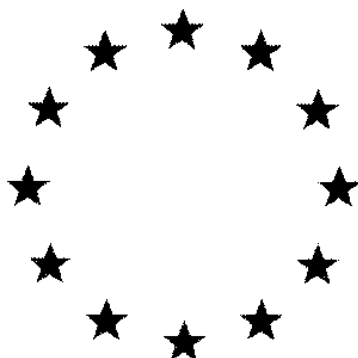


# European Commission



**Draft (Renewal) Assessment Report prepared  
according to the Commission Regulation (EC) No  
1107/2009**

**Daminozide (ISO); 4-(2,2-  
dimethylhydrazino)-4-oxobutanoic  
acid; *N*-dimethylaminosuccinamic  
acid**

**Volume 3 – B.9 (AS)**

Rapporteur Member State: Czech Republic  
Co-Rapporteur Member State: Hungary

**Version history page:**

Date	Version	Reason for revision
May 2018	Version 1	First draft
October 2018	Version 2	Revision after Notifier's and co-RMS comments
June, 2019	Version 3	Update following the ECHA accordance check

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## B.9 Ecotoxicology

This document is a summary of the information presented for this section for the first inclusion and for the purpose of AIR 3.

Daminozide (ISO); 4-(2,2-dimethylhydrazino)-4-oxobutanoic acid; N-dimethylaminosuccinamic acid (hereafter referred to as 'daminozide') is a plant growth regulator currently registered for use on indoor ornamental plants. The mode of action is through interference with gibberellic acid biosynthesis. It is absorbed by the leaves and translocated throughout the treated plant. As a result more compact plants (by inhibition of intermodal elongation) are produced.

Daminozide was included in Annex I to Directive 91/414/EEC by Commission Directive 2005/53/EC and entered into force on 1<sup>st</sup> March 2006. The inclusion Directive only authorised uses as a growth regulator in non-edible crops and noted that Member States (MS) should pay particular attention to the protection of aquatic organisms, bees and non-target arthropods (risk mitigation measures to be applied where appropriate) and operator safety (authorisation should include protective measures, where appropriate). A Review Report for Daminozide (SANCO/3043/99 – Final (15 February 2005)) is available. The rapporteur Member State (RMS) was the Netherlands and there is no EFSA conclusion. The agreed EU endpoints for use in the risk assessment are therefore detailed in Appendix II to the Review Report (and reproduced in Annex I). The Monograph (Draft Assessment Report) was published in June 1999. Subsequent Addenda to the DAR relevant to ecotoxicology were published in June 2002 (Addendum 1), June 2003 (Addendum 4 – List of endpoints) and September 2004 (Addendum 5).

The application for renewal of approval of daminozide will fall within the same rates and timings as the GAP considered for Annex I inclusion. The representative formulations for renewal will be water soluble granules (SG) containing 850 g/kg daminozide. The use pattern is summarised in Table B.9-1.

**Table B.9-1 Intended application pattern**

Crop	Timing of application BBCH	Method of application	Number of applications	Interval between applications (min.)	Maximum application rate, individual treatment	
					Product [kg/ha]	Daminozide [kg a.s./ha]
Ornamentals (Protected)	<50	Over spray (Gantry)	1 - 5	7 days	9	7.65
Ornamentals (Outdoor)	<50	Foliar*	1 - 5	7 days	5	4.25

\* Application using a knapsack sprayer

In the draft renewal assessment report, new data for the renewal of the approval of daminozide has been evaluated. Studies and investigations already assessed within the EUDAR (1997) and Addendum (2002) have been re-evaluated in this report. The conclusions have been updated to meet current scientific standards.

A literature review was carried out for daminozide and its' metabolites according to the requirements of the Regulation (EU) No 844/2012, which itself refers to Article 8(5) of Regulation (EC) No 1107/2009. The review itself is in accordance with the EFSA Guidance document as published in EFSA Journal 2011; 9(2):2092. In case where reliable and adequate literature was found during the literature search, summaries are integrated in the respective sections of the RAR.

#### Metabolites

In addition to the active substance daminozide, the metabolite methanol was addressed in the assessment since it was identified as an environmental occurring residue requiring further assessment in e-fate section.

**Table B. 9- 2      Residues requiring further assessment**

Soil: daminozide, methanol
Surface water: daminozide, methanol
Sediment: daminozide, methanol
Ground water: daminozide, methanol
Air: daminozide, methanol

### B.9.1            Effects on birds and other terrestrial vertebrates

#### B.9.1.1        Effects on birds

##### B.9.1.1.1      Acute oral toxicity to birds

##### i)            Acute toxicity in mallard duck

<b>Reference:</b>	██████████ (1992) Alar technical: an acute oral toxicity study with the mallard
Report No.:	A7.4.2.9
Guideline:	U.S. EPA 71-1(1982)
GLP:	Yes
<b>Previous evaluation:</b>	In DAR (1999)
<b>Material and methods:</b>	
Test material:	Daminozide technical
Lot/Batch No:	BFI 1576
Purity:	99.8%

An acute oral toxicity test with daminozide was performed on mallard ducks (5 males / 5 females per group). Five test concentrations, 292, 486, 810, 1350 and 2250 mg a.s./kg bw, plus vehicle control (gelatine capsule). Birds were acclimated for 5 weeks prior to test initiation. The birds were fasted for at least 15 hours prior to dosing. At initiation of the test , each bird was administered an oral dose of the test substance by gelatin capsule. Each bird was individually weighed and dosed on the basis of milligrams of test substance per kilogram of body weight. The control birds received two blank gelatin capsules, simulating the highest test dosage. Post-dosing observation lasted for 14 days. Body weights were measured individually at initiation of the test and by group on Days 3, 7 and 14. Average estimated feed consumption was determined for each dosage group and the control for Days 0-3, 4-7 and 8-14. Feed consumption was determined by measuring the change in the weight of the feed presented

to the birds over a given period of time. However, feed consumption is presented as an estimate due to the unavoidable wastage by the birds.

### Results:

No mortalities, no influence on body weight and feed consumption were noted in control or treatment group. The LD<sub>50</sub> was determined to be greater than 2250 mg a.s./kg bw, the highest dose tested.

**Table B 9.1.1-1 Average body weight and estimated food consumption**

Dosage		Average Body Weight in Grams								Estimated Feed Consumption Grams/Bird/Day		
mg/kg	Sex	Day 0	Change	Day 3	Change	Day 7	Change	Day 14	Total Change	Days 0-3	Days 4-7	Days 8-14
Control	M	1183	32	1215	-17	1198	10	1208	25	155	127	119
	F	1007	8	1015	-15	1000	-9	991	-16	144	100	101
292	M	1098	51	1149	13	1162	-30	1132	34	159	130	127
	F	962	13	975	-8	967	4	971	9	216	174	162
486	M	1127	3	1130	20	1150	-30	1120	-7	112	122	111
	F	1062	5	1067	1	1068	-36	1032	-30	142	110	109
810	M	1192	35	1227	-3	1224	-44	1180	-12	158	135	116
	F	1046	14	1060	3	1063	-14	1049	3	149	140	118
1350	M	1226	6	1232	0	1232	-25	1207	-19	128	111	109
	F	1117	-1	1116	-1	1115	-40	1075	-42	156	130	109
2250	M	1111	17	1128	25	1153	-47	1106	-5	145	143	109
	F	1136	-2	1134	5	1139	-43	1096	-40	109	108	95

Remark from previous review: The result LD<sub>50</sub> > 2250 mg a.s./kg bw is used for risk evaluation.

### RMS comments and conclusion:

The reported study is GLP compliant and was conducted according U.S. EPA 71-1 guideline (1982). The test results are in compliance with the current guidelines' validity criteria (OECD 223 (2010); OCSPP 850.2100 (2012)).

The study is acceptable for regulatory use.

The acute oral LD<sub>50</sub> for mallard duck is > 2250 mg as/kg bw and the no observed effect level is 2250 mg a.s./kg bw.

### ii) Acute toxicity in bobwhite quail

Reference:	(2006) Daminozide technical: An acute oral toxicity study with the Japanese quail
Report No.:	429-104
Guideline:	U.S. EPA 71-1(1982); OPPTS 850.2100 (1996)
GLP:	Yes

**Previous evaluation:** Submitted for the purpose of renewal

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### Executive Summary

The acute oral toxicity of daminozide to Japanese quail (*Coturnix coturnix japonica*) was determined in the laboratory, with groups of 10 birds exposed to a limit dose of 2250 mg a.s./kg bodyweight, in parallel with an untreated control group. The acute LD<sub>50</sub> was determined to be above 2250 mg a.s./kg bw, the highest dose applied. The no-mortality and the no-observed-effect-levels (NOEL) were 2250 mg a.s./kg bw.

### Materials and methods:

#### A. MATERIALS

1. **Test material:** Daminozide technical  
**Description:** Solid  
**Lot/Batch:** 2003-10-01; Lot 3  
**Purity:** 100.2% wt

#### B. STUDY DESIGN AND METHODS

1. **Test animals:** Japanese quail (*Coturnix coturnix japonica*)  
**Age/growth stage:** Approximately 6 weeks. Individual body weights at the start of the test ranged from 93 to 117 g.  
**Source:** [REDACTED] production flock  
**Acclimation:** 18 days prior to dosing. Food was withheld for approximately 18 hours prior to dosing.  
**Diet:** Fed a game bird ration formulated to [REDACTED] specifications by Cargill Animal Nutrition, Shippensburg, PA. Water was available *ad libitum*, except during the fasting period.
2. **Test concentration:** 0, and 2250 mg a.s./kg bw
3. **Housing:** Birds were housed by sex (2 pens per test group) in indoor pens manufactured by GQF Manufacturing Co.. Each pen had a floor space that measured approximately 78 x 51 cm. Floors were sloping so that ceiling heights ranged from 20 to 25 cm. External walls, ceilings and floors were constructed of wire mesh and side walls were constructed of galvanized sheeting.
4. **Environmental conditions:**  
**Temperature:** 24.6 ± 0.5°C  
**Relative humidity:** 77 ± 2%  
**Photoperiod:** 8 hrs light / 16 hrs dark; light intensity: approximately 316 lux
5. **Animal assignment and treatment:**



Ten Japanese quail, five males and five females, were assigned to the treatment group and the control group by indiscriminate draw. At experimental start, a single dose of the test substance (2250 mg/kg bw) in diluent was orally intubated directly into the crop or proventriculus of each bird. Each bird was individually weighed and dosed on the basis of milligrams of daminozide technical per kilogram of body weight (mg/kg). The control birds received a corresponding volume of diluent only.

#### 6. Dose preparation:

The test substance was dispersed in reverse deionized water. The concentration of the test substance in the diluent was adjusted to provide a constant volume to body weight dosage for all treatment birds.

#### 7. Measurements and observations:

From test initiation until termination, all birds were observed at least twice a day. A record was maintained of all mortality, signs of toxicity, and abnormal behaviour. Body weights were measured individually on Days 0, 3, 7 and 14 of the test. Average feed consumption was determined by pen for the test group and the control group for days 0-3, 4-7 and 8-14.

#### 8. Statistics:

There were no mortalities in this study, therefore, it was not possible to perform the calculation of an LD<sub>50</sub>. No statistical analyses were applied to separate mean responses for the treatment group and control group for the endpoints of food consumption and body weight.

### Results:

#### A. Mortality / clinical observations:

There were no mortalities in the control group, and all control birds were normal in appearance and behaviour throughout the test. Additionally, there were no mortalities in the 2250 mg/kg treatment group.

In the 2250 mg/kg treatment group, one male was noted with feather loss and bruising to the head on the afternoon of Day 4 of the test. This male was also noted to have a slight loss of coordination and a slight ruffled appearance that were attributed to the head injury. The head injury may have been the result of pen mate aggression. This bird continued to exhibit a slight loss of coordination and/or a slight ruffled appearance through the morning of day 7. With the exception of the feather loss and bruising on the head, the bird had recovered by the afternoon of Day 7 and was normal in appearance and behaviour for the remainder of the test. All clinical signs noted were attributed to the head injury and were not considered to be treatment related. All other birds in the 2250 mg/kg treatment group were normal in appearance and behaviour throughout the test.

**Table B 9.1.1-2 Mortality data for Japanese quail exposure to daminozide via a single oral dose**

Treatment (mg/kg bw.)	Mortality (n° death / n° dosed)			Time of death after dosing
	Male	Female	Combined	
Vehicle control	0/5	0/5	0/10	-
2250	0/5	0/5	0/10	-

**B. Body weight / feed consumption:**

When compared to the control group, there were no apparent treatment related effects on body weight among the males and females in any of the treatment dosage group.

**Table B 9.1.1-3 Group mean body weights and total body weight change (g/bird)**

Treatment (mg/kg bw.)	No. of birds	Mean body weight (g/bird)				
		Test day 0	Test day 3	Test day 7	Test day 14	Total body weight change <sup>a</sup>
Vehicle control	10	108 (m)	118 (m)	126 (m)	134 (m)	26 (m)
		108 (f)	117 (f)	128 (m)	130 (f)	22 (f)
2250	10	104 (m)	112 (m)	121 (m)	124 (m)	20 (m)
		107 (f)	118 (f)	127 (f)	129 (f)	23 (f)

<sup>a</sup> The mean change is calculated separately from the mean body weights using the individual changes in body weights  
m = male, f = female

Also, when compared to the control group, there were no apparent treatment related effects on feed consumption among the birds in the treatment dosage group at any feed consumption interval.

**Table B 9.1.1-4 Estimated mean food consumption of combined sexes (g/bird/day)**

Treatment (mg/kg bw.)	Estimated mean food consumption (g/bird/day)		
	Days 0-3	Days 4-7	Days 8-14
Vehicle control	36	34.5	23
2250	39.5	34.5	24

**Conclusion:**

The acute oral LD<sub>50</sub> value for Japanese quail exposed to daminozide as a single oral dose was determined to be greater than 2250 mg a.s./kg. The no-mortality level and the no-observed effect level were 2250 mg a.s./kg.

**RMS comments and conclusion:**

The reported study is GLP compliant and was conducted according U.S. EPA 71-1 (1982) and OPPTS 850.2100 (1996) guidelines. The test results are in compliance with the current guidelines' validity criteria (OECD 223 (2010); OCSPP 850.2100 (2012)).

The study is acceptable for regulatory use.

The acute oral LD<sub>50</sub> for bobwhite quail is > 2250 mg as/kg bw and the no observed effect level is 2250 mg/kg bw.

**B.9.1.1.2 Short-term dietary toxicity****i) Short-term dietary toxicity in bobwhite quail**

<b>Reference:</b>	██████████ (1977) Eight-day dietary LC <sub>50</sub> – bobwhite quail: technical Alar
<b>Report No.:</b>	A.7.4.2.4
<b>Guideline:</b>	Not stated
<b>GLP:</b>	No

**Previous evaluation:** In DAR (1997)

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**Material and methods:**

Test material: Daminozide (technical Alar)

Lot/Batch No: BL8189

Purity: Not stated

An 8 day dietary toxicity test with daminozide (purity not reported) was performed with bobwhite quail (10 birds in 5 pens per group). Five test concentrations, 464, 1000, 2150, 4640 and 10,000 mg/kg feed, plus control. The birds were exposed to the appropriate dietary concentrations for five days, and then maintained on toxicant-free diet for an additional three-day observation period. The negative control birds received the basal diet throughout the study.

Body weights were recorded by pen at initiation and termination of the study. Food consumption was recorded by pen during the five-day exposure period. Food consumption was measured accurately, but is presented as an estimate due to the unavoidable wastage by the birds.

Symptoms of toxicity and mortality were recorded daily throughout the study.

Mortality was analyzed statistically by the method of Litchfield and Wilcoxon 1949.

**Results:**

There was no mortality in the negative control groups and the birds appeared normal throughout the study. However, there was some evidence of toe picking in Control Group 3, which appears to have effected weight gain. There was no mortality in the daminozide treatment groups. Extensive toe picking was evident at the 1000 ppm dose level, which is reflected in the reduced body weight gain at this level. All birds at all other dose levels appeared normal throughout the test period.

The 8-day  $LC_{50}$  > 10,000 mg/kg feed for quail.

Table B 9.1.1-5 Average body weight and estimated food consumption

Material	Concentration ppm	Average Body Weight (g)		Total Estimated Food Consumption During Five-Day Exposure Period g
		Day 0	Day 8	
Technical Alar	464	29	50	372
	1,000	29	39	320
	2,150	31	52	384
	4,640	28	48	381
	10,000	30	48	397
Dieldrin Controls	15.9	35	39	500
	25.1	33	38	425
	39.8	33	33	275
	63.1	32	30	250
	100	30	30	100
Negative Controls	0	32	54	409
	0	28	49	382
	0	31	52	446
	0	32	50	423
	0	30	52	450

Remark from previous review: Purity daminozide not reported. According to the test report from the acute study, the purity of GMS # 4791 is 100%. The result 8-days  $LC_{50} > 10,000$  mg/kg fd is used for risk evaluation.

#### RMS comments and conclusion:

The reported study is non-GLP and no test guideline is stated. Several deviations from the current OCSP 850.2200 (2012) and OECD 205 (1984) guidelines were noted. No diet analysis was performed during the study, therefore the homogeneity and stability of test substance could not be verified. Except for the room temperature, no data on housing conditions were reported (type, size and material of pen, relative humidity, photoperiod and lighting intensity). According to OECD 205 guideline, body weights should be monitored on day 0, 5 and 8 while data on day 0 and 8 were only reported in the study. In addition, food consumption should be measured on days 0-5 and 5-8 while it only was reported for 0-5 day period in the study. Further, no statistical analysis of data to obtain NOEC and LOEC was performed.

Given a lot of shortcomings, the study is not considered valid. Moreover, bodyweights over the exposure period were not reported, therefore, the concentrations could not be converted to daily doses.

## ii) Short-term dietary toxicity in mallard duck

<b>Reference:</b>	██████ (1974) Eight-day dietary LC <sub>50</sub> – mallard ducks: technical Alar
Report No.:	A.7.4.2.5
Guideline:	Not stated
GLP:	No
<b>Previous evaluation:</b>	In DAR (1997)
<b>Material and methods:</b>	
Test material:	Daminozide (technical Alar)
Lot/Batch No:	12-PCD-38
Purity:	Not stated

An 8 day dietary toxicity test with daminozide (purity not reported) was performed with mallard duck (10 birds in 5 pens per group). Five test concentrations, 464, 1000, 2150, 4640 and 10,000 mg/kg feed, plus control. The birds were exposed to the appropriate dietary concentrations for five days, and then maintained on toxicant-free diet for an additional three-day observation period. The negative control birds received the basal diet throughout the study.

Body weights were recorded by pen at initiation and termination of the study. Food consumption was recorded by pen during the five-day exposure period. Food consumption was measured accurately, but is presented as an estimate due to the unavoidable wastage by the birds.

Symptoms of toxicity and mortality were recorded daily throughout the study.

Mortality was analyzed statistically by the method of Litchfield and Wilcoxon 1949.

**Results:**

There was no mortality in the negative control groups and the birds appeared normal throughout the study. There was no mortality in the daminozide treatment groups. There were no symptoms of toxicity or behavioral abnormalities noted.

The 8-day LC<sub>50</sub> > 10,000 mg/kg feed for quail.

Table B 9.1.1-6 Average body weight and estimated food consumption

Material	Concentration ppm	Average Body Weight (g)		Total Estimated Food Consumption During Five-Day Exposure Period g
		Day 0	Day 8	
Technical ALAR	464	200	385	3950
	1,000	202	370	2825
	2,150	195	395	4050
	4,640	187	375	3350
	10,000	202	367	3625
Dieldrin Controls	68	185	320	2600
	100	187	325	2600
	147	182	225	1825
	215	195	*	950
	316	187	*	800
Negative Controls	0	172	370	3325
	0	192	385	3700
	0	192	380	3025
	0	173	375	3225
	0	175	370	3100

\* Data not available due to total mortality.

Remark from previous review: Purity daminozide not reported. According to the test report from the acute study, the purity of GMS # 4791 is 100%. The result 8-days  $LC_{50} > 10,000$  mg/kg fd is used for risk evaluation.

