity if the test system.

The surviving mites were observed for any abnormalities at the end of the test and missing adult mites were recorded as dead. Juvenile mites at day 14 after application were counted after extraction. These results were assessed and no statistical significance was determined caused by Moncut 40SC. The NOEC value was based on the results of this evaluation and was determined to be 1000 mg/kg artificial soil (dry weight) for adult mortality and reproduction. The LOEC as well as the $LC_{10, 20, 50}$ and $EC_{10, 20, 50}$ were estimated to be greater than 1000 mg MONCUT 40SC/kg artificial soil (dry weight), equivalent to 407 mg a.s.(flutolanil)/kg artificial soil.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test material:**	MONCUT 40SC
	Batch no.:	A111019002
	Purity:	40% w/w, nominal flutolanil, 40.7% w/w, analysed content
2.	**Reference material:**	Perfekthion
	Ref. concentration:	400.0 g dimethoate/L (nominal), 420.3 g/L (analysed)
3.	**Test organism:**	Predatory mite (*Hypoaspis aculeifer*)
	Age:	Adult females (*ca*. 9 days after reaching adult stage)
	Source:	Ibacon Laboratory culture
	Feeding:	Cheese mites (*Tyrophagus putrescentiae*) *ad libitum*
4.	**Treatment:**	Control, 62.5, 125, 250, 500 and 1000 mg/kg soil d.w. (nominal)
	Replicates:	4 per treatment; 8 per control
	Vehicle:	Deionised water
5.		**Test vessels:** Glass vessels (100 mL, diameter 5 cm) with tight screw top closure, filled with 20 g ± 1 g artificial soil dw
	Test soil:	Artificial soil substratum based on OECD 226, max WHC 43% of dw
6.		**Environmental conditions**
	Temperature:	18-22°C
	pH:	5.8-6.3
	Water content:	20.4-22.4%
	Water Holding Capacity:	47.4 – 52.2% of max WHC
	Photoperiod:	16 h light : 8 h darkness (400-800 lux)

B. STUDY DESIGN AND METHODS

1. In-life phase: 28 Oct. 2015 – 13 Nov. 2015

2. Test organism assignment and treatment

Adult female mites (*ca*. 9 days after reaching adult stage) from an in house culture were used in the study. The mites were introduced into the test vessels, after the treated artificial soil was filled in into

the test vessels (n=10). Feeding of the mites *ad libitum* started at the beginning of the test and on days 2, 5, 7 and 9 of the study where additional feed was added to the test vessels.

3. Dose preparation

A stock solution of 8.9286 mg of test item/g was prepared and then serially diluted to prepare the five test concentrations. 22.4g of the stock solution and the corresponding dilutions were added to artificial soil, equivalent to 200 g dry weight to prepare the target concentrations and mixed with a laboratory mixer to ensure a homogeneous distribution. The control was not treated and was moistened with deionised water.

4. Measurements and observations

The number of surviving adult female predatory mites were counted (after extraction) 14 days after the initiation of the test. Missing adult predatory mites were recorded as being dead as it was assumed they would have died and degraded during the test period. Living predatory mites were observed for any morphological or behavioural abnormalities at the end of the test. The number of juvenile mites at day 14 after application were counted twice or three times after extraction.

The temperature and light intensity were recorded. Water content and pH were determined according to ISO 10390 ($CaCl_2$) and ISO 11465 at test start and test end.

5. Statistics

Mortality data were statistically analysed using Fisher's Exact Binomial Test (multiple comparison, with Bonferroni Correction, α = 0.05, one-sided greater). Reproduction data were tested for normal distribution and homogeneity of variance using Shaprio-Wilk's test and Levene's test (α = 0.05). As data were normally distributed and homogeneous, the further statistical evaluation was performed using Williams t-test (multiple comparison, α = 0.05, one sided smaller). ToxRat Professional, v. 2.10.05, was the software used for the statistical analysis.

II. RESULTS AND DISCUSSION

A. Biological data

A mortality of up to 5% was observed in the test item treated groups, which was not statistically significantly different compared to the control, where 3% of the adult mites died (Fisher's Exact Test, α = 0.05, one-sided greater). Reproduction of the predatory mites exposed to Moncut 40SC was not statistically significantly different compared to the control up to and including the highest test concentration of 1000 mg/kg soil (Williams t-test, α = 0.05, one-sided smaller).

No behavioural abnormalities were observed in any of the treatment groups. Th biological test results are presented in the table below:

Test item concentration (mg / kg soil)	Adult Mortality		Reproduction	
	Number of dead adults (# of adults introduced)	Adult mortality (%)	Juveniles per test vessel (mean ± sd)	% of control
Control	2 (80)	3%	273 ± 32	-
62.5	0 (40)	0%	315 ± 40	116
125	0 (40)	0%	337 ± 33	124
250	0 (40)	0%	324 ± 19	119
500	2 (40)	5%	338 ± 22	124
1000	0 (40)	0%	327 ± 17	120

Note: Statistical analysis showed no significant difference concerning the number of juveniles between the control and all concentrations of the test item tested.

The LC_{50} value for the reference item was calculated to be 3.25 mg dimethoate/kg artificial soil (dw). The EC_{50} for reproduction was 3.9 mg dimethoate/kg soil and therefore, the observed effect is within the range expected from the guideline and hence acceptable sensitivity of the test system is assured.

The number of juvenile mites per replicate was 218 to 312 and the mean mortality for the control level was 3%. The coefficient of variation was also acceptable. All validity criteria for the study were met and therefore the study was considered to be acceptable.

B. Toxicity endpoints

The endpoints of Moncut 40SC on the predatory mite *Hypoaspis aculeifer* in a 14-day reproduction study are presented below:

Endpoints	mg MONCUT 40 SC/kg soil (dw)	mg a.s.(flutolanil)/kg soil (dw)
NOEC (Mortality and Reproduction)	1000	407
LOEC (Mortality and Reproduction)	> 1000	> 407
$LC/EC_{10, 20, 50}$	> 1000	> 407

III. CONCLUSION

Moncut 40SC caused no statistical significant effects on mortality or reproduction of *Hypoaspis aculeifer* in any of the concentrations tested up to 1000 mg test item/kg soil.

The No Observed Effect Concentration (NOEC) was determined to be 1000 mg test item/kg soil and the Low Observed Effect Concentration (LOEC), the $LC_{10, 20, 50}$ and the $EC_{10, 20, 50}$ were estimated to be greater than 1000 mg test item/kg soil. This endpoint is equivalent to 407 mg flutolanil/kg soil dry weight.

Evaluation RMS

The study was conducted according to OECD 226 and there were no deviations. The validity criteria for the controls were met: mean adult female mortality was 3% (< 20%) at the end of the test; the mean number of juveniles per replicate (with 10 adult females introduced) was 273 (> 50) at the end of the test; the coefficient of variation calculated for the number of juvenile mites per replicate was 12% (< 30%) at the end of the test.

The NOEC was 407 mg a.s./kg soil dw; the L(E)Cx were all > 407 mg a.s./kg soil dw.

According to Regulation (EU) No 283/2013, the statistical power of the NOEC shall be assessed in case no ECx values can be derived. However, this is not possible as long as default type I and II errors have not been fixed, default variance for any response variable have not been established and typical detectable effect sizes of each parameter have not been calculated (EFSA Supporting publication 2015:EN-924). Survival and reproduction were not reduced at any tested concentration, and therefore the NOEC is considered reliable and sufficiently protective.

The NOEC of 407 mg a.s./kg soil dw may be used in the risk assessment.

Ganßmann M.	2015	MONCUT 40SC (EU): Effects on reproduction of the predatory mite *Hypoaspis aculeifer* in artificial soil with 5% peat	Report 105911089
Reliability			
General information			
Is a guideline method or modified guideline used?*		Yes	
Is the test performed under GLP conditions?*		Yes	
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?		Yes	
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?		Yes	
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?		Yes	
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?		Yes	
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?		The a.s. content is known. The effects should be considered the result of the formulated product.	
Test organism			

Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	Yes
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	Yes
Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Not applicable
Is a correct spacing between exposure concentrations applied?	Yes
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	Not applicable
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	No
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	

Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Yes
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable
Concluding weight of evidence/proposed action	Fully acceptable
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable
Consideration/concluding score	Fully acceptable

Study CA 10.6.2c Litter bags

Report:	**Meister, A. (2002)**
Title:	Effect of EXP10066A on the decomposition of organic material enclosed in litter bags in the field. Institut für Biologische Analytik und Consulting GmbH., Rossdorf, Germany
Report no.:	N-3009
Published:	No
GLP:	Yes (OECD, BBA/Germany)
Guidelines:	Draft Method for Litter-bag test on decomposition, 2001, Deviation: None
Previous evaluation	In DAR (2006) for original approval.
Remark by RMS	Considered acceptable at the time of original inclusion. Evaluation from the DAR is copied as a whole without changes.
Conclusion	22.6 to 30% decomposition reduction with EXP10066A (application rate 15000 and 11300 g a.s./ha)

Material and methods

Effect of EXP10066A containing 454 g/L flutolanil on decomposition of the organic matter was investigated under worst case exposure scenario. The 1st application was conducted on arable site (in Germany; altitude 210 m above sea level, latitude 49°52', longitude 08°44') with mustard at 15 kg a.s./ha, followed by second application with the bags enclosing 3 g oat straw on soil surface at 11.3 kg a.s./ha. The size of the field was 1160 m^2 subdivided into 12 experimental plots, each of 25 m^2 size, 4 plots per treatment group and 2 m distance between the plots. As the toxic standard, Benlate (50% Benomyl) was applied at 4kg a.s./ha in parallel. The bags were buried into the ground after 2nd application and exposed for 30, 66, 138, 244 days. The weight changes of ash-free organic material after straw ignition were examined at the end of exposure period.

Results

The decomposition in the control resulted in 64.0 ± 7.9 % at the end of the study period, thus the validity criteria was fulfilled. Decomposition of organic matter exposed by the test item was significantly lower (22.6 to 30% reduction) compared to control. Exposure by Benlate, on the other hand, did not exert evident effect.

Evaluation RMS (DAR 2006)

The study was well performed and reported and in compliance with GLP. The study was performed before the Lisbon workshop proceedings (EPFES 2002) draft appeared wherefore there are some minor deviations from that draft.

Study CA 10.6.2d Litter bags

Report:	**Rosenkranz, B. 2006**
Title:	Field study on the effects of Flutolanil 40SC on the breakdown of organic matter in litter bags. Institut für Biologische Analytik und Consulting IBACON GmbH., Rossdorf, Germany
Report no.:	20301081
Published:	No
GLP:	Yes
Guidelines:	Draft test guideline: Breakdown of organic matter in litter bags developed during the EPFES workshop, Lisbon, April 2002.
Previous evaluation	In DAR (Addendum 1, October 2006) for original approval.
Remark by RMS	Considered acceptable at the time of original inclusion. Evaluation from the DAR is copied as a whole without changes.
Conclusion	Test duration 616 days. Moncut 40SC (670.5 g a.s./ha) induced significant decomposition reduction only at a second sampling (90 days, 11.6 % reduction).