#### RMS comments and conclusion:

The reported study is non-GLP and no test guideline is stated. Several deviations from the current OECD 205 (1984) guideline were noted. No diet analysis was performed during the study, therefore the homogeneity and stability of test substance could not be verified. Except for the room temperature, no data on housing conditions were reported (type, size and material of pen, relative humidity, photoperiod and lighting intensity). According to OECD 205 guideline, body weights should be monitored on day 0, 5 and 8 while data on day 0 and 8 were only reported in the study. In addition, food consumption should be measured on days 0-5 and 5-8 while it was only reported for 0-5 day period in the study. Further, no statistical analysis of data to obtain NOEC and LOEC was performed.

Given a lot of shortcomings, the study is not considered valid. Moreover, bodyweights over the exposure period were not reported, therefore, the concentrations could not been converted to daily doses.

#### B.9.1.1.3 Subchronic and reproductive toxicity

**i) Subchronic and reproductive toxicity in bobwhite quail**

<b>Reference:</b>	(2012) Daminozide: a reproduction study with the northern bobwhite
Report No.:	616-104
Guideline:	U.S. EPA 71-1(1982); OPPTS 850.2300 (1996), OECD 206 (1984)
GLP:	Yes
<b>Previous evaluation:</b>	Submitted for the purpose of renewal

## Executive Summary

A laboratory study was conducted to evaluate the effects upon the adult northern bobwhite (*Colinus virginianus*) of dietary exposure to daminozide over a period of approximately 21 weeks. Each treatment (160, 400 and 1000 ppm a.s.) and control group contained 16 pairs of birds with one male and one female per pen.

Effects on adult health, body weight, and feed consumption were evaluated. In addition, the effects of adult exposure to daminozide on the number of eggs laid, fertility, embryo viability, hatchability, offspring survival, and egg shell thickness were evaluated.

There were no treatment-related mortalities, overt signs of toxicity or treatment-related effects upon body weight or feed consumption at any of the concentrations tested. Additionally, there were no treatment-related effects upon any of the reproductive parameters measured at the 160, 400 or 1000 ppm a.s. test concentrations. The no-observed-effect concentration for northern bobwhite quail exposed to daminozide in the diet during the study was 1000 ppm a.s. (79.7 mg a.s./kg bw/day), the highest concentration tested.

## Material and methods:

### A. MATERIALS

1. **Test material:** Daminozide  
**Batch number:** 101218026  
**Purity:** 100%  
**Appearance:** Solid

- ## 2. Vehicle:Diet

## B. STUDY DESIGN AND METHODS

- 1. Test animals** Northern Bobwhite (*Colinus virginianus*)  
**Source:** [REDACTED]

- Age at test initiation:** 32 weeks
- Weight at test initiation:** 185 – 235 g
- Acclimation period:** Approx. 14 weeks
2. **Diet/water:** All adults and offspring were given food and water *ad libitum*. Basal diet contained at least 27% protein and 2.5% crude fat, and no more than 3.8% crude fibre. Adult diet contained a calcium supplement (1.34%).
3. **Treatment groups:** 0, 160, 400 and 1000 ppm a.s.
4. **Housing:** Adults: pens of 25 x 51 cm with sloping floors that resulted in ceiling height ranging from 20 to 26 cm, constructed of galvanized wire mesh and galvanized sheeting  
Hatchlings: pens of 72 x 90 x 23 cm constructed of galvanized wire mesh and galvanized sheeting
5. **Environmental conditions:**
- Temperature:** Average temperature adult study room:  $22.7 \pm 0.5$  °C  
Incubator:  $37.4 \pm 0.0$  °C  
Hatching compartment:  $37.3 \pm 0.0$  °C  
Hatchling pens: *ca.* 38 °C  
Average temperature hatchling study room:  $27.1 \pm 1.4$  °C
- Humidity:** Average for adults:  $65 \pm 9\%$   
Incubator:  $55 \pm 0\%$   
Hatching compartment:  $57 \pm 1\%$   
Average for hatchlings:  $33 \pm 12\%$
- Air changes:** Vent up to 15 room air volumes every hour and replace them with fresh air
- Photoperiod:** First seven weeks: 8 hours of light per day (mean of 133 lux)  
Beginning of week 8: 17 hours of light per day (mean of 133 lux)  
For hatchlings: 16 hours of light per day

6. **Animal assignment and treatment:**

Each treatment and control group contained 16 pairs of birds with one male and one female per pen. Three treatment groups were fed diets containing either 160, 400 or 1000 ppm a.s. of daminozide for approximately 21 weeks. The control group was fed diet comparable to the treatment groups, but without the addition of the test substance.

The primary phases of the study and their approximate durations were:

1. Acclimation – approximately 14 weeks.
2. Pre-photostimulation – approximately 10 weeks.
3. Egg laying - Approximately 11 weeks.



4. Post-adult termination (final incubation, hatching, and 14-day offspring rearing period) -6 weeks.

#### **7. Dose preparation:**

Test diets were prepared by mixing daminozide into a premix that was used for weekly preparation of the final diet. Control diet and each of the three treated diets were prepared weekly and presented to the birds on Friday of each week. Dietary concentrations were adjusted for purity of the test substance and are presented as parts per million active substance (ppm a.s.).

#### **8. Measurements/observations:**

All adult birds were observed daily throughout the test for signs of toxicity or abnormal behaviour. Adult body weights were measured at test initiation, at the end of Weeks 2, 4, 6, 8, and at adult termination. Feed consumption was also estimated weekly throughout the test and presented as an estimate of total feed consumption.

At the beginning of Week 8, the photoperiod was increased to induce egg production. Following the start of egg production, eggs were collected daily and prepared for incubation on a weekly basis. All eggs laid in a weekly interval were considered as one lot. Weekly, eggs were selected by indiscriminate draw for egg shell thickness measurement and all remaining eggs were candled prior to incubation to detect egg shell cracks or abnormal eggs. Eggs were also candled twice during incubation to detect infertile eggs or embryo mortality.

On Day 21 of incubation, the eggs were placed in a hatcher and allowed to hatch. Once hatching was completed, hatchlings were removed from the hatcher and the group body weight of the hatchlings by pen was determined. At 14 days of age, the average body weight by parental pen of all surviving offspring was determined.

#### **Diet sampling**

Homogeneity of the test substance in the diet was evaluated by collecting six samples from each of the treated diets and one sample from the control diet 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. Control and treatment group diet samples were also collected from the bin feeders on Day 7 of Week 1 to assess stability of the test substance under actual test conditions. Additionally, samples were collected from the control and treatment group diets during Weeks 2, 3, 4, 8, 12, 16 and 20 of the test to measure/verify test concentrations. The diet samples were stored frozen or analysed immediately. Analysis was conducted using High Performance Liquid Chromatography (HPLC) after samples were extracted with methanol.

#### **Necropsy**

Adult birds that died or were euthanized during the course of the study were subjected to a gross necropsy.

#### **9. Statistics:**

Upon completion of the test, an analysis of variance (ANOVA) was performed to determine statistically significant differences between groups. Dunnett's multiple comparison procedure was used to compare the three treatment means with the control group mean and assess the statistical significance of the observed differences. Sample

units were the individual pens within each experimental group, except adult body weights where the sample unit was the individual bird. Percentage data were examined using Dunnett's method following arcsine square root transformation for reproductive parameters.

## **Results:**

### **A. Mortality:**

While no mortalities occurred in the control group or in the 160 or 400 ppm a.s. treatment groups, one incidental mortality occurred during the test in the 1000 ppm a.s. treatment group. The single mortality in the 1000 ppm a.s. treatment group was a female that was euthanized on Day 3 of Week 19 due to her debilitated condition. Prior to euthanasia, the bird was observed with lesions on the head, right leg and foot, feather loss, depression and a ruffled appearance. At necropsy the bird had a body weight of 176 g. Externally, the bird had a right foot lesion with necrotic tissue and bruising on the right leg. Internally, there was subcutaneous bleeding and bruising in the front region of the skull. The cecal contents were firm, the gizzard contents bile-stained and the kidneys were pale. No other lesions were noted and necropsy of the female's pen mate was not remarkable.

No other mortalities occurred during the course of the study. Due to the nature of the lesions observed at necropsy, the mortalities that occurred were not considered to be related to treatment.

### **B. Abnormal behaviour / symptoms of toxicity:**

No overt signs of toxicity were observed at any of the concentrations tested. Incidental clinical observations noted during the test included those that normally are associated with injuries and pen wear. Such observations included lesions on the head, neck, wing, or feet, ventral-head curl, and feather loss. Clinical signs observed included lameness, a thin appearance and an unkempt appearance, but were typically related to the incidental injuries and limited to only a few birds. Except for the incidental observations, birds were noted as normal in appearance and behaviour.

### **C. Body weight / feed consumption:**

There were no apparent treatment-related effects upon adult body weight at any of the concentrations tested. No statistically significant differences between the control group and the treatment groups were observed at any of the body weight intervals.

There were no apparent treatment-related effects upon feed consumption at any of the concentrations tested. There were slight decreases in feed consumption in the 160 ppm a.s. treatment group during Weeks 2 and 5 that were statistically significant at  $p < 0.01$  and at  $p < 0.05$ , respectively, and in the 400 ppm a.s. test concentration during Week 2 that was statistically significant at  $p < 0.01$ . In the 1000 ppm a.s. treatment group there were slight increases in feed consumption during Weeks 1 and 11 that were statistically significant at  $p < 0.05$  and at  $p < 0.01$ , respectively. Differences represented both decreases and increases in feed consumption. These differences were not considered to be treatment related since they were small and were neither consistent over time, nor concentration responsive.

**D. Gross necropsy:**

All surviving adults were subjected to gross necropsy following adult termination. All findings observed were considered unrelated to treatment.

**E. Reproductive results:**

There were no treatment-related effects upon reproductive performance at the any of the test concentrations. When compared to the control group, there were no statistically significant differences in any of the reproductive parameters measured in the 160 and 400 ppm a.s. treatment groups. In the 1000 ppm a.s. treatment group there was a slight reduction from the control group in offspring survival (14-day olds survivors as a percentage of hatchlings) that was statistically significant at  $p < 0.05$ . However, the statistical difference observed was likely due to a low percentage of survivors in one replicate of the 1000 ppm a.s. treatment group and an exceptional performance by the control group.

When data this one replicate were removed, the mean value for the group changed from  $93 \pm 8\%$  to  $95 \pm 5\%$ , and the difference from the control was no longer significant. The initial value for the 1000 ppm treatment group was comparable to the mean control value of  $94 \pm 6\%$  from the eight most recent Wildlife International, Ltd. northern bobwhite reproduction studies and the historical control value for this parameter of  $90 \pm 7\%$ . The survival for the control group offspring for this study exceeded those values ( $98 \pm 3\%$ ). Since the value was likely influenced by data from one replicate, and comparable to both historical and contemporaneous control data, the slight reduction at the 1000 ppm a.s. test concentration was not considered treatment related. There were no other statistically significant differences in the reproductive parameters measured between the control group and the 1000 ppm a.s. treatment group.

Summary of reproductive effects are presented in the table below.

**Table B 9.1.1-7 Summary of reproductive effects of daminozide on northern bobwhite quail**

Parameter	Experimental group (ppm a.s.)			
	Control	160	400	1000
Number of replicates	16	16	16	15
Total change in adult body weight (g)	8 ♂, 38 ♀	15 ♂, 40 ♀	10 ♂, 35 ♀	9 ♂, 37 ♀
<b>Reproductive parameter</b>				
Total eggs laid <sup>a</sup>	628	701	729	708
Eggs laid/hen	39	44	46	47
Eggs laid/hen/day <sup>b</sup>	0.41	0.46	0.47	0.49
Egg shell thickness (mm)	$0.228 \pm 0.015$	$0.230 \pm 0.014$	$0.227 \pm 0.019$	$0.226 \pm 0.011$
Eggs cracked	21	19	31	16
Eggs cracked/eggs laid (%)	3	3	4	3
Eggs set	486	614	625	623
Viable embryos/eggs set (%)	87	97	97	96
Live 3-wk embryos	438	592	568	597

Parameter	Experimental group (ppm a.s.)			
	Control	160	400	1000
Live 3-wk embryos/viable embryos (%)	100	100	99	100
Hatchlings	392	565	512	534
Hatchlings/live 3-wk embryos (%)	90	95	90	89
Hatchlings/eggs set (%)	78	91	81	86
Body weight of hatchling (mean)	6	6	6	6
14-day old survivors	387	553	496	498
14-day old survivors/hatchlings (%)	98	97	97	93*
14-day old survivors/eggs set (%)	77	89	79	80
14-day old survivors/maximum set (%)	38	54	48	52
14-day old survivors/hen	24	35	31	33
Body weight of 14-day old survivors (mean)	30	29	29	31

\* Significantly different from the control at  $p < 0.05$

<sup>a</sup> Represents the total number of eggs laid in each group

<sup>b</sup> Based on 96 days of egg production

### Egg shell thickness

There were no apparent treatment related effects upon egg shell thickness at the any of the test concentrations. When compared to the control group, there were no statistically significant differences in egg shell thickness in the 160, 400 or 1000 ppm a.s. treatment groups.

### Offspring body weights

There were no apparent treatment related effects upon offspring body weight at any of the concentrations tested. When compared to the control group, there were no statistically significant differences in the body weight of hatchlings or 14-day old survivors from the 160, 400 or 1000 ppm a.s. treatment groups.

### F. Diet analysis:

None of the control samples showed any indication of the presence of the test substance. Diet samples were collected from the 160, 400 and 1000 ppm a.s. test concentrations, and were analysed to evaluate the homogeneity of the test substance in the diet. Means and standard deviations for the test concentrations were  $136 \pm 5.95$  ppm a.s.,  $357 \pm 16.7$  ppm a.s. and  $919 \pm 23.3$  ppm a.s., respectively. Samples collected during the test to verify test substance concentrations for the 160, 400 and 1000 ppm a.s. diets had means and standard deviations of  $137 \pm 6.44$  ppm a.s.,  $365 \pm 19.6$  ppm a.s. and  $890 \pm 61.0$  ppm a.s., respectively. These values represented 86%, 91% and 89% of nominal concentrations. Analysis of diet samples collected from feeders after being held at ambient temperature for seven days averaged 89%, 97% and 98% of the Day 0 values for the 160, 400 and 1000 ppm a.s. test concentrations, respectively.

**Estimated Maximum Mean Daily Dietary Dose of Daminozide**

Estimated test substance intakes, daily dietary dose, for northern bobwhite were calculated by treatment group for the pre-egg production period, the egg production period and the overall adult period using the following formula:

$$\text{Daily Dietary Dose (mg/kg body weight/day)} = \frac{\text{Test Concentration (mg/kg)} \times \text{Daily Feed Consumption (g/bird/day)}}{\text{Body Weight (g/bird)}}$$

The mean body weight value is the mean of both male and female body weights. For the pre-egg production interval the body weights were averaged over Weeks 0, 2, 4, 6 and 8. For the egg-production interval body weights were averaged over Weeks 8 and 21 (adult termination). The accuracy of the estimated mean daily dietary dose may be impacted by differences in individual feed consumption, both within and between pens, and feed wastage. The estimated daily dietary doses are presented in the table below.

**Table B 9.1.1-8 Estimated Maximum Mean Daily Dietary Dose of Daminozide (mg/kg body weight/day)**

Test Interval (test weeks)	Test Concentration (ppm a.i.)	Mean Body Weight (g)	Mean Feed Consumption (g/bird/day)	Estimated Daily Dietary Dose (mg/kg/day)
Pre-Egg Production (Weeks 1 - 9)	0	213	13	0.0
	160	208	12	9.15
	400	209	12	23.4
	1000	213	13	61.2
Egg Production (Weeks 10 - 21)	0	227	19	0.0
	160	223	19	13.8
	400	222	19	35.0
	1000	226	20	90.5
Overall (Weeks 1 - 21)	0	217	16	0.0
	160	212	16	12.1
	400	213	16	30.7
	1000	216	17	79.7

**Conclusion:**

There were no treatment-related mortalities, overt signs of toxicity or treatment-related effects upon body weight or feed consumption at any of the concentrations tested. Additionally, there were no treatment-related effects upon any of the reproductive parameters measured at the 160, 400 or 1000 ppm a.s. test concentrations. The no-observed-effect concentration for northern bobwhite quail exposed to daminozide in the diet during the study was 1000 ppm a.s. (79.7 mg a.s./kg bw/day), the highest concentration tested.

**RMS comments and conclusion:**

The reported study is GLP compliant and was conducted according to EPA 71-4 guideline (1982), OPPTS 850.2300 (1996) and OECD 206 (1984). The test results are in compliance with the current guidelines' validity criteria (OECD 206 (1984): the mortality in controls should be less than 10%: actual value was 0%; the average

number of 14 day-old survivors per hen should be at least 12: actual value was 24; the average egg shell thickness for the control group should be at least 0.19: actual value was 0.228).

The study is acceptable for regulatory use.

Since the reproductive performance in all treatment groups was better than control in all parameters except for 14-day old survivors/hatchlings, the statistically significant effects on 14-day old survivors/hatchlings at 1000 ppm group are not considered biologically relevant.

The no-observed-effect concentration for northern bobwhite quail exposed to daminozide in the diet during the study was 1000 ppm a.s. (79.7 mg a.s./kg bw/day), the highest concentration tested.

### **B.9.1.2 Potential for endocrine disruption**

According to the existing data set for terrestrial animals, daminozide does not show to have an effect on the endocrine system. Avian reproduction studies resulted in no treatment related mortalities, body weight, reproductive parameters, or adverse effects to offspring at the highest OECD 206 test concentration of 1000 ppm. Nor were there any findings from the gross necropsy. Furthermore, based on the mammalian data, there were no effects in the repeat dose toxicity studies that could be interpreted as being mediated via the endocrine system. No further testing is indicated to evaluate the endocrine disrupter potential of daminozide to terrestrial vertebrates.

### **B.9.2 Effects on aquatic organisms**

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

##### **B.9.2.1.1 Acute toxicity of active substance to fish**

##### **i) Acute toxicity to rainbow trout (*Oncorhynchus mykiss*)**

<b>Reference:</b>	██████████ (1987) The toxicity of daminozide to rainbow trout
Report No.:	FAL 0020
Guideline:	OECD 203 (1981)
GLP:	No
<b>Previous evaluation:</b>	In DAR (1999)
<b>Material and methods:</b>	
Test material:	Daminozide technical
Lot/Batch No:	V86.10.2.1.201
Purity:	99.4%

A 96 hours toxicity test with rainbow trout was conducted using seven test concentrations of daminozide; 316, 562, 1000, 1778, 3162, 5623 and 10000 mg/L, plus control, under semi-static conditions. Ten fish (mean weight = 6.53 g, mean length = 7.92 cm) per vessel was used, four replicates per concentration. Tests were started by the direct addition of the weight of daminozide calculated to produced the desired concentration to each aquarium. No

intermediate solvents were found necessary because of the ready solubility of the test material; gentle stirring produced complete solution within one minute. The test solutions were renewed at daily intervals to a maximum period of seven days.

Water temperature ranged from 13.8 to 16.0°C. pH ranged from 7.6 to 6.8, but dropped to 5.5 in the highest dose level (thus daminozide was found to have a mildly acidic reaction) which may have contributed to the mortality. The lowest concentration of dissolved oxygen to be recorded was equivalent to 82% of the saturation value. Statistics based on Granmo and Larsstuvold.

Actual concentrations of test substance were not measured.

### Results:

Data on cumulative mortality data for rainbow trout exposed to daminozide technical are presented in the table below.

**Table B 9.2.1-1 Cumulative mortality data for rainbow trout exposed to daminozide technical**

Mean measured concentrations (mg/L)	Exposure time				
	1 d	2 d	3 d	4 d	7 d
Control	0	0	0	0	0
10000	100%	100%	100%	100%	100%
5623	100%	100%	100%	100%	100%
3162	100%	100%	100%	100%	100%
1778	10%	80%	90%	100%	100%
1000	0	30%	100%	100%	100%
562	10%	30%	30%	30%	50%
316	0	0	0	0	0

LC<sub>50</sub> values are presented in the table below.

**Table B 9.2.1-2 LC<sub>50</sub> values and 95% confidence limit**

Period of exposure		LC <sub>50</sub> values and 95% confidence limit (mg daminozide/L)
Days	Minutes	
1	1440*	2200 (1646 – 2939)
2	2880	1200 (854 – 1686)
3	4320*	960 (690 – 1336)
4	5760	625 (482 – 810)
7	10080	580 (433 – 777)

The 96 hours LC<sub>50</sub> was determined to be 625 mg/L (95% confidence interval 482-810 mg/L).