Material and methods

The effects of Moncut 40SC (Lot No.: 3AE8801F, containing 400.6 g/kg flutolanil) on decomposition of organic matter was investigated in a field experiment in Germany (latitude 49°51'55" north, longitude 8°44*48" east). The study was performed on a middle silty sand soil (clay 6.6-7.6 %, silt 30-32 %, sand 62 %, org. C 0.70 %, pH 4.4-5.0) which had not received pesticides within 2 previous years. The experiment used a randomized block design with six replicates and two variants (12 plots in total). The plot size was 6 x 5 m (30 m^2) with a distance of 3 m between to the adjacent plots and a plot margin (unused) of 1 m.

Moncut 40SC was sprayed onto bare soil with an application rate of 670.5 g flutolanil/ha on May 27, 2004. The application is equivalent to 0.223 mg as/kg soil based on a soil depth of 20 cm or 0.447 mg as/kg based on a soil depth of 10 cm (soil density of 1.5 g/cm^3). The control was left untreated. Clover grass mixture was sown after the application and the soil was mixed with a rotary harrow up to a depth of 10 cm. Control plots were treated in the same way

20 days after the application, on June 15, 2004, litter bags were buried horizontally at a depth of approximately 5 cm in the soil. The bags were filled with 4.0 ± 0.1 g dried wheat straw and they were randomly distributed within the plots with a distance of at least 40 cm from each other. 40 litter bags per plot (32 for scheduled samples plus 8 as a reserve), 230 bags per treatment group were buried.

During the experimental period no fertilizer and no additional pesticide treatments were applied. Approximately 2 months after sowing the vegetation was cut and cut material was left in the field. The test field was mulched four times during the study.

Sampling dates were after 29, 90, 162, 365 and 616 days of exposure and 8 litter bags per plot were retrieved at each sampling date. The sampled bags were dried for 12 h at 35 °C; remaining straw was separated from soil particles, earthworm etc. (manually and by dry sieving) and dry weight was

determined. The straw was then combusted for 30 to 45 min at 600 °C and the ash-free dry weight was determined.

The loss in mass of the organic matter in the treated test plots was determined after each time interval and compared with the loss in mass in control (untreated) plots. The statistical analysis was performed using Kolmogoroff-Smirnov test (check for normal distribution), Cochran test (check for homogeneity of variance) and Student t-test.

(The concentration of flutolanil in the test soil after the application and the deposition rate of the active ingredient following the application were determined by Meinerling & Hermann (2006) (see Study 2 of this Chapter)).

Results

A summary of the results obtained in the litter bag test is presented in Table B.9.7-1.

The decomposition in the test item treated group ranged between 88.4 % of the control (at the 2nd sampling after 90 days of exposure) and 102.3 % (at the 1st sampling after 29 days of exposure). Statistical analysis revealed significance between the mass loss for the control and test item groups only at the 2nd sampling (Student t-test; α=0.05; two-sided). At all other sampling dates deviations to the control were not statistically significant.

The results of the chemical analysis confirmed that the initial exposure concentrations of flutolanil in the soil of the treated plots were within the acceptable range of the nominal values (50 to 150 %, according to EPFES, 2002) after the application (Study 2).

Table B.9.7-1. Effects of Moncut 40 SC on the breakdown of organic matter in litter bags.

Sampling date (days of exposure)	Treatment group	Mean mass loss [%]	SD [%]	Decomposition expressed as % of control (=100)	Decomposition per day[A] [%]	Statistical analysis[B]
July 14, 2004 (29)	Control	11.7	2.0		0.40	
	Test item	12.0	1.9	102.3	0.41	n.s.
September 13, 2004 (90)	Control	42.5	8.5		0.50	
	Test item	37.5	8.4	88.4	0.42	*
November 24, 2004 (162)	Control	48.8	8.4		0.09	
	Test item	45.6	7.8	93.5	0.11	n.s.
June 15, 2005 (365)	Control	56.2	9.5		0.04	
	Test item	52.9	10.2	94.2	0.04	n.s.
February 21, 2006 (616)	Control	73.5	10.6		0.07	
	Test item	69.2	12.0	94.1	0.06	n.s.

The results represent rounded values calculated on the exact raw data.

[A] : between burying the bags and the first sampling and between samplings respectively

[B] : Student t-test, two-sided, α=0.05
n.s.: not significantly different compared to the control
***** : the difference statistically significant

Evaluation RMS (DAR 2006)

Based on the results obtained in the litter bag test it can be concluded that flutolanil 40 SC will not cause long-term adverse impacts on organic matter decomposition under practical field conditions.

According to the recommendations of EPFES workshop special-use patterns should, as far as possible, be assessed in accordance with the proposed agricultural use. In this study the test substance was applied at the amount recommended by EPFES (the plateau concentration plus maximum application rate) but it was not used as seed treatment for potatoes. Otherwise the study was conducted according to recommendations of the EPFES workshop. It was also well reported and in compliance with GLP. Thus the Rapporteur MS considers the results acceptable for risk assessment.

Study CA 10.6.2e Analytical report litter bags

Report:	Meinerling, M. & Hermann, S. 2006
Title:	Final analytical report to: Field study on the effects of Flutolanil 40SC on the breakdown of organic matter in litter bags. Institut für Biologische Analytik und Consulting IBACON GmbH., Rossdorf, Germany
Report no.:	20301081R
Published:	No
GLP:	Yes
Guidelines:	Analytical methods established by the performing laboratory (IBACON GmbH) and the Sponsor (Nihon Nohyaku CO., Ltd.), modified to suit the requirements of this study.
Previous evaluation	In DAR (Addendum 1, October 2006) for original approval.
Remark by RMS	Considered acceptable at the time of original inclusion. Evaluation from the DAR is copied as a whole without changes.
Conclusion	The results of the chemical analysis confirmed that the initial exposure concentrations of flutolanil in the soil of the treated plots were within the acceptable range of the nominal values (50 to 150 %, according to EPFES, 2002) after the application.

Material and methods

The purpose of this study was to verify the application of the test item, Moncut 40SC, in the litter bag study presented in Study 1 of this Chapter. This study was done in two ways:

1. Determination of the deposition rate of flutolanil following application of Moncut 40SC in the field

Test samples

Petri dishes were placed on the test item treated plots during the application. Five samples per plot were taken at the application date in the field. At the end of the application, the Petri dishes were covered and transported into the laboratory. They were rinsed three times with acetone and samples were analysed by HPLC.

Control samples

Stability samples were prepared at the start of the biological phase, in parallel to the test item application in the field. 200 g test item was dissolved in 50 mL tap water. Appropriate volumes of this solution were applied onto Petri dishes to obtain stability samples. Fortified samples were prepared and sample preparation was carried out in parallel to the sample preparation for the test samples. The rate were 779 and 1600 g Moncut 40SC/ha, corresponding to 312 and 641 g flutolanil /ha which was sprayed in only one application. Stability and fortified samples were analysed as test samples.

2. Determination of the concentration of flutolanil in the test soil after the test application

Test samples

After each application, 9 soil samples were drawn from each treated plot. Regarding to storage and processing of the samples, the nine soil cores (0-10 cm) were divided into three lots, each of which were combined and homogenized. This resulted in three mixed soil samples per replicate plot. The mixed samples were stored deep frozen until required for further sample preparation. The active ingredient flutolanil was extracted from the soil by shaking with acetone. After centrifugation and filtration the flutolanil content in the final extract was determined using liquid-chromatography with MS/MS detection.

Control samples

Fortification samples were prepared on the day of analysis. Soil samples were taken from untreated areas of the test field. The fortified level was approximately 0.625, 1.872 and 8.462 mg Moncut 40SC/kg corresponding to 0.25, 0.75 and 3.39 mg flutolanil/kg. Fortification samples were analysed as test samples

Results

The summary of the results is presented in Table B.9.7-2.

The mean recovery rate of the active ingredient from Petri dishes placed on the test item treated plots during the application was 86 % of the nominal value (mean value over all 6 plots). The mean recovery rate of active ingredient in the soil samples taken after the application was 64 % of the nominal value (mean value over all 6 plots).

Table B.9.7-2 Results obtained in analytical part of litter bag test.

Test type	Sample type	Mean recovery of flutolanil (%)	Number of samples	Standard deviation (%)
Petri dish	Test	86	30	24
	Stability	106	4	1
	Fortified	105	6	3
Soil samples	Test	64	18	17
	Fortified	94	18	6

Evaluation RMS (DAR 2006)

The results of the chemical analysis confirmed that the initial exposure concentrations of flutolanil in the soil of the treated plots were within the acceptable range of the nominal values (50 to 150 %, according to EPFES, 2002) after the application.

The study was well performed and in compliance with GLP. The RMS considers it acceptable.

B.9.5 Effects on soil nitrogen transformation

Study CA 8.5-01

Report:	CA 8.5 – 01 Forster, J. 1999
Title:	A laboratory assessment of the effect of EXP10066A (Flutolanil) on soil microflora respiration and nitrogen transformations according to current EU guidelines.
Report no.:	N-3024
Published:	No
GLP:	Yes (UK)
Guidelines:	Directive 96/12/EC, SETAC. EPPO Bulletin 24, 1-16 (1994).
Previous evaluation	In DAR (2006) for original approval.
Remark by RMS	Considered acceptable at the time of original inclusion. Study evaluation from DAR copied as a whole without changes.
Endpoint	0.71% effect at day 42 at 2.09 mg a.s./kg soil (1392 g a.s./ha)

Material and methods

The suspension concentrate EXP10066A (Lot number OP980259) containing 464 g/L flutolanil was applied to silty sand soil (sand 60 %, silt 32 %, clay 7.8 %, organic carbon 1.1 %, pH 6.4) to give a concentration of 4.51 mg formulation/kg soil. This was equivalent to a soil exposure of 3.0 L formulation/ha (1392 g a.i./ha), five times the total annual soil exposure expected when the formulation is used as a potato seed treatment with a maximum planting density of 3 tonnes per ha (276 g as/ha).

The effects of EXP10066A on microbial respiration were investigated using short term respiration experiments conducted after 0, 14 and 28 days in soil amended with ground lucerne grass using an infra red gas analyser.

The effects on nitrogen transformation, ammonification and nitrification were investigated by determining ammonium-N, nitrate-N and nitrite-N concentrations in soil amended with ground lucerne grass. Aliquots of soil were extracted with 2M KCl within 3 hours of treatment and after 14, 28 and 42 days. The concentrations of inorganic nitrogen species in the extracts were detected calorimetrically. The study was continued beyond 28 days (to 42 days) as a result of a slight effect detected at 28 days for nitrite-N concentrations.

Results

Mean values for microbial respiration and nitrogen transformations are presented in the following table B.9.5-1.

Table B.9.5-1 Effects of EXP10066A on soil respiration and nitrogen transformation.

Treatment		Day 0	Day 14	Day 28	Day 42
	Respiration rate (ml CO_2/hr/100g soil)				
Control		1.56	1.79	1.38	
EXP10066A		1.56 (0.00)	1.76 (-1.68)	1.29 (-6.52)	
	Concentration of nitrogen (ammonium, nitrate, nitrite and total, mg/kg soil)				
Control	NH_4^+	1.95	1.64	0.41	0.31
	NO_3^-	5.02	31.04	38.00	55.90
	NO_2^-	<0.26	0.85	1.06	<0.26
	Total	7.21	33.53	39.47	56.21
EXP10066A	NH_4^+	1.70 (-12.82)	1.59 (-3.05)	0.38 (-7.32)	0.39 (+25.81)
	NO_3^-	4.37* (-12.95)	30.73 (-1.00)	44.83 (+17.97)	55.42 (-0.86)
	NO_2^-	0.27 (+12.50)	0.83 (-2.35)	0.77* (-27.36)	<0.26 (ND)
	Total	6.34 (-12.07)	33.15 (-1.13)	45.98 (+16.49)	55.81 (-0.71)

Figures in parenthesis represent % variance from control treatment.
ND: Not determined
*: Statistically significant difference from control

Treatment with EXP10066A did not give rise to any statistically significant effect greater than ±10% of control values on soil microflora respiration after 28 days incubation. A slight reductive effect was detected at 28 days with respect to nitrite concentration but this was shown to be transient since the concentrations at 42 days fell to below the level of detection in both treated and untreated soil. No statistically significant difference was observed on other parameters for nitrogen transformation.

Conclusion: the presence of EXP10066A in soil at 3.0 L/ha (1392 g a.i./ha), a concentration equivalent to five times higher than expected from a total annual maximum use rate of 276 g a.i./ha for the maximum planting density (3 tonnes/ha) of seed potatoes would not be expected to cause any significant effect on either soil microflora respiration or nitrogen transfer.

Evaluation RMS (DAR 2006)

The study was well performed and reported and in compliance with GLP. The test rate was five times higher than the intended use. The study is acceptable.

Table B.9.5-2 EU agreed endpoints on the toxicity of flutolanil to soil micro-organisms

Test type	Time scale	Test material	Endpoint	Data point Author, year
Nitrogen transformation	42 days	EXP10066A*	-0.71% effect at 1392 g a.s./ha (2.09 mg a.s.s/ks soil) after 42 days	CA 8.5-01 Forster, J., 1999

* EXP10066A is equivalent to the representative formulaion MONCUT 40 SC

B.9.6 Effects on terrestrial non-target higher plants

B.9.6.1 Summary of screening data

No data submitted.

B.9.6.2 Testing on non-target plants

Study CA 8.6.1-01

Report:	**CA 8.6.1-01 Spatz, B., 2002**
Title:	Effects of EXP10066A on terrestrial (non-target) plants: seedling emergence and seedling growth test
Report no.:	10695086 (N-3012)
Published:	No
GLP:	Yes
Guidelines:	OECD Guideline for the testing of Chemicals, proposal for updating Guideline 208, Draft Document July 2000
Previous evaluation	In DAR (2006) for original approval
Remark by RMS	Considered acceptable at the time of original inclusion. The study summary from the DAR is replaced with an updated version. Study evaluation from DAR copied as a whole without changes.
Endpoint	NOER = 11200 g a.s./ha

Executive Summary

The purpose of this study was to determine the effects of a single dose of the test item on the seedling emergence of several non-target plant species. Parameters measured include plant dry and fresh weight, germination, mortality and phytoxicity. Four dicotyledoneae and two monocotyledoneae species were chosen representing 6 plant families: *Raphanus sativus* (radish), *Cucumis sativus* (cucumber) *Glycine max* (soybean), *Lycopersicon esculentum* (tomato), *Allium cepa* (onion) and *Avena sativa* (oat). They were tested at a single maximum field rate of 11.2 kg a.s./ha for 21 days, corresponding to a typical exposure scenario for the test item.

The spray mixture was prepared by diluting 68.83 g (nominal 61.13 mL) EXP10066A to 1000 mL with tap water. A singular application (24.45 L product/ha, corresponding to 11.2 kg a.s./ha) was performed according to agricultural practice with a laboratory-spraying equipment. The control consisted of tap water.

The following test parameter were determined: germination rate, phytotoxicity, growth stages, mortality and fresh weight. Data were compared with the control using a statistical test and effective concentrations were calculated based on dry and fresh weight.

The mean recovery of flutolanil in the stock solution was 88% and endpoints were expressed based on nominal rate. After 21 days, the maximum field rate of 11.2 kg a.s./ha of EXP10066A did not cause any effects on germination rate, phytotoxicity and mortality in any of the plants tested. The NOEC and LOEC values (dry and fresh weight) for all plants tested was ≥ 11.2 kg a.s./ha and > 11.2 kg a.s./ha, respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** EXP10066A

Batch no.: OP210100

Purity: 458 g/L Flutolanil

Description: White opaque liquid

2. **Control:** Tap water

3. **Test species:** 4 dicotyledoneae and 2 monocotilidoneae species were chosen representing 6 plant families.

- Dicotyledoneae: *Raphanus sativus* "radish" (Family Brassicaceae), *Cucumis sativus* "Cucumber" (Family Cucurbitaceae), *Glycine max* "Soybean" (Family Fabaceae), *Lycopersicon esculetum* "Tomato" (Family Solanaceae)
- Monocotyledoneae: *Avena sativa* "Oat" (Family Gramineae), and *Allium cepa* "Onion" (Family Alliaceae)

4. **Environmental conditions:**

Soil:	Lufa 2.3 (sandy loam; USDA): all particles smaller than 0.2 cm, 1.2 ± 0.2% organic carbon, pH 6.3 ± 0.2
Temperature:	23°C ± 5°C, night 18° ± 5°C (intended)
Lighting:	16:8 h photoperiod (long day conditions), min 5000 lux (intended)
Relative Humidity:	Day approximately 70 %, night: approximately 85% (intended), mean humidity (24 h) during the test period: 71%
Irrigation:	Maximum 500 mL of tap water
Fertilizer:	Flory 9 (Euroflor) 1 g/L with Sequestren (Ciba-Geigy) 0.05 g/L, once to twice a week after development of the first true leaves.
Lighting:	16:8 h photoperiod (long day conditions)
Irrigation:	Maximum 500 mL of water

B. STUDY DESIGN AND METHODS:

1. **In-life phase:** April 26, 2002 to May 8 and 29, 2002

2. **Test organism assignment and treatment**

Four dicotyledonea and 2 monocotyledonae were chosen representing six families: *Raphanus sativus* (radish), *Cucumis sativus* (cucumber) *Glycine max* (soybean), *Lycopersicon esculentum* (tomato), *Allium cepa* (onion) and *Avena sativa* (oat). They were tested at a single maximum field rate of 11.2 kg a.s./ha for 21 days, corresponding to a typical exposure scenario for the test item.

Eight pots (Ø 16 cm, 9 × 9 cm) were sown for each treatment group (single dose level and control), each containing 5 seeds. Each pot represented one replicate. In total 40 seeds per species and treatment group were sown. The day before the application the seeds were introduced manually into the soil. After sowing the pots were placed on the watering system.

3. **Dose preparation**

The spray mixture was prepared by diluting 68.83 g (nominal 61.13 mL) EXP10066A to 1000 mL with tap water. A singular application (24.45 L product/ha, corresponding to 11.2 kg a.s./ha) was performed

according to agricultural practice with a laboratory-spraying equipment. The control consisted of tap water.

The calibration procedure was done with a glass-plate of a known surface area which was placed at target level and then sprayed with tap water. This glass-plate was weighed immediately before and after application. The amount of spray deposit was calculated as the difference between the weight before and after spraying. This was done five times in order to confirm consistent working of the spraying equipment. The deviation in the spray deposit did not exceed ± 10 % of nominal for the 5 repeats. The uniformity of the deposit distribution was checked visually. One control containing of tap water was used.

Duplicate samples from the freshly prepared and continuously stirred stock solution were taken before application for analytical verification.

4. Measurements and observations

Germination was evaluated by determining the percentage of emerged seedlings at Day 7, 14 and 21 after application. Visual phytotoxicity ratings (e.g. chlorosis, necrosis, abnormal growth) were observed at Day 7, 14 and 21 according to EPPO Standard 135. At Day 21, growth stages were reported according to BBCH-Monograph, as well as the number of living and dead plants and the shoot fresh weight.

5. Statistics

All the test parameters data (dry and fresh weight, germination rate and mortality) were compared to the control using a statistical test at a significance level of $\alpha = 0.05$. Mean were calculated for visual percent of phytotoxicity. Fresh and dry weight data were tested for homogeneity with the Cochran-test. If the data were homogeneous, Student t-test for homogenous data was used. If not, Student-t Test for non-homogenous data was used. For the germination and mortality data Fischer Exact Test was used. The computer program used to perform the statistical analyses was Tox.Rat® SPiRiT Solutions (1999-2001), Version l.09ii and SYSTAT Version 9.

II. RESULTS AND DISCUSSION

A. Biological data

The results of EXP10066A for dry and fresh weight are presented in the following table.

Effects of EXP10066A on dry and fresh weight after 21 days

Species	Treatment (kg a.s./ha)	Fw (g)	SD	Effect* (%)		Dw (g)	SD	Effect* (%)	
A. cepa	Control 11.2	0.32 0.33	± 0.30 ± 0.17	5.1 0	n.s[1]	0.02 0.03	± 0.02 ± 0.03	37.3 7	n.s[1]
A. sativa	Control 11.2	5.60 5.92	± 0.76 ± 0.25	5.7 4	n.s[1]	0.62 0.67	± 0.12 ± 0.04	7.59	n.s[1]
L. esculetum	Control 11.2	3.82 4.02	± 0.67 ± 0.39	4.9 7	n.s[1]	0.39 0.37	± 0.08 ± 0.04	-4.10	n.s[1]
G. max	Control 11.2	11.3 1 13.4 2	± 2.79 ± 3.59	18. 67	n.s[1]	2.29 2.39	± 0.69 ± 0.68	4.16	n.s[1]
C. sativus	Control 11.2	7.86 7.94	± 0.68 ± 0.81	1.0 2	n.s[1]	1.05 0.97	± 0.11 ± 0.09	-7.66	n.s[1]
R. sativus	Control 11.2	5.71 5.87	± 2.26 ± 1.29	2.7 8	n.s[1]	0.53 0.60	± 0.21 ± 0.17	13.7 9	n.s[1]

The results represent rounded values calculated on the exact raw data.
Fw = fresh weight; Dw = dry weight; SD = standard deviation; s = Significant; n.s. = not significant; *= negative values indicate reduction compared to control; [1] Student Test, α = 0.05; [2] Fisher Exact Test, α = 0.05

The summary of results of effects of EXP10066A about germination, mortality and phytotoxicity are presented in the following table**.**

Effects of EXP10066A on germination, mortality and phytotoxicity

	Treatment (kg a.s./ha)	Germination (%)		Mortality (%)		Phytotoxicity (%)			BBCH[3]
Days after application		21		21		7	14	21	21
Species									
A. cepa	Control 11.2	75 78	n.s[2]	6.7 3.2	n.s[2]	0	0	0	12 12
A. sativa	Control 11.2	90 100	n.s[2]	0.0 0.0	n.r	0	0	0	13 13
L. esculetum	Control 11.2	98 95	n.s[2]	0.0 0.0	n.r	0	0	0	12 12
G. max	Control 11.2	88 85	n.s[2]	0.0 0.0	n.r	0	0	0	13-14 13-14
C. sativus	Control 11.2	98 93	n.s[2]	0.0 0.0	n.r	0	0	0	11-12 11-12
R. satius	Control 11.2	80 78	n.s[2]	6.3 0.0	n.s[2]	0	0	0	12-14 13-15

The results represent rounded values calculated on the exact raw data
s = significant; n.s. = not significant; n.r. = not relevant; * = negative values indicate reduction compared to control; [1] Student Test, α = 0.05; [2] Fisher Exact Test, α = 0.05; [3] growth stage

After 21 days, the maximum field rate of 11.2 kg a.s./ha of EXP10066A did not cause any effects on germination rate, phytotoxicity and mortality in any of the plants tested. The NOER (dry and fresh weight) for all plants tested was 11.2 kg a.s./ha.