RMS comments and conclusion:

The reported study is non GLP and was conducted according to OECD 203 guideline (1981). The test results are in compliance with the current guideline's validity criteria (mortality in the control less than 10%, dissolved oxygen concentration at least 60% of the air saturation), except for the maintenance of the concentration of the test substance throughout the test. This criterion could not be checked since no measurements of actual concentration of the test substance were carried out. Due to unclear exposure during the test, no reliable endpoint can be derived from the study.

The study is not considered valid.

**ii) Acute toxicity to rainbow trout (*Oncorhynchus mykiss*)**

<b>Reference:</b>	(1977) Acute toxicity of Alar technical, Lot BL 8190 to the rainbow trout ( <i>Salmo gairdneri</i> )
Report No.:	A.7.4.1.5
Guideline:	EPA (1975)
GLP:	No
<b>Previous evaluation:</b>	In DAR (1999)
<b>Material and methods:</b>	
Test material:	Daminozide technical
Lot/Batch No:	BL 8190
Purity:	Not stated

## Executive Summary

A 96 hours toxicity test with rainbow trout (*Salmo gairdneri*) was conducted using five test concentrations of daminozide; 56, 100, 180, 320 and 560 mg/L, plus control, under static conditions. Ten fish per vessel were used. pH ranged from 4.0 to 7.3, but dropped to 4.3 in the highest dose level at study termination which may have contributed to the mortality. The 96 hours LC50 was determined to be 149 mg/L (95% confidence interval 128-174 mg/L), but the actual test concentrations were not measured.

## Materials and methods:

- Test animals:** Rainbow trout (*Salmo gairdneri*)

**Wet weight:** 0.38 g

**Length:** 36 mm

**Age:** 3 months

**Source:** [REDACTED]

**Acclimation:** 24 hours

**Diet:** Fish were not fed 48 hours before testing and during the test



2. **Dilution water:** Obtained from a well on the test site, treated with a reverse osmosis system and then deionised

**Hardness:** 48 mg CaCO<sub>3</sub>/L

**Alkalinity:** 31 mg CaCO<sub>3</sub>/L

**pH:** 7.25

3. **Test vessels:** 5 gallon test vessels

4. **Environmental conditions:**

**Temperature:** 12 ± 1°C

**pH:** 4.3 – 7.26

**Dissolved oxygen:** 8.2 – 9.8 mg/L

**Photoperiod:** Not stated

5. **Animal assignment and treatment:**

Ten individuals were placed in each of the test vessels for 96 hours under static conditions.

6. **Dose preparation:**

Not stated

7. **Measurements and observations:**

Behavioural responses of the test animals were recorded at 24 hour intervals.

8. **Statistics:**

LC<sub>50</sub> values and 95% Confidence Intervals were determined by the Spearman-Kärber Estimator. Values were based upon nominal concentrations.

## Results:

### A. MORTALITY

Mortalities were observed in the 320 and 560 mg/L test concentrations after 48 hours and in the 180 mg/L test concentration after 96 hours. 100% mortality was seen in the two highest test concentrations after 96 hours. pH dropped to 4.3 in the highest dose level at study termination which may have contributed to the mortality.

**Table B 9.2.1-3 Summary of cumulative mortalities**

Nominal concentrations (mg a.s./L)	Cumulative percent mortality		
	24 hour	48 hour	96 hour
Control	0 %	0 %	0 %
56	0 %	0 %	0 %
100	0 %	0 %	0 %

180	0 %	0 %	80 %
320	0 %	10 %	100 %
560	10 %	100 %	100 %

## B. SUB-LETHAL EFFECTS

Fish exposed at 180 mg/L and higher became lethargic and demonstrated erratic swimming behaviour.

A summary of the toxicity endpoints determined from the study is presented in the table below.

**Table B 9.2.1-4 Summary of endpoints**

	LC <sub>50</sub> (mg a.s./L)	95% confidence interval	
		Lower (mg a.s./L)	Upper (mg a.s./L)
24-hour	N/A	N/A	N/A
48-hour	397.8	354.4	446.4
96-hour	149.3	128.0	174.1
<b>NOEC = 100 mg a.s./L</b>			

## C. ANALYSIS

Not stated

## D. DEFICIENCIES

Test material purity not reported. Actual test concentrations not measured.

### Conclusion:

The 96 hours LC<sub>50</sub> was determined to be 149 mg/L (95% confidence interval 128-174 mg/L), but the actual test concentrations were not measured.

Remark from previous review: Actual concentrations were not measured. At high dose level pH dropped to 4.3, which may have contributed to mortality. The incipient LC<sub>50</sub> is almost reached. The results (LC<sub>50</sub> 149 mg/L) can be used for risk evaluation.

### RMS comments and conclusion:

The reported study is non GLP and was conducted according to EPA guideline (1975). The test results are in compliance with the current guideline's validity criteria (mortality in the control less than 10%, dissolved oxygen concentration at least 60% of the air saturation), except for the maintenance of the concentration of the test substance throughout the test. This criterion could not be checked since no measurements of actual concentration of the test

substance were carried out. Due to unclear exposure during the test, no reliable endpoint can be derived from the study.

The study is not considered valid.

### iii) Acute toxicity to rainbow trout (*Oncorhynchus mykiss*)

<b>Reference:</b>	██████ (1972) Acute toxicity of Alar to bluegill ( <i>Lepomis macrochirus</i> )
Report No.:	A.7.4.1.4
Guideline:	APHA standard methods (1970)
GLP:	No
<b>Previous evaluation:</b>	In DAR (1999)
<b>Material and methods:</b>	
Test material:	Daminozide technical
Lot/Batch No:	GMS#4791
Purity:	100%

A 96 hours toxicity test with bluegill sunfish (*Lepomis macrochirus*) was conducted using five test concentrations of daminozide; 100, 240, 490, 750 and 1000 mg/L, plus control, under static conditions. Ten fish (mean weight = 1.3 g, mean length = 4.3 cm) per vessel (30 in control) was used. Water temperature was 18°C. Initial pH of dilution water was 7.1. Dissolved oxygen (DO) was reported to have dropped from 8.7 to 4.9 mg/L between 0 and 96 hours, which equals 52% of saturation value. Statistics based on using probits. The 96 hours LC<sub>50</sub> was determined to be 423 mg/L (95% confidence interval 226-793 mg/L). The DO was too low after 96 hours, pH was not measured at the end of the study and actual test concentrations were not measured.

### Results

Data on cumulative mortality data for rainbow trout exposed to daminozide technical are presented in the table below.

**Table B 9.2.1-5 Summary of cumulative mortalities**

Nominal concentrations (mg a.s./L)	Cumulative percent mortality	
	24 hour	96 hour
Control	0 %	0 %
56	0 %	0 %
100	0 %	0 %
180	0 %	80 %
320	0 %	100 %
560	10 %	100 %

A summary of the toxicity endpoints determined from the study is presented in the table below.

Test substance	LC50 (confidence interval) (mg a.s./L)		NOEC (mg a.s./L)
	24 h	96 h	
Alar	451 (237 – 856)	423 (226 – 793)	240
DDT	-	0.008 (0.005 – 0.012)	-

Remark from previous review: DO was too low after 96 hours. pH was not measured. Actual concentrations were not measured. Not clear if incipient LC<sub>50</sub> is reached. The result 96h LC<sub>50</sub> 423 mg/L is used for risk evaluation

#### RMS comments and conclusion:

The reported study is non GLP and was conducted according to APHA standard methods (1970). The test results are mostly not in compliance with the current guideline's validity criteria: mortality in the control less than 10%, but dissolved oxygen concentration was less than 60% of the air saturation. Moreover, the criterion on the maintenance of the concentration of the test substance throughout the test could not be checked since no measurements of actual concentration of the test substance were carried out. Due to unclear exposure during the test, no reliable endpoint can be derived from the study.

The study is not considered valid.

#### B.9.2.1.2 Acute toxicity of metabolites to fish

i) **Metabolite methanol: Acute toxicity to rainbow trout (*Oncorhynchus mykiss*, syn. *Salmo gairdneri*), bluegill sunfish (*Lepomis macrochirus*) and fathead minnow (*Pimephales promelas*)**

<b>Reference:</b>	Poirier, S.H., Knuth, M.L., Anderson-Buchou, C.D., Brooke, L.T., Lima, A.R., Shubat, P.J. (1986) Comparative toxicity of methanol and N,N-dimethylformamide to freshwater fish and invertebrates.  Bulletin of Environmental Contamination and Toxicology (1986) Vol. 37, pp. 615-621.
Report No.:	-
Guideline:	Not stated
GLP:	Not stated
<b>Previous evaluation:</b>	Submitted for the purpose of renewal
<b>Material and methods:</b>	
Test material:	Methanol (metabolite of daminozide)
Lot/Batch No:	Not stated
Purity:	Not stated

#### STUDY DESIGN AND METHODS

- Test animals:** Rainbow trout (*Salmo gairdneri*), bluegill (*Lepomis macrochirus*), fathead minnow (*Pimephales promelas*)  
**Wet weight:** 0.813 g (rainbow trout), 3.07 g (bluegill), 0.126 g (fathead minnow)

**Length:** Not stated

**Source:** [REDACTED]  
[REDACTED]

**Acclimation:** 24 hours

**Diet:** Fish were not fed 24 hours before testing and during the test

2. **Dilution water:** Directly from Lake Superior

**Alkalinity:** 41.7 mg CaCO<sub>3</sub>/L

3. **Test vessels:** 53 L fibreglass holding tanks with proportional dilutor

4. **Environmental conditions:**

**Temperature:** 19.8 ± 2.3°C

**pH:** 7.04 – 7.97

**Dissolved oxygen:** 78.8%

**Photoperiod:** Not stated

5. **Animal assignment and treatment:**

Ten (rainbow trout, bluegills) or twenty (fathead minnows) individuals were placed in each of the test vessels for 96 hours under flow-through conditions.

6. **Dose preparation:**

Not stated

7. **Measurements and observations:**

Mortality.

Exposure samples containing methanol for bluegill and rainbow trout exposures were analysed by direct aqueous injection gas-liquid chromatography.

Methanol was monitored in the fathead minnow acute exposure by using Rhodamine B dye at 27.0 g/L as a tracer added to the stock solution. Actual methanol concentrations were then calculated by the known ratio of dye to methanol. Rhodamine B was analysed using a Baird-Atomic model SFRI00 spectrofluorimeter with excitation and emission wavelengths of 554 and 578 nm, respectively.

8. **Statistics:**

LC<sub>50</sub> and EC<sub>50</sub> values and 95% confidence intervals were determined using the Trimmed Spearman-Kärber method.

## Results

**A. MORTALITY**

Rainbow trout and bluegills were affected immediately at the two highest concentrations and remained affected through the exposure period with mortalities occurring within 3 hours of initial exposure. Fathead minnows were affected at the two highest concentrations throughout the test with mortalities occurring within the first 12 hours of exposure.

**B. SUBLETHAL EFFECTS**

Not stated.

**Table B 9.2.1-6 Summary of LC<sub>50</sub> values**

Table B-7.2.1-6 Summary of LC <sub>50</sub> values			
Time point	LC <sub>50</sub> (mg a.s./L)	95% confidence interval	
		Lower (mg a.s./L)	Upper (mg a.s./L)
Rainbow trout			
24 hour	20,300	19,800	20,700
48 hour	20,100	19,500	20,700
96 hour	20,100	19,500	20,700
Fathead minnow			
24 hour	29,700	29,000	30,500
48 hour	29,700	29,000	30,500
96 hour	29,400	28,500	30,400
Bluegill			
24 hour	19,100	17,400	21,000
48 hour	19,100	17,300	21,100
96 hour	15,400	13,500	17,600

**C. ANALYSIS**

Methanol-spiked exposure water for the rainbow trout and bluegill acute tests were  $102.9 \pm 3.6\%$  (n=5) and  $101.5 \pm 2.7\%$  (n=5), respectively.

**D. DEFICIENCIES**

Not reported.

**Conclusion**

The 96 hours LC<sub>50</sub> values for methanol were determined to be 20,100 mg/L (rainbow trout), 29,400 mg/L (fathead minnow) and 15,400 mg/L (bluegill).

**RMS comments and conclusion:**

The reported study is a study review taken from the open literature. No full study report was available and, therefore, a lot of information is missing, e.g. batch number and purity of the tested substance, the exact

concentrations tested, the length of tested fish, period of acclimatization, results were presented as LC<sub>50</sub> calculations only – no data in form of effects on mortality of fish for each treatment level were available, no raw data were available. Further, it is not clear if the analytical measurements of methanol were carried out throughout the test and no such data were presented. The validity criteria on mortality in control and on maintenance of the concentration of the test substance throughout the test could not be checked.

The study is not suitable for regulatory use.

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

#### B.9.2.2.1 Long-term and chronic toxicity of active substance to fish

##### i) Fish early life stage toxicity test

<b>Reference:</b>	(2015) Daminozide: an early life stage toxicity test with the fathead minnow ( <i>Pimephales promelas</i> )
Report No.:	616A-123
Guideline:	OECD 210 (1992) and U.S. EPA OPPTS 850.1400
GLP:	Yes
<b>Previous evaluation:</b>	Submitted for the purpose of renewal
<b>Material and methods:</b>	
Test material:	Daminozide technical
Lot/Batch No:	101218026
Purity:	99.8%

### Executive Summary

The objective of this study was to determine the effects of daminozide technical on the time to hatch, hatching success, survival, and growth of fathead minnows, *Pimephales promelas*, during early life-stage development. The study was conducted under flow through conditions for 33 days, a 5-day hatching period plus a 28-day post-hatch growth period). The nominal test concentrations were: 0, 0.26, 0.64, 1.6, 4.0 and 10 mg a.s./L. Observations were made at least daily to determine hatching rate and the number of mortalities and signs of toxicity in each treatment group.

The mean measured concentrations ranged from 97.8 to 103% of nominal concentrations, nonetheless, the result were all expressed in terms of mean measured concentration. There were no statistically significant treatment-related effects on hatching success, growth or survival at any of the concentrations tested. Consequently, the NOEC was 10 mg a.s./L and the LOEC was >10 mg a.s./L.

### Material and methods:

#### A. MATERIALS

1. **Test material:** Daminozide technical  
**Batch number:** 101218026  
**Purity:** 99.8% (w/w)  
**Appearance:** Solid

2. **Vehicle:** Dilution water
3. **Dilution water:** Filtered and aerated well water (*ca.* 40 m deep well at test facility)

## B. STUDY DESIGN AND METHODS

### 1. Test organism

**Species:** Fathead minnow (*Pimephales promelas*)

**Age of test organism:** Embryos (<24 hours old)

**Weight:** Mean 0.33 g wet weight

**Source:** [REDACTED]

**Diet:** Live brine shrimp nauplii (*Artemia sp.*)

2. **Treatment groups:** 0 (control & solvent control), 0.26, 0.64, 1.6, 4.0 and 10 mg a.s./L (nominal)  
0 (control & solvent control), 0.26, 0.62, 1.7, 4.2 and 10 mg a.s./L (mean measured)

### 3. Environmental conditions:

**Test vessels:** 9 litre glass aquaria, containing 7 litres of test solution. Depth of the test water was 16.1 cm

**Temperature:**  $25 \pm 1$  °C

**pH:** 7.7 to 8.1

**Dissolved oxygen:**  $\geq 8.1$  mg/L ( $\geq 99\%$  of saturation)

**Photoperiod:** 16 hours light : 8 hours dark (1034 lux)

### 4. Animal assignment and treatment:

Fathead minnow embryos were exposed to a geometric series of five test concentrations and a negative (dilution water) control under flow-through conditions. Nominal test concentrations were 0.26, 0.64, 1.6, 4.0 and 10 mg a.s./L.

Four replicate test chambers were maintained in each treatment and control group, with one incubation cup in each test chamber. Each incubation cup contained 20 embryos, resulting in a total of 80 embryos per treatment. At test initiation, embryos were impartially distributed to incubation cups and exposed to test solution in the test chambers. After a five-day embryo hatching period, the larvae were released into the test chambers, where exposure continued during a 28-day post-hatch juvenile growth period. Fish were not fed for approximately 48 hours prior to the termination of the test to allow for clearance of the digestive tracts before weight measurements were made.

### 5. Dose preparation:

A continuous-flow diluter was used to deliver each concentration of the test substance and a negative (dilution water) control. Calibrated syringe pumps (Harvard Apparatus, Holliston, Massachusetts) were used to deliver the



five test substance stock solutions into mixing chambers assigned to each treatment. The diluter flow rate was adjusted to provide approximately 10 volume additions of test water in each test chamber per day.

1. Stock solutions were prepared four times during the test. At each preparation, a primary stock solution was prepared in dilution water (well water) at a nominal concentration of 100 mg a.s./mL. The primary stock solution was sonicated for approximately 45 minutes followed by inversion and appeared clear and light yellow in colour. Proportional dilutions of the primary stock were made in dilution water to prepare additional stock solutions at nominal concentrations of 2.6, 6.4, 16 and 40 mg a.s./mL. The secondary stock solutions were mixed by inversion and ranged in appearance from clear and nearly colourless to clear and light yellow. Stock solutions were stored under ambient conditions and fresh aliquots were placed in the syringe pumps every two days during the test. The stock solutions were delivered to the diluter mixing chambers (at a rate of 20 µL/minute) where they were mixed with dilution water (at a rate of 200 mL/minute) to achieve the desired test concentrations of 0.26, 0.64, 1.6, 4.0 and 10 mg a.s./L. The resultant test concentrations were adjusted for the purity of the active ingredient in the test substance (99.8%). The negative control was dilution water.

#### **6. Measurements/observations:**

During the first day of exposure, embryos were observed twice for mortality and the presence of fungus. Thereafter, until hatching was complete, observations of embryo mortality and the removal of dead embryos were performed once daily. During the 28-day post-hatch exposure period, the larvae were observed daily to evaluate the numbers of mortalities and the numbers of individuals exhibiting clinical signs of toxicity or abnormal behaviour. From these observations, time to hatch, hatching success, and post-hatch growth and survival were evaluated. Post-hatch growth of the fathead minnows was evaluated at the conclusion of the 28-day post-hatch exposure period. Total length for each surviving fish was measured, and wet and dry weights were measured. Fish were placed in an oven at approximately 60°C for approximately 48 hours to obtain dry weight data.

Water samples were collected from alternating replicate test chambers of each treatment and control group on Days 0, 7, 14, 21, 28 and 33 (test termination) to determine concentrations of the test substance in the test chambers. All samples were collected at mid-depth in the test chambers, placed in vials and processed immediately for analysis. Analysis was conducted using High Performance Liquid Chromatograph (HPLC) with tandem mass spectrometric detection (LC/MS/MS).

#### **7. Statistics:**

Test endpoints analysed statistically were hatching success, larval survival and growth (total length, wet weight and dry weight). EC<sub>10</sub> and EC<sub>20</sub> and the 95% confidence intervals based on hatching success, survival, total length, wet and dry weight were not reported. Since the conditions for determining the EC<sub>x</sub> and 95% confidence interval were outside of the range of data used for the calculation according to OECD 210 guideline and were considered to be extrapolation rather than interpolation, the calculated values were not reported. The results of the statistical analyses were used to aid in the determination of the NOEC and LOEC.

Hatching success was calculated as the percentage of embryos that hatched successfully. Post-hatch survival was calculated from the number of larvae that survived to test termination as a percentage of the number of embryos that hatched successfully. Hatching success and survival data were considered to be discrete-variable data, while growth data were considered continuous-variable data. Discrete-variable data were analysed using Chi-square and Fisher's Exact test to identify treatment groups that showed a statistically significant difference ( $\alpha = 0.05$ ) from the control. All continuous-variable data were evaluated for normality using Shapiro-Wilk's test, and for homogeneity of variance using Levene's or Bartlett's tests ( $\alpha = 0.01$ ). Since the data of all parameters passed the assumptions of normality and homogeneity of variances, the treatments that were significantly different from the negative control means were identified using Dunnett's one-tailed test ( $\alpha = 0.05$ ) (5). All statistical tests were performed using a personal computer using SAS software.

## Results:

### A. Time to hatch and hatching success:

Daily observations of the embryos indicated that there were no apparent differences in time to hatch between the control groups and any of the daminozide treatment groups. The majority of fathead minnow embryos in the control and treatment replicates hatched on Days 4 and 5 of the test. Hatching reached >90% in the control groups on Day 5 of the test, at which time the larvae were released to their respective test chambers. A few embryos in the 0.26 and 4.2 mg a.s./L treatment group remained in the incubation chambers until they hatched on the Day 6 of the test.

Hatching success in the negative control group was 99 %. Hatching success in the 0.26, 0.62, 1.7, 4.2 and 10 mg a.s./L treatment groups was 100, 100, 99, 100 and 99%, respectively. Fisher's Exact test indicated no statistically significant decrease in hatching success in any of the treatment groups in comparison to the negative control ( $p > 0.05$ ). Consequently, the NOEC for hatching success was 10 mg a.s./L and the LOEC was > 10 mg a.s./L. Hatching success of the fathead minnow embryos is summarised in the table below.

**Table B 9.2.2-1 Summary of hatching success, larval survival and growth of fathead minnow exposed to daminozide technical**

Mean measured conc. (mg a.s./L)	Percent hatchling success <sup>1</sup>	Percent survival to Day 28 post-hatch	Growth Parameters at Day 28 Post-Hatch		
			Mean total length $\pm$ SD (mm)	Mean wet weight $\pm$ SD (mg)	Mean dry weight $\pm$ SD (mg)
Negative control	99	85	24.3 $\pm$ 0.80	113.8 $\pm$ 7.79	22.8 $\pm$ 2.18
0.26	100	80	24.6 $\pm$ 0.48	112.0 $\pm$ 6.69	22.6 $\pm$ 1.63
0.62	100	89	24.5 $\pm$ 0.32	112.8 $\pm$ 2.53	22.6 $\pm$ 0.91
1.7	99	87	23.8 $\pm$ 0.29	104.4 $\pm$ 4.50	20.6 $\pm$ 0.90
4.2	100	79	24.4 $\pm$ 0.54	109.3 $\pm$ 4.21	21.7 $\pm$ 0.68
10	99	75	23.9 $\pm$ 0.47	111.5 $\pm$ 7.66	21.6 $\pm$ 1.54

<sup>1</sup> There were no statistically significant decreases in comparison to the negative control for hatching success and survival (Fisher's Exact test,  $p > 0.05$ ) or in growth (Dunnett's one-tailed test,  $p > 0.05$ ).

**B. Larval survival and clinical observations:**

Survival of the fathead minnow larvae through Day 28 post-hatch is summarised in the table above. Larval survival in the negative control group was 85%. Larval survival in the 0.26, 0.62, 1.7, 4.2 and 10 mg a.s./L treatment groups was 80, 89, 87, 79 and 75%, respectively. Fisher's Exact test indicated no statistically significant decrease in survival in any of the daminozide treatment groups in comparison to the negative control ( $p > 0.05$ ). Consequently, the NOEC for larval survival was 10 mg a.s./L and the LOEC was  $>10$  mg a.s./L.

The results of the biological observations of sub-lethal effects during the 28-day post-hatch period showed the majority of fish in the control groups and in the 0.26, 0.62, 1.7, 4.2 and 10 mg a.s./L treatment groups appeared normal throughout the test. The sublethal signs of toxicity noted during the test including weak, lethargy, discoloration (dark and pale), erratic swimming, loss of equilibrium, morphologically deformity (e.g. curled, curved, crooked spine) and smaller in stature. The occurrence of the sublethal signs of toxicity in the treatment groups was infrequent and comparable to those in the control.

**C. Growth:**

Growth measurements at the end of the 28-day post-hatch period are summarized in the table above. Dunnett's one-tailed test indicated there were no statistically significant reductions in total length, wet weight and dry weight among fish in any of the daminozide treatment groups in comparison to the negative control ( $p > 0.05$ ). Consequently, the NOEC for growth was 10 mg a.s./L and the LOEC was  $>10$  mg a.s./L.

**D. Analytical verification:**

The test solutions in the mixing chambers and test chambers appeared clear and colourless at test initiation and test termination, with no evidence of precipitation observed in any control or treatment solution. On Day 33 of the test, it was discovered that the stock solution of the 10 mg a.s./L treatment group was not delivering to the mixing chamber. Based on the stock volume remaining for the 10 mg a.s./L treatment group, the interruption lasted approximately 13 hours. The measured concentration of the 10 mg a.s./L treatment level on Day 33 was 5.55% of nominal test concentration. Since the interruption was brief and it occurred just prior to termination of the study, the measured concentration of this treatment level on Day 33 was excluded from the calculation of mean measured test concentration. Samples of the test solutions collected during the test had measured concentrations that ranged from 93.6 to 110% of nominal concentrations (Table 8.2.2.1/01-02). When the measured concentrations of test solution samples collected on Days 0, 7, 14, 21, 28 and 33 of the test were averaged for each treatment group, the mean measured test concentrations were 0.26, 0.62, 1.7, 4.2 and 10 mg a.s./L, which represented 100, 97, 106, 105 and 100% of nominal concentrations, respectively. The results of the study were based on the mean measured concentrations.

Table B 9.2.2-2 Measured concentrations of daminozide in test samples

Nominal Test Concentration (mg a.i./L)	Rep.	Sample Number (616A-123-)	Sampling Time (Days)	Measured Concentration (mg a.i./L) <sup>1,2</sup>	Percent of Nominal <sup>2</sup>	Mean Measured Concentration (mg a.i./L)	Mean Measured Percent of Nominal
Negative Control	B	1	0	< LOQ	--	--	--
	C	7	7	< LOQ	--		
	D	13	14	< LOQ	--		
	A	19	21	< LOQ	--		
	B	25	28	< LOQ	--		
	C	31	33	< LOQ	--		
0.26	B	2	0	0.254	97.5	0.26 ± 0.0124 CV = 4.85%	100
	C	8	7	0.257	98.8		
	D	14	14	0.258	99.1		
	A	20	21	0.256	98.6		
	B	26	28	0.275	106		
	C	32	33	0.236	90.6		
0.64	B	3	0	0.620	96.8	0.62 ± 0.0158 CV = 2.53%	97
	C	9	7	0.629	98.3		
	D	15	14	0.619	96.8		
	A	21	21	0.629	98.2		
	B	27	28	0.647	101		
	C	33	33	0.599	93.6		
1.6	B	4	0	1.60	100	1.7 ± 0.0480 CV = 2.89%	106
	C	10	7	1.71	107		
	D	16	14	1.67	105		
	A	22	21	1.71	107		
	B	28	28	1.68	105		
	C	34	33	1.61	100		
4.0	B	5	0	4.17	104	4.2 ± 0.175 CV = 4.16%	105
	C	11	7	4.37	109		
	D	17	14	4.25	106		
	A	23	21	4.01	100		
	B	29	28	4.00	99.9		
	C	35	33	4.41	110		
10	B	6	0	10.0	100	10 ± 0.13 CV = 1.27%	100
	C	12	7	10.1	101		
	D	18	14	9.87	98.7		
	A	24	21	9.83	98.3		
	B	30	28	10.1	101		
	C	36	33 <sup>3</sup>	0.555*	5.55		

<sup>1</sup> The limit of quantitation (LOQ) was 0.0500 mg a.i./L, calculated as the product of the concentration of the lowest calibration standard (0.0250 mg a.i./L) and the dilution factor of the matrix blank samples (2.00).

<sup>2</sup> Results were generated using Analyst version 1.6. Manual calculations may differ slightly.

<sup>3</sup> Due to a diluter malfunction, the delivery of the 10 mg a.i./L treatment concentration stock solution was interrupted for approximately 13 hours. Since the interruption was brief in comparison to the length of the study and the stock was delivered to the system continuously for approximately 32.5 days this analytical result did not represent the test concentration in the study. Therefore, it was excluded from the calculation of mean measured test concentration.

\* Since the peak area of the sample was less than the peak area of the lowest standard, the result was extrapolated.

**Table B 9.2.2-3 Mean measured concentrations of daminozide technical in test samples**

Nominal test concentration (mg a.s./L)	Mean measured concentration (mg a.s./L)	Mean measured percent of nominal
0 (negative control)	-	-
0.26	0.26	100 %
0.64	0.62	97 %
1.6	1.7	106 %
4.0	4.2	105 %
10	10* (8.81**)	100 %* (88.1%**)

\* Due to a diluter malfunction, the delivery of the 10 mg a.i./L treatment concentration stock solution was interrupted for approximately 13 hours. Since the interruption was brief in comparison to the length of the study and the stock was delivered to the system continuously for approximately 32.5 days this analytical result did not represent the test concentration in the study. Therefore, it was excluded from the calculation of mean measured test concentration.

\*\* Value taking into account also the measured concentration of the 10 mg a.s./L treatment level on Day 33 of 5.55% of nominal test concentration.

### ***Conditions for the Validity of the Test***

The following criteria were used to judge the validity of the test:

- 1) the dissolved oxygen concentration was  $\geq 60$  percent of the air saturation value ( $\geq 8.1$  mg/L) throughout the test;
- 2) the water temperature measurements were not to differ by more than  $\pm 1.5^{\circ}\text{C}$  between test chambers or between successive days at any time during the test, and were within  $25 \pm 1^{\circ}\text{C}$ ;
- 3) the concentrations of the test substance in solution were satisfactorily maintained within  $\pm 20\%$  of the mean measured values;
- 4) the percentages of embryos in the negative control that hatched successfully was 99%, and the post-hatch survival in the negative control was 85%. The criteria for validity as outlined by the guidelines was  $>70\%$  control hatchability and  $>75\%$  control larval survival;

### **Conclusion:**

Fathead minnows (*Pimephales promelas*) were exposed to daminozide at mean measured concentrations of 0.26 to 10 mg a.s./L under flow-through conditions for 33 days (a 5-day hatching period plus a 28-day post-hatch growth period). There were no statistically significant treatment-related effects on hatching success, survival or growth at concentrations  $\leq 10$  mg a.s./L. Consequently, the NOEC was 10 mg a.s./L. The LOEC was  $>10$  mg a.s./L.

### **RMS comments and conclusion:**

The reported study is GLP compliant and was conducted according to OECD 210 guideline (1992) and U.S. EPA OPPTS 850.1400 guideline. The test results are in compliance with the guidelines' validity criteria. The study is acceptable for regulatory use.

The NOEC set by the study author was 10 mg a.s./L based on no statistically significant treatment-related effects on hatching success, survival or growth at any concentration tested. However, there is a clear dose response at the highest concentration levels of 4.2 and 10 mg a.s./L in survival to day 28 post-hatch and less pronounced dose

response in mean dry weight. Therefore, the NOEC of 1.7 mg a.s./L is set by RMS, based on survival to day 28 post-hatch.

The lowest-observed-effect concentration (LOEC) is 4.2 mg daminozide/L and the no-observed-effect concentration (NOEC) is 1.7 mg daminozide/L, based on mean measured concentrations.

#### **B.9.2.2.1.1 Bioconcentration in fish**

The log Pow of daminozide is low (log Pow = -1.5), therefore, no bioaccumulation study in fish is required.

#### **B.9.2.3 Endocrine disrupting properties**

A full review of the data as well as any other additional information on the toxicity profile, and mode of action has been undertaken to determine if daminozide is a potential endocrine disruptor in aquatic organisms. Based on the evaluation of the available aquatic data (fish ELS study and chronic *Daphnia*) there is no evidence that daminozide causes endocrine disruption.

No further testing is indicated to evaluate the endocrine disrupter potential of daminozide to fish.

#### **B.9.2.4 Acute toxicity to invertebrates**

##### **B.9.2.4.1 Acute toxicity to *Daphnia magna***

##### **B.9.2.4.1.1 Acute toxicity of active substance to *Daphnia magna***

##### **i) Acute toxicity to *Daphnia magna* in a flow-through test**

<b>Reference:</b>	<b>Lintott, D. R. (1992)</b> Alar technical: Acute toxicity to the water flea, <i>Daphnia magna</i> , under flow-through test conditions
Report No.:	A.7.4.1.8
Guideline:	U.S. EPA 72-2 (1975)
GLP:	Yes
<b>Previous evaluation:</b>	In DAR (1999)

#### **Executive Summary**

A 96 hours toxicity test with *Daphnia magna* was conducted using six test concentrations of daminozide; 7.8-100 mg/L, plus control, under flow-through conditions (flow rate 2 mL/min). Ten daphnids < 24 hour old per vessel were tested, two vessels per concentration were used. Actual concentrations were measured by LC at initiation and termination. Actual concentrations ranged from 101-112% of nominal. Water temperature ranged between 19-21°C. pH of dilution water ranged from 5.1-7.7. Statistics was based on the binomial method. Based on mean measured concentrations the 96-hours EC<sub>50</sub> was 75.5 mg/L (95% confidence interval 66.2-101 mg/L).

#### **Material and methods:**

#### **A. MATERIALS**

- 1. Test material:** Alar Technical
- Description:** White crystalline solid

**Lot/Batch:** BFI 1576

**Purity:** 99%

## **B. STUDY DESIGN AND METHODS**

### **1. Test animals:** *Daphnia magna*

**Age:** < 24 hours at test initiation

**Source:** Toxikon Environmental Sciences

**Diet:** *Selenastrum capricornutum* and a yeast-Cerophyll-trout chow (fed daily throughout the test)

### **2. Dilution water:** Town of Jupiter water

**Hardness:** 72 - 84 mg CaCO<sub>3</sub>/L

**Alkalinity:** 14 - 53 mg CaCO<sub>3</sub>/L

### **3. Test vessels:** 300 mL glass crystallising dishes (10 cm x 5 cm) with a Nitex screen collar

### **4. Environmental conditions:**

**Temperature:** 19.1 – 21.1°C

**pH:** 5.1 – 7.7 (test solutions); 7.5 – 7.7 (controls)

**Dissolved oxygen:** 8.2 – 9.0 mg/L (saturation in freshwater is 9.1 mg/L at 20°C)

**Photoperiod:** 16 hours light: 8 hours darkness (83 to 158 lux)

### **5. Animal assignment and treatment:**

Daphnids (< 24 hours old) were added into the test chambers in pairs until ten animals were added to each test chamber containing 20mL water. Test chambers were duplicated resulting in twenty daphnids per treatment.

### **6. Dose preparation:**

Each test solution was prepared by directly adding the appropriate quantity of test material to 8 L of dilution water.

### **7. Measurements and observations:**

Survival of daphnids was monitored daily and any dead or immobilised daphnids were removed. Any abnormalities in the behaviour or physical appearance of the daphnids were also noted.

### **8. Statistics:**

EC<sub>50</sub> values and 95% confidence intervals were estimated by a computer program (Wheat, 1989) using the following statistical methods: moving average angle, probit, logit and non-linear interpolation. Confidence intervals for EC<sub>50</sub> values determined by non-linear interpolation were calculated by binomial probability. The method selected for reporting the test results was determined by the characteristics of the data.

**Results:****A. BIOLOGICAL EFFECTS****Table B 9.2.4-1 Summary of mortality of *Daphnia magna* exposed to Alar Technical under flow-through test conditions**

Mean measured concentration (mg a.s./L)	Cumulative number dead (percent mortality)			
	0 hour	48 hours	72 hours	96 hours
Control	0 (0)	0 (0)	0 (0)	1 (5)
8.57	0 (0)	0 (0)	0 <sup>d</sup> (0)	2 (10)
14.6	0 (0)	1 <sup>a</sup> (5)	2 (10)	2 (10)
23.6	0 (0)	0 (0)	1 (5)	1 (5)
39.0	0 <sup>a</sup> (0)	0 <sup>b</sup> (0)	4 (20)	4 (20)
66.2	0 (0)	2 <sup>a</sup> (10)	3 <sup>b</sup> (15)	4 (20)
101	0 (0)	7 <sup>c</sup> (35)	19 <sup>a</sup> (95)	20 (100)

<sup>a</sup> one daphnid was lethargic compared to the controls<sup>b</sup> two daphnids were lethargic compared to the controls<sup>c</sup> nine daphnids were lethargic compared to the controls<sup>d</sup> three daphnids were lethargic compared to the controls**Table B 9.2.4-2 Summary of endpoints (based on mean measured concentrations)**

Time scale (hours)	EC <sub>50</sub> (mg a.s./L)	95% Confidence intervals	
		Lower (mg a.s./L)	Upper (mg a.s./L)
24	>101 <sup>a</sup>	-	-
48	>101 <sup>a</sup>	-	-
72	78.8	66.2	101
96	75.5	66.2	101
NOEC = <8.57 mg a.s./L			

<sup>a</sup> The EC<sub>50</sub> could not be calculated due to <50% mortality occurring at all exposure concentrations.**B. ANALYSIS****Table B 9.2.4-3 Measured concentrations of Alar Technical in the exposure solutions**

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)			Percent of nominal (%)
	0 hour	96 hours	Mean	
Control	<1.5	<1.5	-	-
7.8	8.60	8.53	8.57	110
13	14.2	15.0	14.6	112
22	23.7	23.5	23.6	107
36	38.2	39.8	39.0	108
60	63.5	68.8	66.2	110



Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)			Percent of nominal (%)
	0 hour	96 hours	Mean	
100	95.0	107	101	101

### C. DEFICIENCIES

The temperature range was greater than the protocol requirement of  $20 \pm 1^\circ\text{C}$ . Test temperature deviations were slight (maximum deviation of  $0.1^\circ\text{C}$ ) and occurred on only one occasion for approximately two hours. This deviation is not considered to affect the test results.

### Conclusion:

The 96 hour  $\text{EC}_{50}$  for Alar technical was 75.5 mg/L with 95% confidence intervals of 66.2 and 101 mg/L. Due to mortality at all test concentrations, the NOEC was estimated at <8.57 mg/L.

Remark from previous review: Actual concentrations were 101-112% of nominal. Based on mean measured concentrations the 96-hours  $\text{EC}_{50}$  75.5 mg/L (95% confidence interval 66.2-101 mg/L) calculated by binominal method.

Note that the endpoint was reported as a 48 hour value in the Review Report for daminozide (2005).

### RMS comments and conclusion:

The reported study is GLP compliant and was conducted according U.S. EPA 72-2 guideline (1982). The test results are in compliance with the current guidelines' validity criteria. OECD 202 TG recommends that the daphnids should be preferably grouped into 5 organisms per replicate while they were grouped into 10 organisms per replicate in the present study. However, the recommendation of the OECD 202 TG, that at least 2 ml of test solution should be provided for each animal, was fulfilled (10 ml test solution per daphnid was provided in the study).

The study is considered valid and acceptable for regulatory use.

The 96-hour  $\text{EC}_{50}$  is 75.5 mg daminozide /L and the 96-hour no-observed-effect concentration (NOEC) is 8.57 mg daminozide /L, based on mean measured concentrations.

It is noted that the test duration of 48 hours is recommended by OECD 202 and U.S. EPA OPPTS 850.1010. However, since the endpoint for 96 hour is available and it is a worst case, it will be used in the risk assessment.

### ii) Acute toxicity to *Daphnia magna* in a static test

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Reference:	LeBlanc, G. (1976) Acute toxicity of Alar standard to <i>Daphnia magna</i>
Report No.:	A.7.4.1.3
Guideline:	U.S. EPA 72-2 (1975)

GLP: No

<b>Previous evaluation:</b>	In DAR (1999)
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**Material and methods:**

Test material: Daminozide technical

Lot/Batch No: 7506-1

Purity: 99%

A 48 hours toxicity test with *Daphnia magna* was conducted using six test concentrations of daminozide ; 42, 56, 75, 100, 140 and 180 mg/L, plus control, under static conditions. Five daphnids < 24 hour old per vessel were tested, three vessels per concentration was used. The bioassay was conducted in 250 mL beakers which contained 150 mL of test solution. Water temperature was  $22 \pm 1.0$  °C, pH of dilution water ranged from 6.3-7.4 and dissolved oxygen ranged from 6.8 to 8.2 mg/L (76 – 92% of saturation). Test solutions were not aerated during the test. Statistics based on using probits.

**Results:****Table B 9.2.4-4 Summary of mortality of *Daphnia magna* exposed to Alar Technical under static test conditions**

Nominal concentration (mg a.s./L)	Cumulative percent mortality	
	24 hour	48 hours
Control	0	0
Control (acetone)	0	0
42	0	0
56	0	0 <sup>a</sup>
75	0	20
100	0	93
140	60	93
180	100	100

<sup>a</sup> Test animals were lethargic.