B. Analytical Verification

The mean recovery of flutolanil in the stock solution was 88%.

C. Toxicity Endpoints

Endpoints	Effect concentration (dry and fresh weight)	
Raphanus sativus *Cucumis sativus* *Glycine max* *Lycopersicon esculentum* *Allium cepa* *Avena sativa*	NOER	11.2 kg a.s./ha

III. CONCLUSIONS

The substance EXP10066A was tested for effects on seedling emergence on six terrestrial non-target plant species representing six plant families. Effective concentrations were calculated based on dry and fresh weight.

Effects of EXP10066A on plant growth were not observed. The NOER (dry and fresh weight) for all plants tested was 11.2 kg a.s./ha.

Evaluation RMS (DAR 2006)

The study was well performed (but rather briefly reported) and in compliance with GLP. Only one and very high concentration was used (at least 3 concentrations should be tested according to the OECD 208). The study can however be considered as a screening test and is acceptable for the risk assessment.

B.9.7 Effects on other terrestrial organisms (flora and fauna)

Tests on other terrestrial organisms for the active substance were not required and therefore no further testing was conducted. Further information found in the open literature is included below.

Study CA 8.7-01

Report:	**CA 8.7-01. Buysens, C., Dupré de Boulois, H., Declerck, S., 2015**
Title:	Do fungicides used to control *Rhizoctonia solani* impact the non-target arbuscular mycorrhizal fungus *Rhizophagus irregularis*?
Report no.:	Not applicable
Published:	Yes
GLP:	No
Guidelines:	In vitro methodology (Zocco et al. 2011)
Previous evaluation	Submitted for the purpose of renewal
Remark by RMS	Supporting information. See below comments of the RMS.
Endpoint	The IC_{50} based on dry weight for flutolanil and Monarch on *R. solani* were 0.13 mg a.s./L (95% CI 0.13-0.13 mg a.s./L) and 0.15 mg a.s./L (95% CI 0.14-0.15 mg a.s./L), respectively.

Executive Summary

The present study investigated, under *in vitro* controlled conditions, the impact of fungicides, including flutolanil (a systemic Basidiomycota-specific fungicide) and its respective formulation, Monarch, on the growth and development of the arbuscular mycorrhizal fungi (AMF) *Rhizophagus irregularis* MUCL

41833. *R. irregularis* is a biocontrol organism of which the application is a feasible option to reduce incidence of plant pathogens. The spore germination, root colonisation, extraradical mycelium development, and spore production of *R. irregularis* was assessed when exposed at doses of 0.1, 1, 10 and 1000 mg a.s./L.

Spore germination was impacted by flutolanil and Monarch at concentrations above or equal to 10 mg a.s./L. Most of the non-germinated spores (50 to 100 %), washed-free of the fungicides, were able to germinate on fungicide-free medium. This suggests that the fungicide from 10 to 100 mg/L had a fungistatic effect on *R. irregularis* spore germination rather than a fungicidic effect.

Flutolanil and its formulation Monarch at threshold value for the control of *Rhizoctonia solani* (IC_{50} dry weight = 0.13 and 0.15 mg a.s./L for flutolanil and Monarch, respectively), did not affect spore production, germination or extra-radical development. However, Monarch significantly reduced root colonisation of potato plants by the AMF at concentrations above or equal to 0.1 mg a.s./L, possibly because of accumulation of the fungicide in the root zone. Monarch at 0.1 mg a.s./L and above also affected intraradical structures (hyphae and arbuscules) of *R. irregularis*, possibly because of its high systemic activity.

The field applications of Monarch at 9.2 g a.s./dt and 25 g a.s./dt would correspond to 0.09 and 0.25 mg a.s./L, respectively. These values are equal or higher than the threshold values measured *in vitro* to control *R. solani*, and where Monarch impacted the intraradical phase of the fungus. It was concluded that even if fungicides at high doses seem to impact AMF enzymatic pathways, the enzymes of the respiration pathway or cell division pathway in AMF are probably less sensitive to flutolanil, than those of phytopathogenic fungi.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** Flutolanil active ingredient and Monarch® (Belchim Crop Protection sa/nv (Londerzeel, Belgium))

Batch no.: Not reported

Purity: Not reported

2. **Test organism:** *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler comb. nov. MUCL 41833

Age: Spores of a 2 months old culture (in a mycelium donor plant (MDP) in vitro culture system

Source: Glomeromycota in vitro collection (GINCO)

3. **Treatment:** Control, solvent control, 0.1, 1, 10 and 1000 mg a.s./L

Test vessels: 24 Well Cell Culture Cluster (Corning Incorporated, USA)

Test water: MSR medium lacking sucrose and vitamins and solidified with 4 g/L Phytagel

Loading: 2 mL MSR medium containing 48 spores per treatment

B. STUDY DESIGN AND METHODS

1. Test organism assignment and treatment

Spore germination of *R. irregularis*:

Spores of a 2-month-old culture of *R. irregularis* MUCL 41833 were extracted from the MSR medium by citrate buffer solubilisation (Doner and Bécard 1991), isolated with a micropipette under a dissecting microscope, and placed singly in each hole of a 24 Well Cell Culture Cluster (Corning Incorporated, USA) containing 2 mL MSR medium lacking sucrose and vitamins and solidified with 4 g L−1 Phytagel (Sigma-Aldrich, Germany) without fungicide (control) or supplemented with increasing concentrations (0.1, 1, 10, and 100 mg a.s./L) of the fungicide (a.s. or formulation). Forty-eight spores were considered per treatment. The 24 Well Cell Culture Clusters were incubated at 27°C in the dark.

Root colonisation of *Solanum tuberosum*, spore production, and fungal regrowth:

Twelve-day-old micropropagated potato plantlets were plated on the extraradical mycelium network extending into the hyphal compartment (HC). The HC was then supplemented with 20 mL MSR medium lacking sucrose and vitamins without fungicide (control) or final concentrations of 0.1, 1, or 10 mg a.s./L flutolanil. The in vitro culture system was then incubated in a growth chamber set at 22 to 18°C (day to night) with 70% relative humidity, a 16 h photoperiod, and a photosynthetic photon flux (PPF) of 300 µmol/m^2/s. The lamps used in the growth chamber covered a spectrum from λ = 400 to 700 nm. After 14 days of contact with the extraradical mycelium, the potato plantlets were carefully collected from the HC. One potato plantlet per system was used for assessing root colonisation while the other was used to evaluate spore production and mycelium regrowth (Voets et al. 2005) after plating on a fresh MSR medium without sugar or vitamins supplemented or not by fungicide (a.s. or formulation).

2. Dose preparation

Stock solutions of flutolanil and its respective formulation Monarch® were prepared in acetone and water, respectively, at 10% a.s. w/v. MSR medium was supplemented with increasing concentrations of the fungicide (a.s. or formulation) at 0.1, 1, 10 and 100 mg/L.

3. Measurements and observations

Spore germination of *R. irregularis*:

Spore germination was monitored under a dissecting microscope (10 – 40 × magnifications) after 3, 6, 14, and 28 days. Spores that did not germinate on the different fungicide concentrations (a.s. or formulation) were washed free from the fungicides in sterilized (121°C for 15 min) deionized water and placed in holes of a 24 Well Cell Culture Cluster containing 2 mL solidified MSR medium without fungicides. Germination was assessed after 6 days.

Root colonisation of *Solanum tuberosum*, spore production, and fungal regrowth:

Root colonisation was assessed following clearing with 10% KOH at 50°C for 90 min and staining with 5% blue ink (Parker®) diluted in vinegar (7° acidity) at 50°C for 60 min. The roots were then rinsed with deionized water and observed under a compound bright field microscope at 125 –

250 × magnification. Root colonisation was assessed by evaluating the percentage of total root colonisation (% RC), arbuscules (% A), and spores/vesicles (% V) following the method of McGonigle et al. (1990). Two hundred intersections were observed per sample.

The second potato plantlet from each MDP *in vitro* culture system was plated in mono-compartmented Petri plates in the absence (control) or presence of the same concentration of fungicide as for root colonisation. After 9 weeks, the number of newly produced spores were counted (Declerck et al. 1996) and extraradical hyphal length estimated under a stereo microscope at 10 – 40 × magnification. Hyphal length was estimated by counting the number of intersects between hyphae and a grid of lines (Voets et al. 2005) and then using the formula of Newman (1966).

4. Statistics

Data on the percentage germinated spores were analysed using chi-square test ($p < 0.05$). AMF root colonisation was analysed with the statistical software SAS Enterprise guide (version 4.3). Data expressed as percentage (%) were normalised by arcsine transformation and analysed using a one-way ANOVA. The Tukey's honest significant difference test was used to identify the significant difference in root colonisation ($p \leq 0.05$) between the fungicide treatments and the control. Nonlinear dose–response curves of active ingredients and formulations were compared using the software JMP version 10 (SAS Institute Inc., Cary, NC, USA).

II. RESULTS AND DISCUSSION

A. Biological data

Spore germination of *R. irregularis*:

No significant differences were observed between the control treatments (water or acetone, without fungicides). About 75 to 88 % of the spores germinated within 3 days and nearly all the spores within 28 days.

Flutolanil and Monarch at 0.1 and 1 mg a.s./L did not impact spore germination, while at 10 and 100 mg a.s./L, the percentage of the germinated spores was significantly lower as compared to the control (Fig. 1 and 2). For flutolanil at 10 mg a.s./L, 58% of the spores were germinated after 3 days and reached 83% after 28 days.

For Monarch at 10 mg a.s./L, only 44% of spores were germinated after 6 days and reached 71% after 28 days. At 100 mg a.s./L, spore germination was strongly impacted by both fungicides; after 28 days, values were 33 and 25 % for flutolanil and Monarch, respectively.

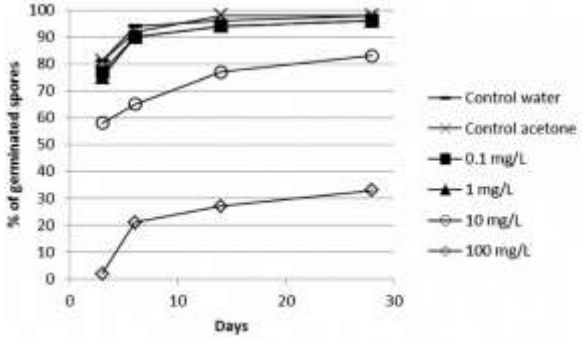


Fig. 1: Dynamics of spore germination of *R. irregularis* MUCL 41833 in the presence of flutolanil. Open symbols (○, ◊) show a significant difference with control (chi-square test, $p < 0.05$; n = 48).