The 48 hours EC<sub>50</sub> was determined to be 98.5 mg/L (95% confidence interval 71.3-136 mg/L), the 48 hours NOEC 42 mg/L.. But the actual test concentrations were not measured.

Remark from previous review: Actual concentration not measured. The result 48 h EC<sub>50</sub> 99 mg/L is used for risk evaluation.

**RMS comments and conclusion:**

The reported study is non-GLP and was conducted according U.S. EPA 72-2 guideline (1975). The test results are in compliance with the current guidelines' validity criteria. OECD 202 TG recommends that at least 20 daphnids should be used at each test concentration and for the controls while 15 daphnids per treatment were used in the present study.

Moreover, it could not be checked if the concentration of the test substance throughout the test was maintained since no measurements of actual concentration of the test substance were carried out. Due to unclear exposure during the test, no reliable endpoint can be derived from the study.

The study is not considered valid.

**iii) Acute toxicity to *Daphnia magna* in a static test**

<b>Reference:</b>	<b>Abram, F. (1987)</b> The toxicity of daminozide to <i>Daphnia magna</i>
Report No.:	FAL 3
Guideline:	OECD 202 (1981)
GLP:	Yes
<b>Previous evaluation:</b>	In DAR (1999)
<b>Material and methods:</b>	
Test material:	Daminozide technical
Lot/Batch No:	V86.10.1.201
Purity:	99.4%

A 48 hours toxicity test with *Daphnia magna* was conducted using six test concentrations of daminozide; 100, 160, 250, 400, 630 and 1000 mg/L, plus control, under static conditions. Five daphnids < 24 hour old per vessel were tested, four vessels per concentration was used. Glass jars containing 50 mL aerated dilution water were used. Water temperature was  $20 \pm 1$  °C, pH of dilution water ranged from 4.1-7.4. At the end of the test, pH at all levels except for control and 1000 mg/L has increased 1-1.5 units. The concentration of dissolved oxygen was kept close to the air saturation value (above 90%) by diffusing air through the aquarium contents. Actual concentrations were not measured.

**Results:**

Table B 9.2.4-5 Summary of pH values and concentrations of dissolved oxygen

Parameter.	Concentration in mg Daminozide/litre, and jar reference.						
	1000	630	400	250	160	100	Control
Start of pH DO* test	A 4.1 >90	A 5.1 >90	A 5.9 >90	A 6.2 >90	A 6.5 >90	A 6.7 >90	A 7.4 >90
	B 4.1 >90	B 5.0 >90	B 5.9 >90	B 6.3 >90	B 6.5 >90	B 6.7 >90	B 7.4 >90
	C 4.1 >90	C 5.2 >90	C 5.9 >90	C 6.3 >90	C 6.6 >90	C 6.7 >90	C 7.4 >90
	D 4.1 >90	D 5.3 >90	D 6.0 >90	D 6.3 >90	D 6.6 >90	D 6.8 >90	D 7.4 >90
End of pH DO* test.	A 4.1 86	A 6.2 82	A 7.0 81	A 7.7 85	A 7.8 85	A 7.9 87	A 7.6 82
	B 4.1 88	B 5.9 80	B 7.1 84	B 7.7 90	B 8.0 82	B 7.8 84	B 7.8 80
	C 4.1 82	C 6.2 81	C 7.2 84	C 7.8 87	C 7.8 88	C 7.8 80	C 7.7 79
	D 4.1 88	D 6.3 90	D 7.3 83	D 7.8 86	D 7.8 81	D 7.8 83	D 7.7 84

\* Concentration of dissolved oxygen in per cent of the air saturation value.

Mean concentration at end of test = 84%ASV, standard deviation  $\pm$  3.08% ASV.

Table B 9.2.4-6 Immobilization of Daphnia by daminozide

	Concentration in mg a.s./L						
	1000	630	400	250	160	100	Control
No. of immobile	20	16	9	5	1	2	1
No. of active	0	4	11	15	19	18	19
% immobilised	100	80	45	25	5	10	5

48 hour EC<sub>50</sub> 379 mg/L (95% C.I. 302-476 mg/L) calculated with probits.

Remark from previous review: Changes in pH were rather large. Actual concentrations not measured. Control mortality 5% and mortality was not monotonically increasing, therefore the result is recalculated with the Trimmed Spearman-Kärber method, based on Hamilton *et al.* (1977/78): 48 h EC<sub>50</sub> 412 mg/L (344-494 mg/L). The result 48 h EC<sub>50</sub> 412 mg/L is used for risk evaluation.

**RMS comments and conclusion:**

The reported study is non-GLP and was conducted according OECD 202 guideline (1981). The test results are in compliance with the current guidelines' validity criteria. OECD 202 TG recommends that the pH should not vary by more than 1.5 units in any one test but it was not fulfilled in the present study.

Moreover, it could not be checked if the concentration of the test substance throughout the test was maintained since no measurements of actual concentration of the test substance were carried out. Due to unclear exposure during the test, no reliable endpoint can be derived from the study.

The study is not considered valid.

**B.9.2.4.1.2 Acute toxicity of metabolites to *Daphnia magna*****i) Metabolite methanol:: Acute toxicity to *Daphnia magna***

<b>Reference:</b>	<b>Dom, N., Pennick, M., Knapen, D., Blust, R. (2012)</b> Discrepancies in the acute versus chronic toxicity of compounds with a designated narcotic mechanism. Chemosphere (2012) Vol. 87, pp. 742-749.
Report No.:	-
Guideline:	OECD 202 (1984); U.S. EPA 72-2 (1982)
GLP:	Not stated
<b>Previous evaluation:</b>	Submitted for the purpose of renewal

**Executive Summary**

A 96 hour toxicity test with *Daphnia magna* was conducted using six test concentrations of methanol (1000-32000 mg/L). Ten daphnids < 24 hour old per vessel were tested with three vessels per concentration used. Water temperature was 20±1°C. Based on nominal concentrations the 96 hour EC<sub>50</sub> was 18,260 mg/L.

**Material and methods:****A. MATERIALS**

- 1. Test material:** Methanol
- Description:** Not stated
- Lot/Batch:** Not stated
- Purity:** 99.8%

**B. STUDY DESIGN AND METHODS**

- 1. Test animals:** *Daphnia magna*
- Age:** < 24 hours at test initiation
- Source:** Not stated
- Diet:** a mixture of *P. subcapitata* and *Chlamydomonas reinhardtii* in a 3/1 ratio (4 x 10<sup>5</sup> cells/mL).
- 2. Dilution water:** Reconstituted water
- Hardness:** 250 mg CaCO<sub>3</sub>/L

**Alkalinity:** Not stated

**pH:** Not stated

**3. Test vessels:** 50 mL glass vessels

**4. Environmental conditions:**

**Temperature:**  $20 \pm 1^{\circ}\text{C}$

**pH:** 7.8-8.2

**Dissolved oxygen:** Not stated

**Photoperiod:** 14 hours light: 10 hours dark

**5. Animal assignment and treatment:**

Ten neonates (< 24 hours old) were added into each test chambers per replicate. The nominal concentrations of the test material were 1000, 2000, 4000, 8000, 16,000 and 32,000 mg/L with three replicates per concentration.

**6. Dose preparation:**

Not stated.

**7. Measurements and observations:**

EC<sub>50</sub> values were calculated after 48 and 96 hours of exposure.

**8. Statistics:**

US EPA Probit Analysis Software.

## Results:

### A. BIOLOGICAL EFFECTS

The EC<sub>50</sub> value was determined to be 18260 mg/L (nominal) on the basis of immobilization.

### B. DEFICIENCIES

Not reported.

## Conclusion

The 96 hour EC<sub>50</sub> for methanol to *Daphnia magna* was 18260 mg/L.

## RMS comments and conclusion:

The reported study is a study review taken from the open literature. It was conducted according OECD 202 guideline (1984). No full study report was available and, therefore, a lot of information is missing, e.g. batch number, measurements of dissolved oxygen, results were presented as EC<sub>x</sub> calculations only – no data in form of effects on immobilization or mortality of daphnids for each treatment level were available, no raw data were

available. The control did not seem to be included. Further, no results of analytical measurements of methanol throughout the test were presented. The validity criteria on mortality and dissolved oxygen concentration in control could not be checked.

The study is not suitable for regulatory use.

#### **B.9.2.4.2 Acute toxicity to an additional aquatic invertebrate species**

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

##### **B.9.2.5.1 Reproductive and development toxicity to *Daphnia magna***

##### **B.9.2.5.1.1 Reproductive and development toxicity of active substance to *Daphnia magna***

<b>Reference:</b>	<b>Last, G. (2011)</b> Chronic effects to <i>Daphnia magna</i> from exposure to daminozide and formaldehyde
Report No.:	8252736
Guideline:	OECD 211 (2008)
GLP:	Yes
<b>Previous evaluation:</b>	Submitted for the purpose of renewal

#### **Executive Summary**

The objective of this study was to determine the effects of simultaneous exposure to daminozide and formaldehyde on the survival, growth and reproduction of the cladoceran, *Daphnia magna*, during a 21 day exposure period under daily renewal test conditions.

The nominal test concentrations were 1.0, 2.2, 4.6, 10, 22, 46 and 100 mg daminozide/L, plus formaldehyde at 12.4% the concentration of daminozide (i.e. 0.124, 0.273, 0.570, 1.24, 2.73, 5.70 and 12.4 mg formaldehyde/L, respectively). An untreated control group was tested in parallel. The exposure profile was based on environmental exposure modelling and the concentration range was based on the results of a range-finding test.

Ten replicate test vessels were prepared for each treatment group and twenty replicates prepared for the control group. A single juvenile *Daphnia magna* (<24 hours old) was added to each test vessel. On each daily renewal, parental animals were transferred into freshly prepared test media.

First-generation daphnids were observed daily during the test for mortality, the onset of reproduction, and clinical signs of toxicity. Following the onset of reproduction, the numbers of second-generation daphnids were counted daily. Carapace lengths of the surviving first-generation daphnids were measured at the end of the exposure period.

The results of the study demonstrated the 21 day EC<sub>50</sub> for adult mortality to be 13.9 mg daminozide/L (plus 1.72 mg formaldehyde/L). The 21 day NOECs for effects on reproduction and carapace length were determined to be 100 and 46 mg daminozide/L, respectively (plus 12.4 and 5.70 mg formaldehyde/L).

**Material and methods:****A. MATERIALS****1. Test material:** Daminozide**Batch number:** 2010-08-15**Purity:** 99.3%**Appearance:** Pure white powder**Test material:** 16% Formaldehyde solution (w/v), methanol-free**Batch number:** MH159185**Purity:** 96.7%**Appearance:** Not stated**2. Vehicle:** None**3. Dilution water:** ASTM standard hard water**B. STUDY DESIGN AND METHODS****1. Test organism****Species:** Cladoceran, *Daphnia magna***Age at test initiation:** Neonates < 24 hours at test start**Source:** Covance Laboratories Ltd.**Diet:** Suspension of *Chlorella vulgaris*, plus a seaweed extract of *Ascophyllum nodosum* as a dietary supplement**2. Treatment groups:** Nominal: 1.0, 2.2, 4.6, 10, 22, 46 and 100 mg daminozide/L, plus 0.124, 0.273, 0.570, 1.24, 2.73, 5.70 and 12.4 mg formaldehyde/L, respectively

Mean measured: 0.965, 2.12, 4.40, 9.49, 19.9, 38.9 and 93.8 mg daminozide/L, plus 0.160, 0.244, 0.504, 1.32, 2.50, 5.59 and 12.2 mg formaldehyde /L

**3. Environmental conditions:****Test vessels:** 60 mL glass jars, with plastic screw caps, containing 50 mL of test medium**Temperature:** 18.8 to 21.0 °C**pH:** 6.51 to 8.28**Dissolved oxygen:** 79 to 106 % ASV**Photoperiod:** 16 hours light: 8 hours dark**4. Animal assignment and treatment:**

Daphnids were exposed to a geometric series of seven test concentrations of daminozide, plus formaldehyde at a concentration of 12.4% that of daminozide. A negative control (dilution water) was tested in parallel. Ten replicate



test vessels were prepared for each test concentration. Twenty replicate vessels were prepared for the control group. Two sets of test vessels (set A and set B) were used on alternate test media renewal days during the test. Between renewals, the test vessels were rinsed with dilution water and maintained in the test area. *Daphnia magna* were transferred between vessels using wide bore glass pipettes. At the start of the test, a single juvenile *Daphnia magna* (<24 hours old) was randomly allocated to each test vessel and added using a wide bore pipette. The internal bore of the pipette was sufficiently wide to avoid damage of the animal during transfer.

The *Daphnia magna* were fed daily with a suspension of *Chlorella vulgaris*. The daily feeding rate of *Chlorella vulgaris* was up to a maximum of  $5 \times 10^5$  alga cells per mL, per day. The amount of algae added to each test vessel was based on the age of the *Daphnia magna* and on the amount of precipitated algae present on the base of the vessels or in suspension directly before feeding. A seaweed extract of *Ascophyllum nodosum* was used as a dietary supplement and 0.3 ml was added to each test vessel at each test media renewal.

#### 5. Dose preparation:

The test design was semi-static using sealed test vessels, with renewal of test media on a daily basis. Each day during the test a concentrated stock solution of formaldehyde was prepared by adding *ca.* 387.5 mg of a 16% formaldehyde solution to 500 mL of ASTM to produce a 124 mg/L formaldehyde solution. A 200 ml aliquot of this stock solution was added to 2000 ml of ASTM along with 200 mg of daminozide to produce test media at a nominal concentration of 100 mg/L as daminozide and 12.4 mg/L as formaldehyde. This test media was then serially diluted to produce the remaining test media at nominal daminozide concentrations of 46, 22, 10, 4.6, 2.2 and 1.0 mg/L with respective formaldehyde concentrations of 5.70, 2.73, 1.24, 0.570, 0.273 and 0.124 mg/L. A control treatment was prepared by the addition of ASTM only to the test vessels.

#### 6. Measurements/observations:

At approximately 24-hour intervals, the adult *Daphnia magna* were observed for immobility, the presence or absence of eggs developing in the brood pouch (gravid or non-gravid) and mortality. Following each test media renewal and transfer of parental *Daphnia magna*, the number of juveniles present in the old test vessels (alive or dead) was recorded and then discarded.

A *Daphnia magna* was considered immobile if, when the contents of the test vessel were briefly agitated, they did not swim during a 15-second period of observation. At the end of the test, the carapace lengths of all surviving parental *Daphnia magna* were measured using a microscope under low magnification fitted with a graticule slide.

The pH, temperature and concentration of dissolved oxygen were determined in each freshly prepared test medium at each renewal. At the end of each exposure period, water quality measurements were determined using pooled replicate samples of test media at each of the nominal test concentrations.

At each time point duplicate samples of test medium were taken. The concentrations of daminozide and formaldehyde were measured in samples of freshly prepared test media (20 mL) taken on days 1, 9 and 20 and in

samples taken from old test media (20 mL) on days 2, 10 and 21. The concentrations of daminozide and formaldehyde were determined by HPLC.

## 7. Statistics:

The EC<sub>50</sub> for adult mortality was calculated using Probit analysis. The total number of juveniles produced between days 1 to 14 and days 1 to 21 were analysed using one-way analysis of variance (ANOVA). Pairwise comparisons with the control were made using Dunnett's test. Dunnett's tests were interpreted with one-sided risk for decreased response with increasing dose. The juvenile data were square root transformed prior to analysis.

The actual carapace lengths were analysed using one-way ANOVA and Dunnett's test for pairwise comparisons of test concentrations with the control. The test was interpreted with two-sided risk. Levene's test for equality of variances among the concentrations was also performed and, in all cases, this showed no evidence of heterogeneity ( $P \geq 0.01$ ).

## Results:

### A. Survival and Clinical Observations:

After 14 days there was 0 - 30% mortality of parental *Daphnia magna* across all treatments including the control group. By Day 21 there was 10 - 80% mortality of parental *Daphnia magna* across all treatments including the control group. Two out of the twenty adults in the control did not survive to Day 21, therefore the validity criterion for adult mortality not exceeding 20% over the duration of the test was satisfied. Total parental *Daphnia magna* survival on Day 14 and 21 is presented in the following table.

**Table B 9.2.5-1 Total parental *Daphnia magna* survival on Day 14 and 21**

Nominal concentration as daminozide (mg/L) (formaldehyde)	% Survival of parental <i>Daphnia magna</i>			
	14 Days	21 Days	Control corrected	
			14 Days	21 Days
Control	100	90	-	-
1.0 (0.124)	100	60	100	70
2.2 (0.273)	90	50	90	60
4.6 (0.570)	100	60	100	70
10 (1.24)	80	60	80	70
22 (2.73)	100	20	100	30
46 (5.70)	100	20	100	30
100 (12.4)	70	30	70	40

Mortality was observed in every treatment level including the control. The majority of this mortality occurred between Days 17 and 20 and could therefore not be predicted from the range-finding data (which was conducted over 11 days). By Day 21 the recorded total control corrected mortality at the lowest four treatment levels (1.0, 2.2, 4.6 and 10 mg daminozide/L) was between 30 and 40 %. At concentrations above 10 mg daminozide/L the

control corrected mortality was much greater (between 60 and 70 %) suggesting marked toxicity at concentrations of 22 mg daminozide/L and above.

The majority of the adults that did not survive were observed to have had algal detritus attached to them on at least the day before they were recorded as immobile. A NOEC for parental survival could not be determined from this data but an EC<sub>50</sub> has been reported. The 21 day EC<sub>50</sub> for control corrected adult mortality was 13.9 mg daminozide/L.

### B. Reproduction:

Live juveniles were observed in all treatments by Day 8 therefore there was no treatment related effect on the time to the first brood. Vessels were excluded from the statistical analysis if the parental *Daphnia magna* did not survive to Day 21. Any juveniles produced by these *Daphnia* were excluded from the analysis. The mean number of live juveniles produced per treatment by Day 14 and Day 21, from surviving parental *Daphnia magna* are presented in the table below.

**Table B 9.2.5-2 Mean number of live juveniles produced per treatment by Day 14 and 21**

Nominal concentration as daminozide (formaldehyde) (mg/L)	Mean cumulative live juvenile production		% reduction compared to the control	
	Day 14	Day 21	Day 14	Day 21
Control	73	87	-	-
1.0 (0.124)	67	72	8.22	17.2
2.2 (0.273)	46*	48**	37.0	44.8
4.6 (0.570)	64	74	12.3	14.9
10 (1.24)	66	82	9.59	5.75
22 (2.73)	75	106	-2.74	-21.8
46 (5.70)	79	126	-8.22	-44.8
100 (12.4)	62	115	15.1	-32.2

\* significant difference compared to the control (p <0.05)

\*\* significant difference compared to the control (p <0.01)

- not applicable

Negative values indicate an increase in juvenile numbers relative to the controls

On Day 13 of the test eight aborted eggs were observed in one of the test vessels at the highest treatment level of 100 mg daminozide/L. On comparison to the control group, there was a statistically significant effect on the total numbers of juveniles produced between days 1 to 14 and days 1 to 21 at a nominal daminozide concentration of 2.2 mg daminozide/L. This was not considered to be treatment related, as there was no statistically significant effect on the total numbers of juveniles produced between days 1 to 14 or days 1 to 21 at all other treatment levels. The NOEC for reproduction was therefore considered to be 100 mg daminozide/L.

### C. Growth:

The mean carapace lengths at each treatment are summarised in the table below.

**Table B 9.2.5-3 Mean carapace lengths**

Nominal concentration as daminozide (formaldehyde) (mg/L)	Mean length of surviving parental <i>Daphnia magna</i> after 21 days (mm)	% reduction compared to the control
Control	4.1	-
1.0 (0.124)	4.1	0
2.2 (0.273)	4.0	2.44
4.6 (0.570)	4.1	0
10 (1.24)	4.0	2.44
22 (2.73)	4.3	-4.88
46 (5.70)	3.9	4.88
100 (12.4)	3.4*	17.1

\* significant difference compared to the control ( $p < 0.001$ )

- not applicable

Negative values indicate an increase in juvenile numbers relative to the controls

On comparison to the control group, there was a statistically significant reduction on the mean length of the parental *Daphnia magna* at Day 21 at the highest treatment level of 100 mg daminozide/L. The NOEC for adult carapace length was considered to be 46 mg daminozide/L.

#### **D. Toxicity values:**

The Day 14 and Day 21 EC<sub>50</sub>, NOEC and LOEC toxicity values in terms of adult mortality, juveniles produced per surviving parental *Daphnia magna* and parental carapace length are presented in the following table.

**Table B 9.2.5-4 Summary of toxicity values**

Parameter	Toxicity value based on nominal daminozide concentrations (mg/L)*				
	Adult mortality		Juvenile production		Carapace length
	Day 14	Day 21	Day 14	Day 21	Day 21
EC <sub>50</sub>	>100	13.9 (1.13 - >100)	>100	>100	>100
NOEC	46	<1.0	100	100	46
LOEC	100	1.0	>100	>100	100

\* Exposure included formaldehyde at nominally 12.4% the level of daminozide  
Numbers in brackets are 95% fiducial limits

#### **E. Analytical verification:**

The overall geometric mean measured concentrations of daminozide in samples of new and old test media were 0.965, 2.12, 4.40, 9.49, 19.9, 38.9 and 93.8 mg/L, corresponding to 96.5, 96.3, 95.7, 94.9, 90.5, 84.5 and 93.8 % of the nominal concentrations, respectively (see the table below). The overall geometric mean measured concentrations of formaldehyde in samples of new and old test media were 0.160, 0.244, 0.504, 1.32, 2.50, 5.59

and 12.2 mg/L, corresponding to 129, 89.4, 88.5, 107, 91.7, 98.0 and 98.2 % of the nominal concentrations, respectively (see the table below).

The toxicity of daminozide and formaldehyde to *Daphnia magna* during the reproduction test has been expressed in terms of the nominal concentrations.

**Table B 9.2.5-5 Measured concentrations of daminozide**

Nominal test concentration (mg/L)	Mean measured concentration (mg/L) *	Mean measured percent of nominal
Control	-	-
1.0	0.965	96.5
2.2	2.12	96.3
4.6	4.40	95.7
10	9.49	94.9
22	19.9	90.5
46	38.9	84.5
100	93.8	93.8

\* Mean value determined as the geometric mean of all measured concentrations

**Table B 9.2.5-6 Measured concentrations of formaldehyde**

Nominal test concentration (mg/L)	Mean measured concentration (mg/L) *	Mean measured percent of nominal
Control	-	-
0.124	0.160	129
0.273	0.244	89.4
0.570	0.504	88.5
1.24	1.32	107
2.73	2.50	91.7
5.70	5.59	98.0
12.4	12.2	98.2

\* Mean value determined as the geometric mean of all measured concentrations

### Conclusion:

A 21 day daily renewal study was conducted to determine the effects of simultaneous exposure to daminozide and formaldehyde (at 12.4% the level of daminozide) on *Daphnia magna* reproduction and survival. The results of the study demonstrated the 21 day EC<sub>50</sub> for adult mortality to be 13.9 mg daminozide/L (plus 1.72 mg formaldehyde/L). The 21 day NOECs for effects on reproduction and carapace length were determined to be 100 and 46 mg daminozide/L, respectively (plus 12.4 and 5.70 mg formaldehyde/L).

### RMS comments and conclusion:

The reported study is GLP compliant and was conducted according to OECD 211 guideline (2011). The test results are in compliance with the guideline's validity criteria.

Adult survival was shown to be the most sensitive parameter measured and was significantly affected at all concentrations tested. The 21-day no-observed-effect concentration (NOEC) for parental mortality could not be determined (<1.0 mg daminozide/L). The 21-day no-observed-effect concentration (NOEC) for reproduction and growth is 46 mg daminozide /L, based on nominal concentrations.

It is noted that daminozide was tested simultaneously with formaldehyde, therefore the results of the study reflect the combined toxicity of both substances, not only daminozide. For the present evaluation, formaldehyde is a not relevant metabolite. Thus, the study is not suitable for regulatory use.

#### **B.9.2.5.1.2 Reproductive and development toxicity of metabolites to *Daphnia magna***

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#### **B.9.2.5.2 Reproductive and development toxicity to an additional aquatic invertebrate species**

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#### **B.9.2.5.3 Development and emergence in *Chironomus riparius***

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#### **B.9.2.5.4 Sediment dwelling organisms**

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#### **B.9.2.6 Effects on algal growth**

##### **B.9.2.6.1 Effects of active substance on algal growth**

##### **B.9.2.6.1.1 Effects on growth of green algae**

##### **i) Effects on growth of freshwater green alga *Chlorella vulgaris***

<b>Reference:</b>	<b>Douglas, M., Pell, I. (1986)</b> The algistatic activity of Alar Technical
Report No.:	A.7.4.1.7
Guideline:	Not stated
GLP:	Yes
<b>Previous evaluation:</b>	In DAR (1999)
<b>Material and methods:</b>	
Test material:	Daminozide technical
Lot/Batch No:	46036
Purity:	98-99%

The effects of daminozide on the growth of green algae, *Chlorella vulgaris*, were determined in a static system for 72-hours. Eight test concentrations, 5, 10, 20, 40, 80, 160, 320 and 640 mg/L, plus control were tested; three vessels per concentration. Light intensity 6000 lux. Initial cell count was  $2.1 \times 10^5$  cells/mL. Samples for growth measurement were taken at 0, 24, 48 and 72 hours. Vessels were stirred continuously. pH ranged from 7.7 (control)

to 4.7 (640 mg/L) and changed (up to 3 units increase at lower concentrations) over 3 days to 4-8.5. Logarithmic growth was apparent for three days. EC<sub>50</sub> derived from graph according to OECD 201.

#### Results:

The 72 hour E<sub>b</sub>C<sub>50</sub> 160 mg a.s./L, 24-48 hour E<sub>r</sub>C<sub>50</sub> 180 mg a.s./L (no reliable C.I.), and the NOEC was 80 mg a.s./L.

**Table B 9.2.6-1 Inhibition of growth**

Concentration mg/l	Area under curve @ 72 h	% inhibition	Growth rate (24 - 48 h)	% inhibition
Control	10.408	-	0.0491	-
5.0	11.360	<9>	0.0533	<8>
10	11.080	<6>	0.0483	2
20	11.104	<7>	0.0497	<1>
40	10.920	<5>	0.0516	<5>
80	10.460	0	0.0529	<8>
160	5.476	47	0.0260	47
320	1.360	87	-0.0107	122
640	0.312	97	-0.0127	126

< increase >

Remark from previous review: Actual concentrations not measured. pH changes within individual vessels are rather large (up to three units); only at 40 mg/L and lower pH are >7. Initial cell count 21 times above OECD directive. Effects appear to be pH-related. The result NOEC 80 mg/L is used for conclusions.

#### RMS comments and conclusion:

The reported study is GLP compliant, no guideline is stated in the study but it seems to be in line with OECD 201 guideline (1984). However, no measurements of actual concentration of the test substance were carried out, therefore it could not be checked if the concentration of the test substance throughout the test was maintained. Due to unclear exposure during the test, no reliable endpoint can be derived from the study.

The study is not considered valid.

#### ii) Effects on growth of freshwater green alga *Chlorella vulgaris*

<b>Reference:</b>	<b>Abram, F. (1987)</b> The Toxicity of Daminozide to a Green Alga
Report No.:	FAL 4
Guideline:	OECD (1981)
GLP:	No
<b>Previous evaluation:</b>	In DAR (1999)
<b>Material and methods:</b>	
Test material:	Daminozide technical
Lot/Batch No:	V86.10.2.1.201

Purity: 99.4%

The effects of daminozide on the growth of green algae, *Chlorella vulgaris*, were determined in a static system for 72-hours. Six test concentrations, 0.1, 1.0, 10, 100, 1000 and 100000 mg/L, plus control were tested; three vessels per concentration. Light intensity near 5000 lux. Initial cell count was approximately  $1 \times 10^4$  cells/mL. The concentration of cells were recorded at intervals of 1, 2, 3, 4 and 6 days. Vessels were not stirred, but the contents were mixed twice daily by manual agitation. pH ranged from 7.9 (control) to 3.0 (10000 mg/L) and increased slightly (max. 0.6 units) over 6 days. EC<sub>50</sub> derived from graph according to OECD 201.

### Results:

Logarithmic growth was apparent for four days. 6-days E<sub>b</sub>C<sub>50</sub> 241 mg/l (54 - 1060 mg/l) and NOEC 1 mg/l.

Remark from previous review: Actual concentrations not measured. Light intensity <8000 lux. Logarithmic growth in control is observed only in first three days. Concentrations differ factor 10 instead of 1.6. pH test concentrations <7. The graphs with the comparative results and the calculated EC<sub>50</sub> of author do not match the results in the tables. Furthermore, the scales of graphs in figures 1 and 2 are different, but the curves drawn are the same. The statistical method was not described in report. The E<sub>b</sub>C<sub>50</sub> in the heading table is recalculated using tabulated data from author (first three days) with a log-logistic regression: 3.6 mg/l, 95% confidence interval 1.8-7.4 mg/l. At 0.1 mg/l 15, 13, and 12% reduction in biomass after three, four, and six days is calculated. The 72-h NOEC <0.1 mg/l (pH 6.5). Effects are pH-related. The results are not used for risk evaluation.

### RMS comments and conclusion:

The reported study is GLP compliant and was conducted according to OECD 201 guideline (1981). Several deficiencies were identified during the previous review of daminozide (see above). Since no measurements of actual concentration of the test substance were carried out it could not be checked if the concentration of the test substance throughout the test was maintained. Due to unclear exposure during the test, no reliable endpoint can be derived from the study.

The study is not considered valid.

### iii) Effects on growth of freshwater green alga *Pseudokirchneriella subcapitata*

<b>Reference:</b>	<b>Manson, P.S., Scholey, A. (2006)</b> Daminozide Technical: Inhibition of Growth to the alga <i>Pseudokirchneriella subcapitata</i>
Report No.:	2242/049-D2149
Guideline:	OECD 201 (1984)
GLP:	Yes
<b>Previous evaluation:</b>	Submitted for the purpose of renewal

### Executive Summary



The effects of daminozide on the growth of green algae, *Pseudokirchneriella subcapitata*, were determined in a static system. Three replicate algal suspensions were each exposed to nominal concentrations of 4.27, 9.39, 20.7, 45.5 and 100 mg a.s./L for 72 hours. Six replicates without test item were used as control. Observations of cell growth were recorded at 24, 48 and 72 hours to determine the potential effect on area under growth curve and growth rate.

The measured concentrations of daminozide were in the range of 92 to 102% of the nominal values. All study results are therefore based on nominal concentrations. Under the experimental conditions, the 72-hour EC<sub>50</sub> for both areas under growth curve and growth rate of daminozide for *Pseudokirchneriella subcapitata* was higher than 100 mg a.s./L. The NOEC was 100 mg a.s./L for both endpoints.

#### **Material and methods:**

##### **A. MATERIALS**

###### **1. Test material:** Daminozide Technical

**Batch:** 2003-10-01

**Purity:** 100.2% (analysed)

**Description:** Off-white powder

##### **B. STUDY DESIGN AND METHODS**

###### **1. Test animals:** *Pseudokirchneriella subcapitata* (green algae)

**Strain:** Strain 278/4

**Source:** Culture Collection of Algae and Protozoa (CCAP), SAMS Research Services Ltd., Oban, UK

**Initial density:**  $1 \times 10^4$  cells/mL

###### **2. Test concentrations:** 0, 4.27, 9.39, 20.7, 45.5 and 100 mg a.s./L

###### **3. Environmental conditions:**

**Test vessels:** 250 mL Erlenmeyer flask loosely sealed with a cap

**Test water:** growth medium according to OECD 201

**Temperature:** 22.6-23.6°C

**pH:** Test start: 7.2-7.6, test end: 9.9-10.2

**Photoperiod:** Continuous lighting (6890-6950 lux)

###### **4. Animal assignment and treatment:**

Glass test vessels (250 mL Erlenmeyer flasks) were filled with 100 mL of test solution and algae were then added to these vessels that were then incubated. The flasks were positioned randomly. The study consisted of three replicate test chambers in each treatment group and six replicates were tested in the control group. An extra test vessel without algae was prepared for each test group for analyses at the start of the study.

###### **5. Dose preparation:**

First, the highest test item solution of 100 mg/L was prepared, by dispersing test item in the growth medium. The remaining test item solutions were obtained by serial dilution of the highest test item solution. Six concentrations (nominal) were tested: 4.27, 9.39, 20.7, 45.5 and 100 mg a.s./L.

#### 6. Measurements and observations:

Cell density was measured daily using a Z2 Coulter Counter, excitation at 436 nm, emission at 685 nm.

The concentration of daminozide technical in samples of test media taken from each replicate test vessel (and pooled) were analytically verified by HPLC with UV detection at 215 nm at the beginning and the end of the exposure. Separate replicates for the test item analysis at the beginning of the exposure were prepared without algae.

The pH-value and light intensity were measured at the start and the end of the test. The room temperature was recorded continuously.

#### 7. Statistics:

The algal cell density data was evaluated using comparison of areas under the growth curve and comparison of the average specific growth rate.

The NOEC was determined by calculation of statistical significance of growth rate and area under the growth curve using the Dunnett's test ( $\alpha=0.05$ ).

EC<sub>10</sub>-, EC<sub>20</sub>- and EC<sub>50</sub>-values of the growth rate and area under the growth curve after 72 hours could not be calculated because there was no reduction in growth relative to the control treatment.

### Results:

#### A. Growth inhibition:

For the control cultures, biomass increased exponentially by a factor higher than 16 (184), coefficient of variation for section-by-section specific growth rates did not exceed 35% (27%) and coefficient of variation of average specific growth rates during whole test period in replicate control cultures did not exceed 7% (1%).

Mean values for area under the growth curve and growth rate with the corresponding percent inhibition values are presented in Table 8.2.6.1/02-01. Mean cell densities are listed in the table below.

**Table B 9.2.6-2 Mean area under the growth curve (A) and growth rate ( $\mu$ ) with the corresponding percent inhibition over a 72 hour exposure period**

Nominal test item concentration (mg a.s./L)	Area under growth curve (A x 10 <sup>6</sup> )	Inhibition of area under growth curve relative to control (%)	Growth rate ( $\mu$ x 10 <sup>-2</sup> )	Inhibition of growth rate relative to control (%)
Control	35.8	-	7.24	-
4.27	35.0	2.26	7.20	0.50

Nominal test item concentration (mg a.s./L)	Area under growth curve (A x 10 <sup>6</sup> )	Inhibition of area under growth curve relative to control (%)	Growth rate ( $\mu \times 10^{-2}$ )	Inhibition of growth rate relative to control (%)
9.39	35.3	1.31	7.23	0.10
20.7	34.2	4.45	7.21	0.44
45.5	36.6	-2.22	7.29	-0.70
100	34.3	4.14	7.26	-0.32

Negative inhibitions = increase of growth

**Table 8.2.6.1/02-02: Mean cell densities**

Nominal test item concentration (mg a.s./L)	Cell density (cells x 10 <sup>4</sup> /mL)			
	0 hours	24 hours	48 hours	72 hours
Control	1.0	6.03	53.8	184
4.27	1.0	6.15	52.7	179
9.39	1.0	6.14	52.3	183
20.7	1.0	6.13	49.2	179
45.5	1.0	6.06	53.7	191
100	1.0	6.16	46.1	187

#### B. Toxicity endpoint:

The 72-hour toxicity endpoints are presented in the table below.

**Table B 9.2.6-3 Toxicity endpoints for algae exposed to daminozide after 72 hours based on nominal test item concentrations**

	Area under growth curve inhibition
NOEC	100
E <sub>r</sub> C <sub>50</sub>	>100
	Growth rate inhibition
NOEC	100
E <sub>b</sub> C <sub>50</sub>	>100

#### C. Analytical verification:

The concentrations of daminozide were determined in the fresh media (0 h) and old media (72 h) of all tested concentration levels and the control via HPLC. The measured concentrations of daminozide were in the range of 92 to 102% of the nominal values at all tested concentration levels (see the table below).

**Table B 9.2.6-4 Measured concentrations of daminozide in the exposure solutions**

Nominal test item concentration (mg a.s./L)	Measured concentration of daminozide (mg a.s./L)			
	0 hour (without algae)	72 hour (without algae)	72 hour (with algae)	(%) of nominal concentration*
Control	< LOQ	< LOQ	< LOQ	-

4.27	4.18	4.06	3.99	96
9.39	8.40	9.63	8.78	92
20.7	21.0	20.7	21.0	102
45.5	43.9	47.1	47.2	100
100	99.9	104	99.7	100

\* Mean of 0 hour (without algae) and 72 hour (with and without algae)

LOQ = limit of quantification (0.3 mg/L)

### Conclusion

In this study daminozide was not found to inhibit the growth of the freshwater green algae *Pseudokirchneriella subcapitata* after 72 hours. Hence, the EC<sub>50</sub> value for area under the growth curve and growth rate was higher than the highest test concentration of 100 mg a.s./L and the NOEC value was 100 mg a.s./L.

### Validity criteria

The test is considered to be valid if the algal density in the control has increased at least 16 times after 3 days (OECD 1984); cell density increased at least 55.6 times in the controls, which meets the validity criterion of a minimal 16 times increase.

### RMS comments and conclusion:

The reported study is GLP compliant and was conducted according to OECD 201 guideline (1984). It is also in line with current OECD 201 guideline (2011).

To check the validity criteria according to OECD 201 guideline (2011), the coefficients of variation of average specific growth rates in control replicates were calculated and are presented in the table below:

**Table B 9.2.6-5 Average coefficient of variance at 0-72 hours and section-by-section in the control cultures**

Replicates	0-72 h			Section by section (day 0-1, 1-2, 2-3)			
	Average growth rate (day <sup>-1</sup> )	St Dev	CV (%)	Average growth rate (day <sup>-1</sup> )	St Dev	CV (%)	Mean CV (%)
A	1.74	0.0084	0.48	1.74	0.48	27.73	24.69
B				1.73	0.47	27.11	
C				1.74	0.45	25.91	
D				1.73	0.40	23.26	
E				1.75	0.46	26.19	
F				1.74	0.31	17.95	

According to current OECD 201 guideline the validity criteria were met (the specific growth rate in control was 1.74 per day within the 72-hour period (should be greater than 0.92 per day); the mean coefficient of variation for section-by section specific growth rates in the control cultures was 24.69% (should be less than 35%); the coefficient of variation of average specific growth rates during the whole test period in replicate control cultures was 0.48 (should be less than 7%)).

The study is acceptable for regulatory use.

The 72-hour  $E_rC_{50}$  and  $E_bC_{50}$  is  $>100 \mu\text{g daminozide/L}$ , the 72-hour no-observed-effect concentration (NOEC) is  $100 \mu\text{g daminozide/L}$ , based on nominal concentrations.

#### B.9.2.6.1.2 Effects on growth of an additional algal species

##### i) Effects on growth of freshwater blue-green alga *Anabaena flos-aquae*

<b>Reference:</b>	Seeland-Fremer, A., Mosch, W. (2014) Toxicity of daminozide technical to <i>Anabaena flos-aquae</i> in an Algal Growth Inhibition Test
Report No.:	87711210
Guideline:	OECD 201 (2011)
GLP:	Yes
<b>Previous evaluation:</b>	Submitted for the purpose of renewal

#### Executive Summary

The effects of daminozide on the growth of the freshwater green algae *Anabaena flos-aquae* were determined in a static system. Three replicate algal suspensions were each exposed to nominal concentrations of 0.317, 1.00, 3.16, 10.0, 31.6 and 100 mg a.s./L for 72 hours. Six replicates without test item were used as control. Observations of cell growth were recorded at 24, 48 and 72 hours to determine the potential effect on algal growth rate and yield, relative to the control.

The measured concentrations of the test item daminozide at the start of the exposure (0 hours) were in the range of 90 to 101% of the nominal values. The measured concentrations of the test item at the end of the exposure (72 hours) were in the range of 99 to 107% of the nominal value. All study results are therefore based on nominal concentrations. Under the experimental conditions, the 72-hour  $E_rC_{50}$  and the 72-hour  $E_yC_{50}$  of daminozide for *Anabaena flos-aquae* were both higher than 100 mg a.s./L. The NOEC was 100 mg a.s./L for growth rate and for yield.