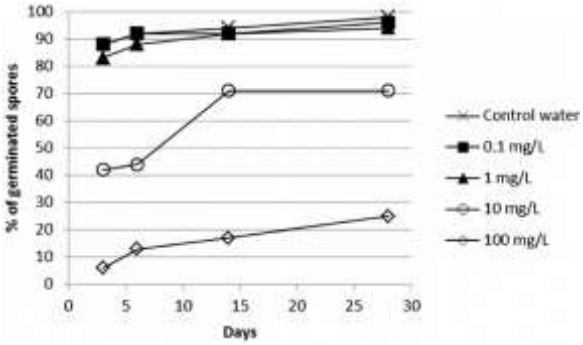


Fig. 2: Dynamics of spore germination of *R. irregularis* MUCL 41833 in the presence of Monarch. Open symbols (○, ◊) show a significant difference with control (chi-square test, $p < 0.05$; n = 48).

Root colonisation of *Solanum tuberosum*, spore production, and fungal regrowth:

Flutolanil at 1 and 10 mg a.s./L significantly reduced the percentage of total root colonisation (% RC) as compared to the acetone control treatment (Fig. 3). The % RC at 10 mg a.s./L was significantly lower than % RC at 1 mg a.s./L (Fig. 3). Values for the percentage of arbuscules (% A) did not differ from the acetone control treatment at 1 mg a.s./L but was significantly lower at 10 mg a.s./L (Fig. 3). The percentage of spores/vesicles (% V) was significantly lower from the acetone control treatment at 1 mg a.s./L but not at 10 mg a.s./L.

At 1 mg a.s./L of Monarch, the % RC, % A, and % V values were significantly reduced as compared to the control water treatment (Fig. 4). At 0.1 mg/L, a significant difference with the control water treatment was found for % RC and % A but not for % V (Fig. 4). It was suggested that effects could be due to the accumulation of the fungicide in the root zone, and because of its high systemic activity.

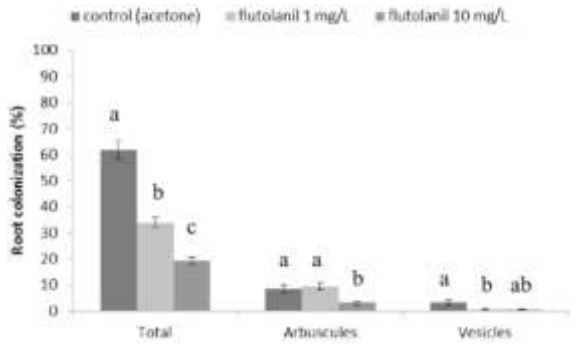


Fig. 3: Percentage of root colonisation (total, arbuscules, and vesicles/spores) of potato by *R. irregularis* MUCL 41833 in the presence of flutolanil. Bars with different letters are significantly different (Tukey's test, $p < 0.05$, n = 7).

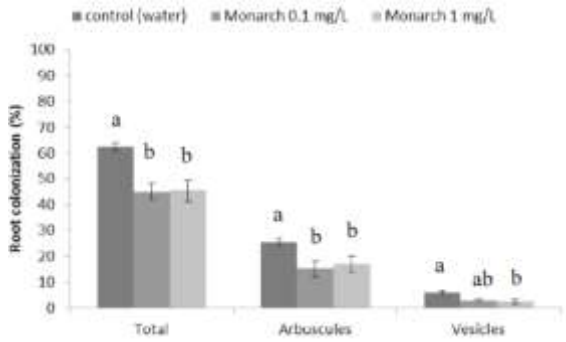


Fig. 4: Percentage of root colonisation (total, arbuscules, and vesicles/spores) of potato by *R. irregularis* MUCL 41833 in the presence of Monarch. Bars with different letters are significantly different (Tukey's test, $p < 0.05$, n=7).

The number of new spores produced from plants colonised in the in vitro culture system in the absence or presence of Monarch at 0.1 mg a.s./L following their transfer on MSR medium in mono-compartmented Petri plates under the same conditions as above, is presented in Fig. 5. No significant reduction in spore production was observed for Monarch at 0.1 mg a.s./L as compared to the control treatment.

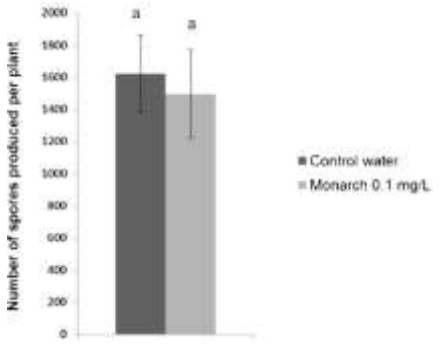


Fig. 5: Hyphal length of *R. irregularis* MUCL 41833 per plant after 9 weeks of culture in the absence or presence of Monarch at 0.1 mg a.s./L Bars with the same letters are not significantly different (Tukey's test, $p < 0.05$, n=5).

Extraradical mycelium regrowth from colonised plants under the in vitro culture system in the absence or presence of Monarch at 0.1 mg a.s./L following their transfer on the MSR medium in mono-compartmented Petri plates under the same conditions as above, is presented in Fig. 6. No significant reduction in mycelium growth was observed for Monarch at 0.1 mg a.s./L compared to the control treatment.

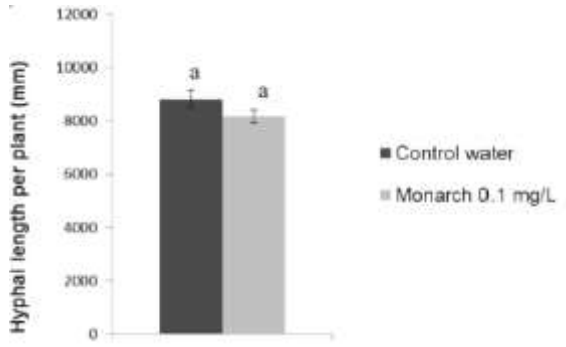


Fig. 6: Spore production by *R. irregularis* MUCL 41833 after 9 weeks of culture in the absence or presence of Monarch at 0.1 mg a.s./L Bars with the same letters are not significantly different (Tukey's test, $p < 0.05$, n=5).

The field applications of Monarch at 9.2 g a.s./dt and 25 g a.s./dt would correspond to 0.09 and 0.25 mg a.s./L, respectively. These values are equal or higher than the threshold values measured in vitro to control *R. solani*, and where Monarch impacted the intraradical phase of the fungus. It was concluded that even if fungicides at high doses seem to impact AMF enzymatic pathways, the enzymes of the respiration pathway or cell division pathway in AMF are probably less sensitive to flutolanil, than those of phytopathogenic fungi.

B. Analytical verification

Analysis of the test solutions were not reported.

III. CONCLUSION

Flutolanil and its formulation Monarch at threshold value for the control of *Rhizoctonia solani* (IC_{50} dry weight = 0.13 and 0.15 mg a.s./L for flutolanil and Monarch, respectively), did not affect spore production, germination or extra-radical development. However, Monarch significantly reduced root colonisation of potato plants by the AMF at concentrations above or equal to 0.1 mg a.s./L, possibly because of accumulation of the fungicide in the root zone. Monarch at 0.1 mg a.s./L and above also affected intraradical structures (hyphae and arbuscules) of *R. irregularis*, possibly because of its high systemic activity. The field applications of Monarch are equal or higher than the threshold values measured *in vitro* to control *R. solani*, and where Monarch impacted the intraradical phase of the fungus. It was concluded that even if fungicides at high doses seem to impact AMF enzymatic pathways, the enzymes of the respiration pathway or cell division pathway in AMF are probably less sensitive to flutolanil, than those of phytopathogenic fungi.

IV. REFERENCES

Declerck S, Strullu DG, Plenchette C (1996) In vitro mass-production of the arbuscular mycorrhizal fungus, *Glomus versiforme*, associated with Ri T-DNA transformed carrot roots. Mycol Res 100:1237–1242

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McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA (1990) A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol 115:495–501

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Zocco D, Van Aarle IM, Oger E, Lanfranco L, Declerck S (2011) Fenpropimorph and fenhexamid impact phosphorus translocation by arbuscular mycorrhizal fungi. Mycorrhiza 21:363–374. doi:10.1007/s00572-010-0344-0

Evaluation RMS

This screening study on the fungicidal activity of flutolanil was not conducted according to a guideline and not under GLP.

Three experiments were performed under *in vitro* controlled conditions with the purpose to investigate whether the growth and development of the beneficial fungi *Rhizophagus irregularis* is affected at doses that are used to control the pathogenic fungi *Rhizophagus solani*:

1. impact of flutolanil and its formulation Monarch on the growth of *Rhizophagus solani* (derivation of an IC_{50})
2. impact of flutolanil and its formulation Monarch on spore germination of the arbuscular mycorrhizal fungi *Rhizophagus irregularis* at the threshold control value for *R. solani* (IC_{50})
3. impact of flutolanil and its formulation Monarch on potato root colonization by *Rhizophagus irregularis*, spore production and fungal regrowth at the threshold control value for *R. solani* (IC_{50})

The IC_{50} based on dry weight for flutolanil and Monarch on *R. solani* were 0.13 mg a.s./L (95% CI 0.13-0.13 mg a.s./L) and 0.15 mg a.s./L (95% CI 0.14-0.15 mg a.s./L), respectively.

Flutolanil, tested as active substance or formulation, did not affect spore germination, root colonization, spore production and extraradical hyphal length of *R. irregularis* at the IC_{50} for *R. solani*. Significant reduction of spore germination was observed at 10 and 100 mg a.s./L tested as a.s. and formulation.

Root colonization was significantly reduced at 1 mg a.s./L (< 50%) and at 10 mg a.s./L (> 50%) as tested with the active substance; at 0.1 mg a.s./L (< 50%) and at 1 mg/L (< 50%) as tested with the product.

The study of the publication was well conducted and is acceptable with restrictions. As it is not a regulatory endpoint, the study can be used for information only.

Buysens, C., Dupré de Boulois, H., Declerck, S.	2015	Do fungicides used to control *Rhizoctonia solani* impact the non-target arbuscular mycorrhizal fungus *Rhizophagus irregularis*?	Mycorrhiza (2015) 25:277-288
Reliability			
General information			
Is a guideline method or modified guideline used?*	No		
Is the test performed under GLP conditions?*	No		
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?	Not applicable		
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?	Yes		
* these criteria are of minor importance for study reliability, but may support study evaluation			

Test compound	
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?	Yes
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	No
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	The amount of a.s. in the formulation is not known, but the IC50 values to *R. solani* were expressed as mg a.s./L
Test organism	
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	Yes
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	Yes, according to *in vitro* culture system
Exposure conditions	
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?	Yes
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?	Yes
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	Yes
Is a correct spacing between exposure concentrations applied?	Not applicable as no guideline was followed. The spacing factor for the 4 tested concentrations was 10 and included the threshold control value for *R. solani* (IC50). This is acceptable.
Is the exposure duration defined?	Yes
If necessary, are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?	No
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Not applicable as no guideline was followed. As the results for the controls in all experiments were consistent, the biomass loading is considered acceptable.
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	Yes.

Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	No
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	No. It is an *in vitro* test and thus only indicative of field conditions
Is the tested exposure scenario relevant for the species?	Possibly. It is an *in vitro* test and thus only indicative of field conditions
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	No, it is an *in vitro* test.
Are the organisms tested relevant for the tested compound?	Yes, as it is a fungicide.
Are the reported endpoints appropriate for the regulatory purpose?	No, as it is not a regulatory endpoint
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	No, as it is not a regulatory endpoint
Are appropriate life-stages studied?	Yes
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Not applicable
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable
Concluding weight of evidence/proposed action	The study is reliable with restrictions and relevant with restrictions.
Type of information (Fully acceptable, supporting information, not applicable)	Supporting information only
Consideration/concluding score	Supporting information only

B.9.8 Effects on biological methods for sewage treatment

Study CA 8.8-01

Report:	**CA 8.8-01. Barnes, S., 2007**
Title:	Flutolanil: Activated Sludge: Respiration Inhibition Test
Report no.:	NHH 0123/072254 (N-3030)
Published:	No
GLP:	Yes
Guidelines:	OECD guideline 209, (OPPTS) Method 850.6800, 1996
Previous evaluation	In DAR (Addendum 2, April 2007) for original approval
Remark by RMS	The study is fully acceptable. The study summary from the DAR is replaced with an updated version. Re-evaluation for the purpose of renewal is presented below.
Endpoint	EC_{50} >1000 mg a.s./L.

Executive Summary

The objective of this study was to assess the effects of flutolanil on sewage micro-organisms by measuring the rate of oxygen uptake of activated sludge at 20 ± 2°C in its presence at a range of concentrations. Samples of activated sludge (suspended solids 1.6 g/L) fed with synthetic sewage were exposed to the test substance at nominal concentrations of 10, 100 and 1000 mg/L for 3 hours. Single mixtures were prepared at 10 and 100 mg/L and the highest level was prepared in triplicate. Their rates of oxygen consumption were determined and compared with those of controls, containing activated sludge and synthetic sewage alone, which were established at the beginning and end of the culture series.

The reference inhibitor 3,5-dichlorophenol (3,5-DCP) was employed at 3, 10 and 32 mg/l, as a positive control. The specific respiration rate of the control cultures established at the end of the test series (23.9 mg O_2/g/h) was 104% of the rate of that established at the start (22.9 mg O_2/g/h). The thirty-minute 50% effect concentration (EC_{50}) for 3,5-DCP was calculated to be 9.6 mg/L (95% confidence limits 7.9 - 11.8 mg/L). These results show that the test was valid and that the sample of activated sludge employed was sensitive to inhibition.

Flutolanil was considered to have had no biologically significant inhibitory effect on the respiration rate of activated sludge at any of the concentrations employed in the test. The EC_{20}, EC_{50} and EC_{80} of the test substance could not, therefore, be calculated but these must be greater than 1000 mg/L, the highest level tested.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Flutolanil
Batch no.: 2AE0008P
Purity: 98.5%
Description: White powder
Reference inhibitor: 3,5-Dichlorophenol (3,5-DCP)

Purity: 99.9%

2. Control: Dechlorinated tap water (hardness 200 - 250 mg/L as CaCO3)

3. Test micro-organism: Activated sludge

Source: Worlingworth Sewage Treatment Works

4. Environmental conditions:

Temperature: 20.1-20.2°C (initial) 19.9-20.1°C (final)

pH: 7.5 (initial), 7.5-7.7 (final)

B. STUDY DESIGN AND METHODS:

1. In-life phase: 30 January to 01 February 2007

2. Preparation of microbial inoculum

A sample of activated sludge was obtained the day before the start of the test from Worlingworth Sewage Treatment Works, which treats predominantly domestic waste. In the laboratory, the sample was maintained under aerobic conditions until required. The concentration of suspended solids in a homogenised sample was determined on the day of collection and immediately before the start of the test. On the day of collection, aliquots (25 ml) of the activated sludge were filtered through dried and pre-weighed Whatman GF/C filter papers which were then dried again at approximately 105°C for at least one hour, allowed to cool in a desiccator and reweighed. The mixed liquor suspended solids (MLSS) content of the activated sludge was then calculated. Synthetic sewage (50 mL/L) was added to the sample of activated sludge and the mixture aerated overnight. On the day of the test, the MLSS content of the sludge was determined and adjusted to 4 g/L by the addition of dechlorinated tap water. The pH and temperature of the sludge was also measured.

3. Dose preparation

The dilution water used to prepare solutions of synthetic sewage and the reference substance was tap water that had been softened and treated by reverse osmosis. The water used to prepare test mixtures was dechlorinated tap water (hardness 200 - 250 mg/L as $CaCO_3$).

A concentrated solution of 3,5-DCP (500 mg/L) was prepared by dissolving 0.5 g in 10 mL of 1N sodium hydroxide and diluting to approximately 30 mL with ultrapure water. Sulphuric acid (1N) was added to the point of incipient precipitation and the solution made up to a final volume. Nominal concentrations of 3, 10 and 32 mg/L were prepared.

At test initiation, appropriate weights were established in 1 L silanised test beakers, dechlorinated tap water (284 mL) was added and the mixtures treated with ultrasound for one hour. The pH of a preparation at the highest concentration indicated adjustment was unnecessary. Additions of synthetic sewage, silicone oil in water antifoam, to suppress foam from the sludge, and the inoculum were made at 15-minute intervals. Nominal concentrations tested were 10 and 100 mg/L (single) and 1000 mg/L (in triplicate).

The prepared mixtures were covered loosely with aluminium foil and aerated for thirty minutes in a thermostatically-controlled water bath, using a glass aerator connected to a laboratory supply of oil-free compressed air (*ca.* 1 L/minute).

4. Measurements and observations

Following the exposure period, a well-mixed sample of each mixture was transferred to a biochemical oxygen demand (BOD) bottle (nominal capacity, 270 mL). The rate of oxygen consumption was measured, over a period of approximately ten minutes or until the dissolved oxygen concentration fell below 2 mg O_2/L, using a Yellow Springs Instruments (YSI) dissolved oxygen meter, with temperature probe and self-stirring bottle probe, connected to a chart recorder. The pH and temperature of the samples were measured at the start and end of the test. The oxygen consumption rate of each test, reference and control mixture was calculated.

5. Statistics

The EC_{50} and 95% confidence limits (Donaldson and Schnabel, 1985) of the reference substance were calculated using the SAFEstat curvefit programme (SAS Institute, 1999).

II. RESULTS AND DISCUSSION

A. Biological data

Sludge respiration rates were progressively reduced in the presence of increasing concentrations of 3,5-DCP. The three-hour 50% effect concentration (EC_{50}) for 3,5-DCP was calculated to be 9.6 mg/L (95% confidence limits 7.9 - 11.8 mg/L).

The specific respiration rate of the control culture established at the end of the test (23.9 mg O_2/g/h) was 104% of the rate of that established at the start (22.9 mg O_2/g/h).

These results show that the test was valid and that the sample of activated sludge employed was sensitive to inhibition.

Flutolanil was considered to have had no biologically significant inhibitory effect on the respiration rate of activated sludge at any of the concentrations employed in the test. The specific respiration rate was decreased, at most, by 3% of the mean control rate in one mixture at 1000 mg/L. The EC_{20}, EC_{50} and EC_{80} of the test substance could not, therefore, be calculated but these must be greater than 1000 mg/L, the highest level tested.

Summary of the effects of flutolanil on the respiration of activated sludge after three hours exposure

Test mixture concentration (mg/L)	Specific respiration rate (mg oxygen/g/h)	Respiration inhibition (%)
Control (1)	22.9	-
Flutolanil		
10	23.3	0
100	25.0	0
1000	24.3	0
1000	22.7	3
1000	24.5	0
3,5-DCP		
3	19.8	16
10	11.4	51
32	4.3	82
Control (2)	23.9	-

B. Toxicity Endpoints

The 3-hour effect endpoints for flutolanil to the respiration rate of activated sludge are presented in the following table.

Endpoints of the test item flutolanil

Endpoint	Effect concentration (mg/L)	
Activated sludge	NOEC	≥ 1000
	LOEC	> 1000
	ER_{50}	> 1000

III. CONCLUSIONS

Flutolanil was considered to have had no biologically significant inhibitory effect on the respiration rate of activated sludge at any of the concentrations employed in the test. The EC_{20}, EC_{50} and EC_{80} of the test substance could not, therefore, be calculated but these must be greater than 1000 mg/L, the highest level tested.

The three-hour EC_{50} for 3,5-DCP (9.6 mg/L) fulfilled the validity criterion relating to sensitivity to inhibition (acceptable EC_{50} range 5 to 30 mg/L), and that relating to the respiration rates in the control (variation not greater than 15%) was also satisfied.

Evaluation RMS

The study was conducted according to OECD 209 and the deviation specified in the summary did not invalidate the study. The validity criteria were met: the blank controls oxygen uptake rates were 22.9 and 23.9 mg O_2/g/h (> 20 mg O_2/g/h); the coefficient of variation of oxygen uptake rate in control replicates was 3% (< 30%) at the end of the test and the 30 min-EC_{50} of 3,5-DCP was 9.6 mg/L (between 2 mg/L and 25 mg/L for total respiration).
The EC_x values were all above 1000 mg a.s./L.
The study is fully acceptable.

Barnes, S.	2007	Flutolanil: Activated Sludge: Respiration Inhibition Test	Report NHH 0123/072254 (N-3030)
Reliability			
General information			
Is a guideline method or modified guideline used?*		Yes	
Is the test performed under GLP conditions?*		Yes	
If applicable, are validity criteria fulfilled (e.g. control survival, growth, etc.)?		Yes	
Are appropriate controls performed (e.g. solvent control, negative and/or positive control)?		Yes	
* these criteria are of minor importance for study reliability, but may support study evaluation			
Test compound			
Is the test substance clearly identified with name or CAS-number? Are test results reported for the appropriate compound?		Yes	
Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?		Yes	
If a formulation is used or if impurities are present: do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?		Not applicable	
Test organism			
Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?		Yes	
Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?		Yes.	
Exposure conditions			
Is the experimental system appropriate for the test substance, taking into account its physicochemical characteristics?		Yes	
Is the experimental system appropriate for the test organism? Have conditions been stable during the test?		Yes	
If appropriate, were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?		Test substance solutions were applied by direct addition	
Is a correct spacing between exposure concentrations applied?		Yes	
Is the exposure duration defined?		Yes	
If necessary, are chemical analyses adequate to verify concentrations of the test substance		Not applicable	

over the duration of the study?	
Where applicable, is the biomass loading of the organisms in the test system within the appropriate range?	Yes
Statistical Design and Biological Response	
Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	Yes
Are appropriate statistical methods used?	Yes
Is a concentration-response curve observed? Is the response statistically significant?	No effects were observed at the highest tested concentration
Are sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, concentration-response curves)?	Yes
Relevance of the study for Environmental Risk Assessment, appropriateness of study endpoints	
Exposure Relevance	
Is the substance tested representative and relevant for the substance being assessed?	Yes
Is the tested exposure scenario relevant for the substance?	Yes
Is the tested exposure scenario relevant for the species?	Yes
Biological relevance	
Is the species tested relevant for the compartment under evaluation?	Yes
Are the organisms tested relevant for the tested compound?	Yes
Are the reported endpoints appropriate for the regulatory purpose?	Yes
Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	Yes
Is the effect relevant on a population level?	Yes
Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	Yes, ECx values > 1000 mg a.s./L
Are appropriate life-stages studied?	Not applicable
Are the experimental conditions relevant for the tested species?	Yes
Is the exposure duration relevant and appropriate for the studied endpoints and species?	Yes
If recovery is studied, is this relevant for the framework for which the study is evaluated?	Not applicable

Concluding weight of evidence/proposed action	The study is reliable without restrictions and relevant without restrictions.
Type of information (Fully acceptable, supporting information, not applicable)	Fully acceptable
Consideration/concluding score	Fully acceptable

B.9.9 Monitoring data

No data submitted.