#### Material and methods:

##### A. MATERIALS

###### 1. Test material: Daminozide Technical

**Batch:** 1596-84-5

**Purity:** 99.8% (analysed)

**Description:** White solid

**Reference Item:** 3,5-dichlorophenol

(Tested in a separate study in July 2014)

##### B. STUDY DESIGN AND METHODS

###### 1. Test animals: *Anabaena flos-aquae*

**Strain:** UTEX B1444

**Source:** University of Texas, UTEX Culture Collection of Algae, Austin, Texas, USA

**Initial density:**  $1.5 \times 10^4$  cells/mL

2. **Treatment:** 0, 0.317, 1.00, 3.16, 10.0, 31.6 and 100 mg a.s./L

3. **Environmental conditions:**

**Test vessels:** 50 mL Erlenmeyer flask covered with glass dishes

**Test water:** 20X AAP medium

**Temperature:** 21.0-22.0°C

**pH:** test start: 7.3-7.6, test end: 8.7-8.8

**Photoperiod:** Continuous lighting (3600-4140 lux)

4. **Animal assignment and treatment:**

Algal cells were taken from an exponentially growing pre-culture. The test was started with a nominal algal cell density of 15000 algal cells per mL test medium. The study consisted of three replicate test chambers in each treatment group and six replicates were tested in the control group. An extra test vessel without algae was prepared for each test group at the start of the test, to provide a blank for the analyses. Just before introduction of the algae in the test vessels, the test media were prepared.

5. **Dose preparation:**

First, the highest test item solution of 100 mg/L was prepared, by dispersing 50.2 mg test item in 502 mL growth medium by intense stirring for 15 minutes. The remaining test item solutions were obtained by serial dilution of the highest test item solution. Six concentrations (nominal) were tested: 0.317, 1.00, 3.16, 10.0, 31.6 and 100 mg a.s./L. Total volume in the test vessels was 50 mL.

6. **Measurements and observations:**

After 72 hours the shape of the algal cells was examined by microscope for all replicates. In addition, samples from all replicates were taken daily for spectrophotometrical measurements. These spectrophotometrical measurements were used to calculate the cell densities. A linear regression was used for this calculation, which was based on counted cell densities (by microscope) and absorption (by spectrophotometer) in a number of samples from the pre-culture before test start.

Duplicate samples from the three highest test item concentrations (10.0, 31.6 and 100 mg/L) and the control (with algae) were taken at the beginning and the end of the test. These samples were centrifuged before analysis of the daminozide concentration by LC-MS/MS. One additional blank control without algae was taken.

The pH-value was measured at the start and the end of the test. The room temperature was recorded daily. Light intensity was measured once during the test.

## 7. Statistics:

The 72-hour NOEC and LOEC values were determined by testing the significant differences of growth rate and yield for the test item groups compared to the control, by Williams' test.  $EC_{10}$ ,  $EC_{20}$  and  $EC_{50}$  values were calculated by Probit analysis where possible. ToxRat Professional, Version 2.10.05 (ToxRat Solutions GmbH) was used to perform the statistical analysis.

## Results:

### A. Growth inhibition:

The control group showed an increase in cell density of 29.2, a coefficient of variation of sectional growth rates of 28.1% and a coefficient of variation of growth rates between replicates during the whole test period of 1.5%. No statistically significant differences were observed for growth rate and yield of test item groups compared to the control.

Mean growth rates and yield with the corresponding percent inhibition values and mean cell densities are presented in the tables below.

**Table B 9.2.6-7 Mean growth rate and yield, with corresponding percent inhibition over a 72 hour exposure period**

Nominal test item concentration (mg a.s./L)	Growth rate (d <sup>-1</sup> )	Inhibition of growth rate (%)	Yield (cells x 10 <sup>4</sup> /mL)	Inhibition of yield (%)
Control	1.124	-	42.25	-
0.316	1.123	0.1	42.14	0.3
1.00	1.123	0.1	42.03	0.5
3.16	1.104	1.8	39.61	6.3
10.0	1.147	-2.1	45.34	-7.3
31.6	1.092	2.8	38.28	9.4
100	1.172	-4.2	48.99	-15.9

Negative inhibitions = increase of growth

**Table B 9.2.6-8 Mean cell densities**

Nominal test item concentration (mg a.s./L)	Cell density (cells x 10 <sup>4</sup> /mL)			
	0 hours	24 hours	48 hours	72 hours
Control	1.5	4.19	12.36	43.75
0.317	1.5	4.69	12.86	43.64
1.00	1.5	6.12	14.18	43.53
3.16	1.5	4.25	12.75	41.11
10.0	1.5	5.79	15.06	46.84
31.6	1.5	4.58	9.55	39.78
100	1.5	5.35	18.37	50.49

Microscopic evaluation of the cells at end of the test period revealed no abnormalities in cell shape, for the control and any of the test item concentrations.

### B. Toxicity endpoint:

EC<sub>10</sub>, EC<sub>20</sub> and EC<sub>50</sub> values could not be calculated due to the lack of effects. Due to the lack of effects, endpoints for growth rate and yield inhibition were the same. The 72-hour toxicity endpoints are presented in the table below.

**Table B 9.2.6-9 Toxicity endpoints for growth rate and yield of algae exposed to daminozide for 72 hours based on nominal test item concentrations**

	<b>Growth rate Nominal test item concentration (mg a.s./L)</b>
NOEC	100
LOEC	>100
E <sub>r</sub> C <sub>50</sub>	>100
	<b>Yield Inhibition Nominal test item concentration (mg a.s./L)</b>
NOEC	100
LOEC	>100
E <sub>y</sub> C <sub>50</sub>	>100

The reference item 3,5-dichlorophenol was found to inhibit the growth of algae significantly. The EC<sub>50</sub> values for inhibition of growth rate (E<sub>r</sub>C<sub>50</sub>) and for inhibition of yield (E<sub>y</sub>C<sub>50</sub>) were 4.19 mg/L and 2.66 mg/L, respectively. These values fall within the validity range.

### C. Analytical verification:

The concentrations of daminozide were determined in the fresh media (0 h) and old media (72 h) of all tested concentration levels and the control via LC-MS/MS. The measured concentrations of daminozide in the fresh media (0 h) were in the range of 90 to 101% of the nominal values at all test item concentrations. In the old media (72 h) the measured concentrations were in the range of 99 to 107% of the nominal values for all test item concentrations (see the table below). As the measured concentrations were within 80-120% of the nominal concentration, results were based on nominal concentrations.

**Table B 9.2.6-10 Mean measured concentrations of daminozide in the exposure solutions**

<b>Nominal test concentration (mg a.s./L)</b>	<b>Daminozide</b>			
	<b>Test start (0 h)</b>		<b>Test end (72 h)</b>	
	<b>Measured concentration (mg a.s./L)</b>	<b>(%)</b>	<b>Measured concentration (mg a.s./L)</b>	<b>(%)</b>
Control	< LOD	-	< LOD	-
10.0	9.63	96.5	10.23	102.5
31.6	31.15	99	33.86	107
100	92.53	92.5	101.6	101.5

% = Percent of the nominal concentration of the test item

LOD = limit of detection (3 µg test item/L)



**Validity criteria:**

Cell Density Increase in Control Cultures: 29.2-fold increase within 72 hours and thus, validity criterion (at least 16-fold increase) was met.

Coefficient of Variation of Sectional (Daily) Growth Rates in Control Cultures: 28.1 % and thus, validity criterion (should not exceed 35%) was met.

Coefficient of Variation of Average Growth between Control Replicates: 1.5 % and thus, validity criterion (should not exceed 10%) was met.

**Conclusion:**

In this study daminozide was not found to inhibit the growth of the freshwater blue-green algae *Anabaena flos-aquae* after 72 hours up to and including the highest test item concentration. Hence, the EC<sub>50</sub> values for inhibition of growth rate (E<sub>r</sub>C<sub>50</sub>) and yield (E<sub>y</sub>C<sub>50</sub>) were both >100 mg a.s./L. The NOEC-value for inhibition of growth rate and yield after 72 hours was determined to be 100 mg a.s./L.

**RMS comments and conclusion:**

The reported study is GLP compliant and was conducted according to OECD 201 guideline (2011).

To check the validity criteria according to OECD 201 guideline (2011), the coefficients of variation of average specific growth rates in control replicates were calculated and are presented in the table below:

**Table B 9.2.6-11 Average coefficient of variance at 0-72 hours and section-by-section in the control cultures**

Replicates	0-72 h			Section by section (day 0-1, 1-2, 2-3)			
	Average growth rate (day <sup>-1</sup> )	St Dev	CV (%)	Average growth rate (day <sup>-1</sup> )	St Dev	CV (%)	Mean CV (%)
A	1.12	0.016	1.46	1.11	0.16	13.95	28.08
B				1.11	0.36	31.90	
C				1.15	0.47	41.04	
D				1.11	0.55	49.90	
E				1.14	0.20	17.41	
F				1.13	0.16	14.30	

According to current OECD 201 guideline the validity criteria were met (the specific growth rate in control was 1.12 per day within the 72-hour period (should be greater than 0.92 per day); the mean coefficient of variation for section-by section specific growth rates in the control cultures was 28.08% (should be less than 35%); the coefficient of variation of average specific growth rates during the whole test period in replicate control cultures was 1.46 (should be less than 10%)).

The study is acceptable for regulatory use.

The 72-hour E<sub>r</sub>C<sub>50</sub> and E<sub>y</sub>C<sub>50</sub> is >100 µg daminozide/L, the 72-hour no-observed-effect concentration (NOEC) is 100 µg daminozide/L, based on nominal concentrations.

**B.9.2.6.2 Effects of metabolites on algal growth****B.9.2.6.2.1 Effects on growth of green algae****i) Metabolite methanol: Effects on growth of freshwater green alga *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*)**

<b>Reference:</b>	<b>Cho, C-W., Heon, Y-C., Pham, T.P.T., Vijayaraghavan, K., Yun, Y-S. (2008)</b> The ecotoxicity of ionic liquids and traditional organic solvents on microalga <i>Selenastrum capricornutum</i> . Ecotoxicology and Environmental Safety (2008) Vol. 71, pp. 166-171.
<b>Report No.:</b>	-
<b>Guideline:</b>	OECD 202 (1984); U.S. EPA 72-2 (1982)
<b>GLP:</b>	Not stated.
<b>Previous evaluation:</b>	Submitted for the purpose of renewal

**Executive Summary**

The effects of methanol on the growth of green algae, *Selenastrum capricornutum*, were determined in a static system. Two replicate algal suspensions were each exposed to nominal concentrations of 0.1 M to 1.26  $\mu$ M (389,000 to 4,900 mg/L) for 96 hours. Three replicates without test item were used as control. The 96 hour EC<sub>50</sub> was determined to be  $5.85 \pm 0.08 \mu$ M (approximately 22,000 mg/L) based on growth rate.

**Material and methods:****A. MATERIALS**

- 1. Test material:** Methanol
- Batch:** Not stated
- Purity:** >99.5%
- Description:** Not stated

**B. STUDY DESIGN AND METHODS**

- 1. Test animals:** *Selenastrum capricornutum*
- Strain:** ATCC-22662
- Source:** National Institute Environmental Research, Korea
- Initial density:** Not stated
- 2. Test concentrations:** 0.1 M to 1.26  $\mu$ M (389,000 to 4,900 mg/L)
- 3. Environmental conditions:**
- Test vessels:** 250 mL Erlenmeyer flask
- Test water:** sterilised culture medium
- Temperature:** 25°C
- pH:** Not stated

**Photoperiod:** Continuous lighting (average illumination of  $30 \pm 5 \mu\text{E m/s}$ )

**4. Animal assignment and treatment:**

Experiments were performed in 250 mL Erlenmeyer flasks containing 55 mL sterilised culture medium inoculated with 5 mL samples of 7 day cultured algae. The test material was subsequently added to the flasks and a concentration range of 0.1 M and 1.26  $\mu\text{M}$  established. All experiments were performed in duplicate except for the control tests which were performed in triplicate.

**5. Dose preparation:**

Test material directly added to the test flasks.

**6. Measurements and observations:**

At each determined exposure date, the optical density of the algal biomass was estimated at 438 nm using a spectrophotometer.

**7. Statistics:**

The dry cell weight was determined from the linear relationship  $\text{dry cell weight (g/L)} = 0.329 \times \text{optical density}$ .

**Results:**

The 96 hour  $\text{EC}_{50}$  was determined to be  $5.85 \pm 0.08 \mu\text{M}$  (approximately 22,000 mg/L) based on growth rate.

**Conclusion:**

The 96 hour  $\text{EC}_{50}$  for methanol was determined to be  $5.85 \pm 0.08 \mu\text{M}$  (approximately 22,000 mg/L) based on growth rate.

**RMS comments and conclusion:**

The reported study is a study review taken from the open literature. It was conducted according OECD 201 guideline (2001) and OPPTS 850.5400 guideline (1996). No full study report was available and, therefore, a lot of information is missing, e.g. batch number, the exact concentrations tested, initial algal density, results were presented as  $\text{EC}_x$  calculations only – no data in form of effects on inhibition of algal growth for each treatment level were available. Further, no results of analytical measurements of methanol throughout the test were presented. No raw data were available, therefore the validity criteria on the coefficients of variation of average specific growth rates in control replicates could not be checked.

The study is not suitable for regulatory use.

**B.9.2.7 Effects on aquatic macrophytes**

**B.9.2.7.1 Effects of active substance on aquatic macrophytes**

**i) Effects on *Lemna gibba* in laboratory test**

<b>Reference:</b>	<b>Palmer, S.J., Kendall, T.Z., Krueger, H.O. (2001)</b> Daminozide: A 7-day toxicity test with duckweed ( <i>Lemna gibba</i> G3)
Report No.:	117A-1197
Guideline:	OECD 221 (2000), U.S. EPA OPPTS 850.4400 (1996)
GLP:	Yes
<b>Previous evaluation:</b>	In Addendum (2002)

### Executive Summary

The objective of this study was to determine the toxicity of daminozide to the aquatic macrophyte, *Lemna gibba*, under static test conditions during a seven-day exposure. Fronds of duckweed were exposed to a geometric series of six concentrations and a negative control (20X AAP medium). Three replicate test chambers were maintained in each treatment and control group. At initiation, five plants with a total of 15 fronds were added to each replicate test chamber. Effects upon the duckweed were assessed through direct counts of duckweed frond numbers, chlorosis, necrosis, dead fronds, root destruction and break-up of colonies conducted on days 3, 5 and 7 of the test.

Mean measured concentrations were 95-106% of nominal test concentrations.

The 7 day IC<sub>50</sub> value for *Lemna gibba* G3 exposed to daminozide, based on mean frond numbers and group biomass data was >127 mg daminozide/L, the highest concentration tested. Based on frond numbers, biomass and the general health of plants, the NOAEC was 127 mg daminozide/L.

### Material and methods:

#### A. MATERIALS

- Test material:** Daminozide B-Nine Technical  
**Batch number:** GE1D17H101  
**Purity:** 99.8%  
**Appearance:** White powder
- Vehicle:** Dimethylformamide (DMF) at 100 µL DMF/L
- Dilution water:** 20X AAP medium
- Test organism**  
**Species:** Duckweed (*Lemna gibba* G3)  
**Source:** In-house culture (Wildlife International Ltd).
- Treatment groups:** 3.8, 7.5, 15, 30, 60 and 120 mg test item/L and a control.
- Environmental conditions:**

**Test vessels:** 250 mL glass beakers, containing 100 mL of test solution, covered with disposable Petri dishes

**Temperature:** 24.5 to 25.6°C

**pH:** 7.0 to 8.9

**Photoperiod:** Continuous (4550 to 5510 lux)

## **B. STUDY DESIGN AND METHODS**

### **1. Organism set up and treatment:**

Fronds of duckweed, *Lemna gibba* G3, were exposed to a geometric series of six test concentrations and a negative (20X AAP medium) control under static conditions for seven days. Three replicate test chambers were maintained in each treatment and control groups. At initiation, five plants with a total of 15 fronds were added to each replicate test chamber.

### **2. Dose preparation:**

A primary stock solution was prepared by dissolving daminozide in 20X AAP medium at a nominal concentration of 120 mg daminozide/L, equivalent to the highest test concentration. The stock solution was sonicated for approximately one hour and mixed by inversion. Aliquots of the stock solution were diluted with 20X AAP medium to achieve final volumes of 500 mL of the test solution at the appropriate test concentrations. After mixing, 100 mL of the test solution were measured into each replicate test chamber.

### **3. Measurements/observations:**

Growth was determined through direct counts of fronds on days 3, 5 and 7 of the test. In addition, the total number of plants in each replicate test chamber was determined at test termination. Observations of effects such as were performed on days 3, 5 and 7 of the test. Biomass (dry weight in mg) was determined at the end of the exposure period. Plants were pooled by concentration and dried at approximately 50-60°C for approximately 48 hours and then weighed.

### **4. Statistics:**

Calculations of mean frond numbers, percent inhibition values and the percentages of necrotic, chlorotic and dead fronds were performed using Excel. Statistical analyses were conducted using TOXSTAT Version 3.5. Percentages of dead, chlorotic and necrotic fronds were calculated to the total number of fronds in each test chamber at each observation period. Percent inhibition values were calculated for each treatment group as the percent reduction in mean frond number and group biomass relative to the mean frond number or group biomass in the negative control replicates.

Day 7 frond numbers were evaluated for normality and homogeneity of variances ( $p = 0.05$ ) using the Shapiro-Wilk's and Levene's tests, respectively. Statistically significant differences between the control and treatment groups were identified using ANOVA and Dunnett's test.

**Results:****A. Observations and measurements**

No treatment-related growth inhibition was observed in any treatment groups. Percent inhibition of growth based on day 7 frond number was -9.2 to 9.8%. Dunnett's test showed that frond production was not significantly reduced in any treatment group compared to the negative control. Percent inhibition of growth based on day 7 biomass data was -2.7 to 8.9%. Consequently the 7 day IC<sub>50</sub> value for frond number and biomass was estimated to be >127 mg/L, the highest concentration tested. A few necrotic and chlorotic frond were observed but these observations accounted for less than 1% of the total number of fronds on day 7 and the numbers did not increase with concentration.

The findings are summarised in the following tables.

**Table B 9.2.7-1 Mean frond numbers, group biomass and percent inhibition at test termination**

Mean measured concentration (mg test item/L)	Mean frond number $\pm$ SD	Frond number percent inhibition	Group biomass (mg)	Biomass percent inhibition
Negative control	220 $\pm$ 10	-	112	-
3.6	232 $\pm$ 35	-5.6	102	8.9
7.2	240 $\pm$ 11	-9.2	115	-2.7
15	223 $\pm$ 14	-1.4	110	1.8
31	217 $\pm$ 29	1.5	105	6.3
61	198 $\pm$ 21	9.8	108	3.6
127	202 $\pm$ 9.0	8.0	109	2.7

**Table B 9.2.7-2 IC<sub>50</sub> values**

Time (days)	Frond number		Biomass	
	IC <sub>50</sub> (mg/L)	95% CI	IC <sub>50</sub> (mg/L)	95% CI
7	>127	-	>127	-

95% CI could not be statistically calculated from the data obtained

**C. Analytical verification**

The quantification of the test material was performed using HPLC with UV detection. Samples of test solutions were collected on day 0 (from individual batches of test solution prepared for each treatment and control group prior to the addition of *Lemna*) and day 7 (from each replicate and pooled by treatment group). All samples were analysed immediately without storage.

**Table B 9.2.7-3 Summary of analytical results**

Nominal test concentration (mg a.s./L)	Measured concentration on (mg a.s./L)		Mean measured concentration (mg a.s./L)	Mean measured percent of nominal
	Day 0	Day 7		

0 (negative control)	<LOQ	<LOQ	-	-
3.8	3.72	3.51	3.6	95
7.5	7.44	7.03	7.2	96
15	15.0	14.5	15	100
30	30.3	31.2	31	103
60	60.6	62.2	61	102
120	120	134	127	106

**Conclusion:**

The 7 day IC<sub>50</sub> value for *Lemna gibba* G3 exposed to daminozide, based on mean frond numbers and group biomass data was >127 mg daminozide/L, the highest concentration tested. Based on frond numbers, biomass and the general health of plants, the NOAEC was 127 mg daminozide/L.