B.9.10 Biological activity of metabolites potentially occurring in groundwater

No data submitted.

B.9.11 Literature search

A search of the public literature was performed on 01 August 2016. The search was performed according to EFSA (2011; 9(2): 2092), and was considered acceptable by the RMS. Of 85 references returned in the search, 70 references were excluded following an initial “rapid” screening for relevance. Of the 15 references which were screened in detail (full text screening), 10 were eliminated from consideration according to the notifier, as shown in the table below.

References proposed as non-relevant after full-text screening

Author(s)	Year	Title	Source	Reason for exclusion
Anasco, N.C., Koyama, J., Uno, S.	2010	Pesticide Residues in Coastal Waters Affected by Rice Paddy Effluents Temporarily Stored in a Wastewater Reservoir in Southern Japan	Arch Environ Contam Toxicol 58:352–360	No ecotoxicological test or impact assessments were performed with flutolanil or its metabolites.
Bro, E., Millot, F., Decors, A., Devillers, J.	2015	Quantification of potential exposure of grey partridge (*Perdix perdix*) to pesticide active substances in farmlands	Science of the Total Environment 521–522 315–325	Neither flutolanil nor its metabolites were investigated.
Campillo, J.A.,*et al*.	2013	Impact assessment of agricultural inputs into a Mediterranean coastal lagoon (Mar Menor, SE Spain) on transplanted clams (*Ruditapes decussatus*) by biochemical and physiological responses	Aquatic Toxicology 142–143 365–379	The impact of flutolanil or its metabolites was not Investigated.
Henning-De Jong,*et al*.	2008	Ranking of Agricultural Pesticides in the Rhine-Meuse-Scheldt Basin Based on Toxic Pressure in Marine Ecosystems	Environmental Toxicology and Chemistry 27(3) 737–745	Neither flutolanil nor its metabolites were investigated.
Padilla, S., *et al*.	2012	Zebrafish developmental screening of the ToxCastTM Phase I chemical library	Reproductive Toxicology 33 174– 187	No analytical verification was performed. The study does not provide a NOEC for the reproduction test.
Rice, P.J.,	2010	Evaluation of core cultivation	Environmental	Run-off from golf courses

Horgan, B.P. and Rittenhouse, J.L.		practices to reduce ecological risk of pesticides in runoff from A*grostis palustris*	Toxicology and Chemistry 29(6) pp. 1215–1223	are not representative of run-off from agricultural lands owing to different practices. The endpoints used are from ECOTOX database (US EPA).
Rotroff, D.M., *et al*	2014	Predictive Endocrine Testing in the 2008 21st Century Using *in Vitro* Assays of Estrogen Receptor Signalling Responses	Environ. Sci. Technol. 48, 8706−8716	A more recent paper (Yang *et al*. 2016) provides more details on this topic and has been included in the dossier.
Speck-Planche, A., *et al*.	2012	Predicting multiple ecotoxicological profiles in agrochemical fungicides: A multi-species chemoinformatic approach	Ecotoxicology and Environmental Safety 80 308–313	Neither flutolanil nor its metabolites were investigated.
Tsuda, T., Nakamura, T., Inoue, and A., Tanaka, K.	2009	Pesticides in Water, Fish and Shellfish from Littoral Area of Lake Biwa	Bull Environ Contam Toxicol 82:716-721	Concentrations of flutolanil in water, fish and shellfish were very low and did not allow the calculation of field BCF values.
Werner, S.J., *et al*	2008	Registered Pesticides and Citrus Terpenes as Blackbird Repellents for Rice	Journal of Wildlife Management 72(8):1863–1868	Obscure test substance (GWN-4770) with unknown composition and purity.

The RMS has checked this list and has the following comments:

(1) The study by Padilla, et. al. (2012) shows that flutalonil is toxic in the zebrafish embryo assay. This is supported by the more detailed data presented in the study of Yang, et. al. (2016), which has been included in this dossier. As a result, the study by Padilla et. al. (2012) does not need to be submitted and further evaluated.

(2) The study by Rotroff, et. al. (2014) does not relate to the study of Yang et. al. (2016), as it is a screening of *in vitro* estrogen receptor assays compared to in vivo data for certain substances. It suggests that the results from the in vitro ER HTP screening were in-line with the results from the uterotrophic assays in the same substances. Nonetheless, the RMS agrees that this study does not need to be submitted, as it does not provide information that is directly relevant to the risk assessment, as a uterotrophic assay has already been presented.

(3) The study by Werner, et. al. is from 2010, rather than 2008 and is a repellency study using a formulation of flutalonil. According to the abstract the manufacturer applied for a US patent for the use of flutalonil as a bird repellant, though it is not on the market as a repellent in the US as of 2017. Although this information is interesting from a risk perspective in general, we do not consider it relevant and useful for the dossier at present and therefore agree with the notifier that it should not be submitted and evaluated.

The remaining five studies which were deemed relevant after the in-depth screening of the full text reports were submitted and evaluated by the RMS. They can be found in the relevant sections of this document and are listed in the references. For clarity, they are also listed in the table below.

References proposed as relevant after full-text screening and evaluated in this dossier

Author(s)	Data point	Year	Title	Source
Buysens, C., Dupré de Boulois, H. and Declerck, S.	CA 8.7-01	2015	Do fungicides used to control *Rhizoctonia solani* impact the non-target arbuscular mycorrhizal fungus *Rhizophagus irregularis*?	Mycorrhiza 25:277–288
Matsumoto, K.-I., Hosokawa, M., Kuroda, K. and Endo, G.	CA 8.2.8-02	2009	Toxicity of Agricultural Chemicals in *Daphnia magna*	Osaka City Med. J. Vol. 55, 89-97
Millot, F., Berny, P., Decors, A., Bro, E.	CA 8.1.4-02	2015	Little field evidence of direct acute and short-term effects of current pesticides on the grey partridge	Ecotoxicology and Environmental Safety 117 41–61
Wang, Y., Wu, S., Chen, L., Wu, C., Yu, R., Wang, Q. and Zhao, X.	CA 8.4.1-03	2012	Toxicity assessment of 45 pesticides to the epigeic earthworm *Eisenia fetida*	Chemosphere 88 484–491
Yang, Y., Qi, S., Chen, J., Liu, Y., Teng, M. and Wang, C.	CA 8.2.8-01	2016	Toxic Effects of Bromothalonil and Flutolanil on Multiple Developmental Stages in Zebrafish	Bull Environ Contam Toxicol 97:91–97

B.9.12 References relied on

Note: Some studies from the DAR (2006) which have been included in this document for completeness sake, have been added to the reference list in the appropriate location, however, they are in grey and are not numbered according to this RAR, as they are not used in the risk assessment.

Data point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner
CA 8.1.1.1-01	[redacted].	1987a	The acute oral toxicity (LD_{50}) of Flutolanil to the bobwhite quail. Nihon Nohyaku, Report No.: W-3003. GLP, Non Published	Y	N	-	Nihon-Nohyaku Co. Ltd.
CA 8.1.1.1-02	[redacted]	1987b	The acute oral toxicity (LD_{50}) of Flutolanil to the mallard duck. Nihon Nohyaku, Report No.: W-3004. GLP, Non Published	Y	N	-	Nihon-Nohyaku Co. Ltd.
CA	[redacted].	1987c	The subacute	Y	N	-	Nihon-

8.1.1.2-01			dietary toxicity (LD_{50}) of Flutolanil to the bobwhite quail. Nihon Nohyaku, Report No.: W-3006. GLP, Non Published				Nohyaku Co. Ltd.
CA 8.1.1.2-02	[redacted]	1987d	The subacute dietary toxicity (LD_{50}) of Flutolanil to the mallard duck. Nihon Nohyaku, Report No.: W-3006. GLP, Non Published	Y	N	-	Nihon-Nohyaku Co. Ltd.
CA 8.1.1.3-01	[redacted]	1993a	Flutolanil technical : A one-generation reproduction study with the bobwhite (*Colinus virginianus*). Nihon Nohyaku, Report No.: W-3025. GLP, Non Published	Y	N	-	Nihon-Nohyaku Co. Ltd.
CA 8.1.1.3-02	[redacted]	1993b	Flutolanil technical : A one-generation reproduction study with the mallard (*Anas platyrhynchos*). Nihon Nohyaku, Report No.: W-3026. GLP, Non Published	Y	N	-	Nihon-Nohyaku Co. Ltd.
CA 8.2.1-01	[redacted]	1987a	Acute toxicity of flutolanil technical to rainbow trout (*Salmo gairdneri*) Report No.: 35378 (W-3008) Test facility: [redacted], GLP: Y / Published: N	Y	N		Nihon-Nohyaku Co. Ltd.

CA 8.1.1.3-03	Palmer, D.A.	2016	EAG Laboratories letter – To whom it may concern. Test code: 244-108 I 18-7606-6837 Test facility: EAG Laboratories (formerly Wildlife International) GLP: N / Published: N	N	N	-	Nihon-Nohyaku Co. Ltd.
CA 8.1.2.2-01	Thomas, G.D., Myers, D.P.	2016	Request to determine reliable ECx values Report No: 17 October 2016 Test facility: Envigo GLP: N / Published: N	N	N	-	Nihon-Nohyaku Co. Ltd.
CA 8.1.4-01	[redacted]	2011	Flutolanil: Amphibian Metamorphosis assay for the detection of Thyroid Active Substances Report No.: 397A-149 (W-3073) Test facility: [redacted]. GLP: Y / Published: N	Y	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon-Nohyaku Co. Ltd.
CA 8.1.4-02	Millot, F., Berny, P., Decors, A., Bro, E.	2015	Little field evidence of direct acute and short-term effects of current pesticides on the grey partridge Report No.: Not applicable Institution: National Game and Wildlife Institute (ONCFS), and College of	Y	N	-	Not applicable

			Veterinary Medicine Lyon, France. GLP: N / Published: Y				
CA 8.1.5-01	Odum J., Roberts, M., Matthiessen, P.	2016	Assessment of flutolanil and its potential for endocrine disruption Report No.: NIC002_001 Test facility: Regulatory Science Associates GLP: N / Published: N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon-Nohyaku Co. Ltd.
CA 8.2.1-02	[redacted]	1987b	Acute toxicity of flutolanil technical to bluegill sunfish (*Lepomis macrochirus*) Report No.: 35377 (W-3009) Test facility: [redacted] GLP: Y / Published: N	Y	N		Nihon-Nohyaku Co. Ltd.
CA 8.2.1-03	[redacted].	1990	Acute toxicity of flutolanil technical to fathead minnow (*Pimephales promelas*) Report No.: 38101 (W-3010) Test facility: [redacted] GLP: Y / Published: N	Y	N		Nihon-Nohyaku Co. Ltd.
CA 8.2.2.1-01	[redacted].	1995	Early life-stage toxicity of flutolanil to the fathead minnow (*Pimephales promelas*) under flow-through conditions Report No.: 41685 (W-3030) Test facility:	Y	N		Nihon-Nohyaku Co. Ltd.

			[redacted] GLP: Y / Published: N				
CA 8.2.2.1-02	Palmer, D.A.	2016	EAG Laboratories letter – To whom it may concern. October 18, 2016 Test facility: EAG laboratories (formerly ABC Laboratories) GLP: N / Published: N	N	N		Nihon-Nohyaku Co. Ltd.
CA 8.2.2.3-01	[redacted].	1991	Uptake, depuration and bioconcentration of ^{14}C-Flutolanil by bluegill (*Lepomis macrochirus*) Report No.: 37902 (W-3013) Test facility: [redacted] GLP: Y / Published: N	Y	N		Nihon-Nohyaku Co. Ltd.
CA 8.2.2.3-02	[redacted]	1991	Characterization of ^{14}C-flutolanil residues in bluegill (Lepomis macrochirus) water and tissues Report No.: 38946 (W-3022) Test facility: [redacted] GLP: Y / Published: N	Y	N		Nihon-Nohyaku Co. Ltd.
CA 8.2.3-01	[redacted]	2011	Flutolanil: Fish Short-Term Reproduction Assay with the Fathead Minnow	Y	Y	Y	Nihon-Nohyaku Co. Ltd.

			(*Pimephales promelas*) Report No.: 397A-148 (W-3072) Test facility: [redacted] GLP: Y / Published: N				
CA 8.2.4.1-01	Forbis, A.D., Young, B.M., Hicks, S.L.	1990	Acute toxicity of flutolanil to *Daphnia magna* Report No.: 38718 (W-3014) Test facility: Analytical Bio-Chemistry Laboratories, Inc. GLP: Y / Published: N	N	N		Nihon-Nohyaku Co. Ltd.
CA 8.2.4.2-01	Forbis, A.D.	1991	Acute toxicity of flutolanil to *Mysidopsis bahia* Report No.: 38720 (W-3015) Test facility: Analytical Bio-Chemistry Laboratories, Inc. GLP: Y / Published: N	N	N		Nihon-Nohyaku Co. Ltd.
CA 8.2.5.1-01	Blakemore G.C., Burgess, D.	1991	21-Day chronic static renewal toxicity of flutolanil to *Daphnia magna* Report No.: 38721 (W-3017) Test facility: Analytical Bio-Chemistry Laboratories, Inc. GLP: Y / Published: N	N	N		Nihon-Nohyaku Co. Ltd.
CA 8.2.5.1-02	Palmer, D.A.	2016	EAG Laboratories letter – To whom it may concern. October 18, 2016 Test facility: EAG	N	N		Nihon-Nohyaku Co. Ltd.

			laboratories (formerly ABC Laboratories) GLP: N / Published: N				
CA 8.2.5.2-01	Kowalski, P.L., Boeri, R.L., Ward, T.J.	1995	Life-cycle Toxicity of Flutolanil to Mysid, *Mysidopsis bahia* Report No.: 481-NI (W-3029) Test facility: T.R. Wilbury Laboratories, Inc. GLP: Y / Published: N	N	N		Nihon-Nohyaku Co. Ltd.
CA 8.2.5.3-01	Desmares-Koopmans, D.	2003	Sediment-water Chironomid toxicity test using water spiked with Flutolanil Report No.: 335431 (N-3025) Test facility: NOTOX B.V. GLP: Y / Published: N	N	N		Nihon-Nohyaku Co. Ltd.
CA 8.2.6.1-01	Migchielsen, M.H.J.	2003	Fresh water algal growth inhibition test with Flutolanil Report No.: 354904 (N-3014) Test facility: NOTOX B.V. GLP: Y / Published: N	N	N		Nihon-Nohyaku Co. Ltd.
CA 8.2.8-01	Yang, Y., *et al.*	2016	Toxic Effects of Bromothalonil and Flutolanil on Multiple Developmental Stages in Zebrafish Report No.: Not applicable Institution: China Agricultural University GLP: N / Published: Y	Y	N		Not applicable

CA 8.2.8-02	Matsumoto, K.-I., *et al.*	2009	Toxicity of Agricultural Chemicals in *Daphnia magna* Report No.: Not applicable Institution: Sakai City Institute & Osaka City University GLP: N / Published: Y	Y	N		Not applicable
CA 8.3.1.1.1-01	Schmitzer, S.	2001	Laboratory testing for toxicity (Acute contact and oral LD_{50}) of Flutolanil tech. on honey bees (*Apis mellifera* L) (Hymenoptera, Apidae) Report No.: 9051036 (N-3015) Test facility: Institut fiir Biologische Analytik und Consulting IBACON GmbH GLP: Y / Published: N	N	N	-	Nihon-Nohyaku Co. Ltd.
CA 8.3.1.2-01	Ruhland, S.	2016	Chronic toxicity of Flutolanil SC to the honey bee *Apis mellifera* L. under laboratory conditions Report No.: 16 10 48 034 B (N-3078) Test facility: BioChem agrar GLP: Y / Published: N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon-Nohyaku Co. Ltd.
CA 8.3.1.3-01	Scheller, K.	2016	Repeated exposure of Flutolanil 40 SC to honey bee (*Apis mellifera*) larvae under laboratory conditions (*in vitro*) Report No.: 16 10 48 035 B (N-3079)	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon-Nohyaku Co. Ltd.

			Test facility: BioChem agrar GLP: Y / Published: N				
CA 8.3.2.1-01	Nienstedt, K.M.	1999a	EXP10066A: A laboratory toxicity test with the parasitic wasp, *Aphidius rhopalosiphi* (Hymenoptera: Braconidae) Report No.: 99-073-1013 (N-3016) Test facility: Springborn laboratories AG GLP: Y / Published: N	N	N	-	Nihon-Nohyaku Co. Ltd.
CA 8.3.2.2-01	Nienstedt, K.M.	1999b	EXP10066A: Laboratory Contact Toxicity Test with the Predacious Mite, *Typhlodromus pyri* Scheuten (Acari: Phytoseiidae) Report No.: 99-074-1013 (N-3017) Test facility: Springborn laboratories AG GLP: Y / Published: N	N	N	-	Nihon-Nohyaku Co. Ltd.
II A, 8.4.1	Wüthrich V.	1990	Acute toxicity (LD_{50}) study of Flutolanil techinical to earthworms. Nihon Nohyaku, Report No: W-3020. GLP, Non Published	N	N		NIH
CA 8.4.1-01	Lührs, U.	2000	Effects of EXP10066A on Reproduction and Growth of Earthworms *Eisenia fetida* (Savigny 1826) in Artificial Soil Report No.: 8411022 (N-3022) & Report amendment, 2016	N	N	-	Nihon-Nohyaku Co. Ltd.

			Test facility: Institut fiir Biologische Analytik und Consulting IBACON GmbH GLP: Y / Published: N				
CA 8.4.1-02	Lührs, U.	2001	Effects of EXP10066A on Reproduction and Growth of Earthworms *Eisenia fetida* (Savigny 1826) in Artificial Soil with reduced organic matter content Report No.: 8414022 (N-3023) & Report amendment, 2016 Test facility: Institut fiir Biologische Analytik und Consulting IBACON GmbH GLP: Y / Published: N	N	N	-	Nihon-Nohyaku Co. Ltd.
CA 8.4.1-03	Wang, Y., *et al.*	2012	Toxicity assessment of 45 pesticides to the epigeic earthworm *Eisenia fetida* Report No.: Not applicable Institutions: Zhejiang Academy of Agricultural Sciences, China GLP: N / Published: Y	N	N	-	Not applicable
CA 8.4.2-01	Meister, A., Lührs, U.	2002	Effects of EXP100066A on Reproduction of the Collembola *Folsomia candida* in Artificial Soil Report No.: 10694016 (N-3010) & Report amendment, 2016 Test facility:	N	N	-	Nihon-Nohyaku Co. Ltd.

			Institut fiir Biologische Analytik und Consulting IBACON GmbH GLP: Y / Published: N				
CA 8.4.2.1-01	Ganßmann, M.	2015	MONCUT 40C (EU): Effects on reproduction of the predatory mite *Hypoaspis aculeifer* in artificial soil with 5% peat Report No.: 105911089 (N-3075) Test facility: Institut fiir Biologische Analytik und Consulting IBACON GmbH GLP: Y / Published: N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon-Nohyaku Co. Ltd.
CA 8.5-01	Forster, J.	1999	A laboratory assessment of the effects of EXP10066A (Flutolanil) on soil microflora respiration and nitrogen transformations according to current EU guidelines Report No.: GOoD 14469 (N-3024) Test facility: Chemex International plc GLP: Y / Published: N	N	N	-	Nihon-Nohyaku Co. Ltd.
III A, 10.6.2b	Meister, A.	2002	Effect of EXP10066A on the decomposition of organic material enclosed in litter bags in the field. Nihon Nohyaku, Report No.: N-3009. Non GLP, Published	N	N		NIH
IIIA,	Meinerling,	2006	Final analytical	N	N		NIH

10.6.2c	M. ; Hermann, S.		report to: Field study on the effects of Flutolanil 40SC on the breakdown of organic matter in litter bags. Institut für Biologische Analytik und Consulting IBACON GmbH., Rossdorf, Germany. Nihon Nohyaky, Project 20301081R. GLP, Non published				
IIIA, 10.6.2d	Rosenkranz, B.	2006	Field study on the effects of Flutolanil 40SC on the breakdown of organic matter in litter bags. Institut für Biologische Analytik und Consulting IBACON GmbH., Rossdorf, Germany. Nihon Nohyaky, Project 20301081. GLP, Non published	N	N		NIH
CA 8.6.1-01	Spatz, B.	2002	Effect of EXP10066A on terrestrial (non-	N	N	-	Nihon-Nohyaku Co. Ltd.

			target) plants: Seedling emergence and seedling growth test Report No.: 10695086 (N-3012) Test facility: Institut fiir Biologische Analytik und Consulting IBACON GmbH GLP: Y / Published: N				
CA 8.7-01	Buysens, C., *et al.*	2015	Do fungicides used to control *Rhizoctonia solani* impact the non-target arbuscular mycorrhizal fungus *Rhizophagus irregularis*? Report No.: Not applicable Institute: Earth and Life Institute, Université Catholique de Louvain, Belgium GLP: N / Published: Y	N	N	-	Not applicable
CA 8.8-01	Barnes, B.	2007	Flutolanil: Activated Sludge: Respiration Inhibition Test Report No.: NHH 0123/072254 (N-3030) Test facility: Huntingdon Life Sciences Ltd. GLP: Y / Published: N	N	Y	Article 59(1) & (2) of Regulation (EC) 1107/2009 applies	Nihon-Nohyaku Co. Ltd.
IIA, 8.7/02	Sharman, M.R.Q.A. and Barnes, S.P.	2007	Flutolanil: Activated sludge – respiration inhibition test Huntingdon Life Sciences Ltd., England,	N	N		NIH

			Nihon Nohyaky, Report No: NHH 0123/072254, Report amendment 1 GLP, Non published				