Remark from previous review: The results of 7-day IC<sub>50</sub> >127 mg/L and NOEC ≥127 mg/L are used for risk assessment.

**RMS comments and conclusion:**

The reported study is GLP compliant and was conducted according to OECD 221 (2000) and U.S. EPA OPPTS 850.4400 (1996) guidelines. According to current OECD 221 (2006) and EPA OCSPP 850.4400 guidelines the following validity criterion should be fulfilled:

„For the test to be valid, the doubling time of frond number in the control must be less than 2.5days (60 h), corresponding to approximately a seven-fold increase in seven days and an average specific growth rate of 0.275 d<sup>-1</sup>.“ Data on growth rates and doubling times in the control replicates throughout the test are presented in the table below.

**Table B 9.2.7-4 Growth rates and doubling times in control replicates.**

Time (days)	Mean growth rate (day <sup>-1</sup> )	T <sub>d</sub> (days)
0-7	0.39	1.81
0-3	0.53	1.31
3-5	0.28	2.54
5-7	0.40	1.72

The validity criteria were fulfilled although the doubling time for section 3-5 days is 2.54 days, it is still considered acceptable.

It is noted that percent inhibition values were calculated for each treatment group as the percent reduction in mean frond number and group biomass relative to the mean frond number or group biomass in the control replicates. Thus, percentage inhibition of growth rate was not calculated.

Further, the current OECD 221 guideline (2006) recommends to base response variables on frond numbers and on one additional parameter of observation (e.g. biomass dry weight). The results of the study did not only allow

calculation based on on biomass - since biomass was only observed on day 7 and no estimate of starting biomass was available.

It is concluded that the endpoint  $IC_{50} > 127$  mg daminozide/L based on mean frond numbers and group biomass data is not suitable for regulatory use.

#### **B.9.2.8 Further testing on aquatic organisms**

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For a risk assessment of daminozide to aquatic organisms, see Vol. 3 CP B.9 for the formulated product.

#### **B.9.3 Effects on arthropods**

##### **B.9.3.1 Effects on bees**

##### **B.9.3.1.1 Acute toxicity to bees**

##### **i) Acute oral toxicity to honeybees**

<b>Reference:</b>	<b>Davies, L. (1987)</b> Report on a laboratory investigation into the toxicity of daminozide to honey bees ( <i>Apis mellifera</i> )
Report No.:	FAL 5
Guideline:	to UK guidelines (1992)
GLP:	No
<b>Previous evaluation:</b>	In DAR (1999)

#### **ORAL TEST:**

##### **Executive Summary**

The purpose of this study was to determine the acute oral toxicity of daminozide to the honey bee (*A. mellifera*). Mortality of the bees was used as the toxic endpoint. The effects of five doses of test item (0, 1, 50, 100, 150 and 200 µg per bee) were compared to a control. To confirm the sensitivity of the test insects, the reference item Guthion (azinphos methyl) was also evaluated in a series of doses. The test item and reference item were applied in 20% w/v sucrose solution, which was used as a carrier (food). For the control, pure 20% w/v sucrose solution was offered to the bees. Four replicate cages, each containing 10 bees (i.e. 40 bees in total), were prepared for each treatment. The treated food was offered by syringe through the top of the test cages. The bees were assessed for mortality at 48 hours. In an oral-exposure laboratory test with the honeybee, *Apis mellifera*, the 48 h median lethal dose ( $LD_{50}$ ) for daminozide was shown to be  $> 200$  µg/bee. The  $LD_{50}$  of Guthion (azinphos methyl) used routinely as the toxic reference in this laboratory was approximately 0.1 µg/bee in the oral test.

##### **Material and methods:**

#### **A. MATERIALS**

- Test material:** Technical grade daminozide  
**Description:** Not stated



**Lot/Batch:** Not stated  
**Purity:** 99.4% w/w

**2. Reference material:**

**Description:** Guthion (azinphos-methyl)  
**Lot/Batch:** Not stated  
**Purity:** 99%

**B. STUDY DESIGN AND METHODS**

**1. Test animals:** Honeybee (*Apis mellifera* L.)

**Age/life stage:** Worker bees (age not stated)

**Source:** Frames of a healthy queen-right colony

**Diet:** 20% w/v solution of sucrose in water

**2. Test units:** Tinned wire-mesh cylinders (120 x 40 mm) closed at both ends with a cork, with a small glass feeding tube (orifice about 1.5 mm) inserted into the top cork

**3. Environmental conditions:**

**Temperature:** 25°C

**Relative humidity:** Not stated

**Photoperiod:** Not stated

**4. Animal assignment and treatment:**

Worker honey bees were collected from frames (without brood) of a healthy queen-right colony. The bees were taken to the laboratory in a 2 gallon bucket with three layers of blotting paper at the bottom to absorb moisture. After anaesthetisation with the minimum amount of carbon dioxide, ten bees were transferred into each experimental cage. At least five doses of the test material and toxic standard were given to four replicates of ten bees per treatment. A 0.2 ml volume of a solution of the appropriate concentration of the test material in 20% sucrose in water was presented to each group of ten bees in a small glass tube inserted into the top cork. When the bees had taken the 0.2 ml of the test material they were fed 20% sucrose in tubes as in the contact test. After 48 hours, the number of dead bees was counted.

**5. Dose preparation:**

Daminozide is highly soluble in water and the oral tests were conducted by dissolving the test material directly in the sucrose feed solution. Test item rates of 1, 50, 100, 150 and 200 µg per bee were studied. A control was carried out using 20% aqueous sucrose solution.

**6. Measurements and observations:**

The number of dead bees was recorded after 48 hours.

## 7. Statistics:

Results obtained from the bees treated with the test and reference items were compared to those obtained from the control. The median lethal dose (LD<sub>50</sub>) was calculated using a Probit Analysis (GLIM, 1977 Royal Statistical Society, London).

## Results:

### A. MORTALITY

Table B 9.3.1-1 Mortality in the oral test

Treatment (µg a.s./bee)	No. of dead bees (total of 40)
Control	0
1	0
50	0
100	2
150	1
200	4

## Conclusion:

In an oral-exposure laboratory test with the honeybee, *Apis mellifera*, the oral 48 h median lethal dose (LD<sub>50</sub>) for daminozide was shown to be > 200 µg test item/bee.

## CONTACT TEST:

### Executive Summary

The purpose of this study was to determine the acute contact toxicity of daminozide to the honey bee (*A. mellifera*). Mortality of the bees was used as the toxic endpoint. The effects of five doses of test item (0, 1, 50, 100, 150 and 200 µg per bee) were compared to a control. Dimethylsulphoxide was used as the solvent. For the control, untreated dimethylsulphoxide was applied to the bees. To confirm the sensitivity of the test insects, the reference item Guthion (azinphos methyl) was also evaluated in a series of doses. Four replicate cages, each containing 10 bees (i.e. 40 bees in total), were prepared for each treatment. Following anaesthetisation, a 1.0 µL droplet of the test-item solution was applied to the surface of the bee ventral thorax. The treated bees were then returned to the test cages. The bees were assessed for mortality at 48 hours. In a contact-exposure laboratory test with the honeybee, *Apis mellifera*, the 48 h median lethal dose (LD<sub>50</sub>) for daminozide was shown to be > 200 µg/bee. The LD<sub>50</sub> of Guthion (azinphos methyl) used routinely as the toxic reference in this laboratory was approximately 0.1 µg/bee in the contact test.

## I. MATERIALS AND METHODS

### A. MATERIALS

1. **Test material:** Technical grade daminozide

**Description:** Not stated  
**Lot/Batch:** Not stated  
**Purity:** 99.4% w/w  
**Solvent:** Dimethylsulphoxide

**2. Reference material:**

**Description:** Guthion (azinphos-methyl)  
**Lot/Batch:** Not stated  
**Purity:** 99%

**B. STUDY DESIGN AND METHODS**

**1. Test animals:** Honeybee (*Apis mellifera* L.)  
**Age/life stage:** Worker bees (age not stated)  
**Source:** Frames of a healthy queen-right colony  
**Number:** 4 replicates with 10 bees each per treatment  
**Diet:** 20% w/v solution of sucrose in water

**2. Test design**

**Duration:** 48 hours  
**Test units:** Tinned wire-mesh cylinders (120 x 40 mm) closed at both ends with a cork, with a small glass feeding tube (orifice about 1.5 mm) inserted into the top cork  
**Concentrations:** 0, 1, 50, 100, 150 and 200 µg per bee  
**Volume:** 1.0 µL

**3. Environmental conditions:**

**Temperature:** 25°C  
**Relative humidity:** Not stated  
**Photoperiod:** Not stated

**4. Animal assignment and treatment:**

Worker honey bees were collected from frames (without brood) of a healthy queen-right colony. The bees were taken to the laboratory in a 2 gallon bucket with three layers of blotting paper at the bottom to absorb moisture. After anaesthetisation with the minimum amount of carbon dioxide, ten bees were transferred into each experimental cage. The bees were re-anaesthetised with carbon dioxide before applying the test compound. The anaesthetised bees were laid ventral surface up on filter paper in a Petri dish and 1.0 µl drops of the test compound dissolved in dimethylsulphoxide were placed on the ventral thorax using an Arnold micro-applicator. In the control treatment the bees were treated with 1.0 µl volumes of dimethylsulphoxide. At least five doses of the test material and toxic standard were given to four replicates of ten bees per treatment. The treated bees were returned to the cages and fed with 20% sucrose solution presented to the bees in glass tubes inserted through the top cork. After 48 hours, the number of dead bees was counted.

**5. Dose preparation:**

The contact tests were conducted using dimethylsulphoxide as the solvent (as the solubility of daminozide in acetone is low). Test item rates of 1, 50, 100, 150 and 200 µg per bee were studied. A control was carried out using dimethylsulphoxide.

**6. Measurements and observations:**

The number of dead bees was recorded after 48 hours.

**7. Statistics:**

Results obtained from the bees treated with the test and reference items were compared to those obtained from the control. The median lethal dose (LD<sub>50</sub>) was calculated using a Probit Analysis (GLIM, 1977 Royal Statistical Society, London).

**Results:****A. MORTALITY****Table B 9.3.1-2 Mortality in the contact test**

Treatment (µg a.s./bee)	No. of dead bees (total of 40)
Control	0
1	0
50	0
100	1
150	1
200	2

**Conclusion:**

In a contact-exposure laboratory test with the honeybee, *Apis mellifera*, the contact 48 h median lethal dose (LD<sub>50</sub>) for daminozide was shown to be > 200 µg test item/bee.

**RMS comments and conclusion:**

The reported study non-GLP and was conducted according to UK guideline (1992). It is also in line with the current OECD 213 and 214 guidelines, except for several deviations. According to the OECD 213, the amount of treated diet consumed per group should be monitored; once consumed (usually within 3-4 hours), the feeder should be removed from the cage and replaced with one containing sucrose solution alone. It is not clear from the study report if this procedure was followed. It is only stated there that when the bees had taken the 0.2 ml of the test material they were fed 20% sucrose. In addition, OECD 213 recommends use of 50% (w/v) sucrose solution as food while 20% (w/v) sucrose solution was used in the study. Further, the mortality was assessed 48 hours after

start of the test while OECD 213 and 214 recommend that mortality should be recorded also at 4 h and at 24 h. It is noted that

The test results of the study are in compliance with the OECD 213 and 214 guidelines' validity criteria (mortality in control less than 10%; LD<sub>50</sub> of toxic standard met the specified ranges).

The 48-hour oral and contact LD<sub>50</sub> is >200 µg daminozide/bee.

### B.9.3.1.2 Chronic toxicity to bees

#### i) Chronic oral toxicity laboratory test

<b>Reference:</b>	<b>Haupt, S. (2014)</b> Chronic oral toxicity of daminozide technical on the honey bee ( <i>Apis mellifera</i> L.) in the laboratory
Report No.:	87715136
Guideline:	OECD 213 (1998); CEB 230 (2014) with modifications and current recommendations of the ring test group (2014)
GLP:	Yes
<b>Previous evaluation:</b>	Submitted for the purpose of renewal

#### Executive Summary

Freshly emerged adult female worker bees were exposed to daminozide for a period of 10 days under laboratory conditions, at a concentration of 3333 mg a.s./kg food. In addition, a control group (pure diet) and one dose level of the toxic reference (Dimethoate) were tested.

The results obtained with the toxic reference substance confirmed the sensitivity of the test system under the conditions of the test and mortality on the control groups was acceptable (100%).

The LD<sub>50</sub> for the test item was determined to be higher than 106.2 µg a.s./bee/day and LC<sub>50</sub> for the test item was higher than 3333 mg a.s./kg food. The NOED was estimated to be 106.2 µg a.s./bee/day and the NOEC was 3333 mg a.s./kg food.

#### Material and methods:

##### A. MATERIALS

- Test material:** Daminozide Technical  
**Batch no.:** 101218026  
**Purity:** 99.8% w/w (analysed)  
**Description:** White solid
- Reference item:** Perfekthion EC  
**Purity:** 400.9 g dimethoate/L (analysed)  
**Description:** Blue liquid

**B. STUDY DESIGN AND METHODS****1. Test organism:** *Apis mellifera* L. *carnica***Stage and sex:** Freshly emerged adult female worker bees**Source:** Disease-free and queen-right honey bee colonies, bred by IBACON**Diet:** 50% (w/v) sucrose solution

**2. Treatment:** 0 (diet), 3333 mg daminozide/kg food (which equals a target dose of 100 µg daminozide/bee/day) and 1 mg dimethoate/kg food (which corresponds to 0.02 µg dimethoate/bee/day).

**3. Test units:** Stainless steel cages (10 x 8.5 x 5.5 cm) with a removable glass sheet at the front side.

**4. Environmental conditions:****Temperature:** 33-34°C**Relative humidity:** 72% (34-83%)**Photoperiod:** Darkness (except during observation)**5. Test organism assignment and treatment:**

A brood comb with sealed brood from one hive was taken from the hive and adult bees were swept out. Then the comb was placed in an excluder box and placed back into the hive. The freshly hatched bees remained in the excluder box. After one day the bees were transferred to the test units. The test included 3 different treatment groups: a control (diet only), one concentration of the test item at 3333 mg daminozide/kg food and one concentration of the reference item at 1 mg dimethoate/kg food. Each treatment group consisted of 5 replicates, with 10 individuals per replicate. The untreated and treated food was offered *ad libitum* in syringes. These syringes were weighed daily before introduction into the cages and after the feeding interval (before replacement with fresh food).

**6. Dose preparation:**

The test item was dissolved in 50% sucrose solution (treated feeding solution). The reference item was dissolved in water before addition to the sucrose solution. All the solutions (including the control solution) were prepared directly before the start of the experiment and were stored tightly closed at 4°C in the dark.

**7. Measurements and observations:**

Mortality and behavioural abnormalities (moribund, affected, cramps, apathy and vomiting) were assessed daily. In addition, the food consumption was calculated by the number of surviving bees and the amount of food taken up.

**8. Statistics:**

Mortality data of the test item was compared to the control, using Fisher's exact test, for the determination of the No Observed Effect Concentration (NOEC). The statistics were performed using the ToxRat Professional, Version 2.10.05 (ToxRat Solutions GmbH).

## Results:

### A. Mortality and observations:

Mean mortality in the control after 10 days was 2.0% and in the reference item group the mortality was 100% after 10 days. All bees exposed to the test item for 10 days survived. No behavioural abnormalities were observed for the bees exposed to the test item.

The mean food consumption in the group exposed to the test item was 106.2 µg daminozide/bee/day. For the bees exposed to the reference item, the food uptake was 0.027 µg dimethoate/bee/day.

The results for food consumption, mortality and behavioural abnormalities are shown in the table below.

**Table B 9.3.1-3 Food consumption, mortality and behavioural abnormalities of *Apis mellifera* exposed to daminozide, the control or the reference item for 10 days**

Treatment	Nominal concentration (µg a.s./bee/day)	Nominal dosage (µg a.s./bee/day)	Measured dosage (µg a.s./bee/day) <sup>2</sup>	Mortality (%) <sup>3</sup>	Behavioural abnormalities (%)
Control	0.0	0.0	0.0	2.0	0.0
Daminozide	3333	100	106.2	0.0	0.0
Reference item	1.0	0.02	0.027	100	5.6 <sup>1</sup>

<sup>1</sup> Mean % of bees with behavioural abnormalities over the 10 day exposure period

<sup>2</sup> Dose measured based on consumed feeding solution

<sup>3</sup> Mortality at study termination 10 days after start of first feeding

### B. Toxicity endpoints:

The 10-day toxicity endpoints are presented in the table below.

**Table B 9.3.1-4 Toxicity endpoints for *Apis mellifera* exposed to daminozide after 10 days based on nominal test item concentration and measured test item dosage**

	Toxicity endpoint
LC <sub>50</sub>	>3333 mg a.s./kg food
LD <sub>50</sub>	>106.2 µg a.s./bee/day
NOEC	3333 mg a.s./kg food
NOED	106.2 µg a.s./bee/day

**Conclusion:**

Exposure of *Apis mellifera* to daminozide for 10 days did not result in mortality at the tested concentration. The  $LC_{50}$  for the test item was  $>3333$  mg a.s./kg food and the  $LD_{50}$  was estimated to be  $>106.2$   $\mu$ g a.s./bee/day. The NOEC and NOED values were determined to be 3333 mg a.s./kg food and 106.2  $\mu$ g a.s./bee/day, respectively.

**RMS comments and conclusion:**

The reported study is GLP compliant. No agreed study protocol for bee chronic toxicity testing is available recently. The reported study was carried out according to OECD 213 and CEB 230. It is in line with the current OECD 245 guideline (2017). The test results are in compliance with the current guideline's validity criteria (mortality in control less than 15%; mortality in the reference substance more than 50% at the end of the test). The study is acceptable for regulatory use.

The 10-day  $LC_{50}$  is  $>3333$  mg daminozide/kg (nominal) and 10-day contact  $LDD_{50}$  is 106.2  $\mu$ g daminozide/bee/day.

**B.9.3.1.3 Effects on honeybee development and other honeybee life stages****i) Larval toxicity laboratory test**

<b>Reference:</b>	<b>Odemer, R. (2015)</b> Daminozide: Toxicity to Honey Bee ( <i>Apis mellifera</i> L.) Larvae after Repeated Exposure under In Vitro Laboratory Conditions
Report No.:	20150038
Guideline:	OECD Draft Test Guideline on Honey bee ( <i>Apis mellifera</i> ) Larval Toxicity Test Repeated Exposure (2014)
GLP:	Yes
<b>Previous evaluation:</b>	Submitted for the purpose of renewal

**Executive Summary**

The toxicity of daminozide to honey bee *Apis mellifera* larvae was determined in a repeated feeding study in the laboratory. First instar larvae were fed on days 3, 4, 5 and 6 with diet containing daminozide at the nominal concentration of 714.6 mg a.s./L food (equivalent to a dose of 100  $\mu$ g a.s./larva), from a repeated exposure. A negative control (feeding solution only) and the reference item dimethoate (at 6.2  $\mu$ g a.s. per larva corresponding to 0.044 g a.s./L diet or approximately 40 mg a.s./kg diet) were tested in parallel. Three replicates for the control and reference item, respectively and five replicates for the test item treatment were set up; each consisted of 16 larvae from three different colonies. Larval mortality was assessed after 4, 5, 6 and 8 days, i.e., at 24, 48, 72 and 120 h after the first administration of the test item treatment, respectively. Assessment of not consumed food was carried out on day 8, pupal development on day 15 and adult emergence on day 22 (test termination).

Chemical analysis demonstrated that the mean recovery for daminozide in the application solution on day 3 and day 6 was 101.3% and 103.8% of the nominal concentrations, respectively.



The mortality of the bee larvae in the control was determined to be 2.2% and the corrected mortality at the concentration of daminozide of 100 µg a.s./larva was 4.1% after 8 days of exposure. Hence, the 8-day no-observed-effect dose (NOED) was determined to be > 100 µg a.s./larva, whereas the lowest-observed-effect-dose (LOED) was determined to be ≥ 100 µg a.s./larva. The 8-day LD<sub>50</sub> value for *Apis mellifera* larvae exposed to daminozide was calculated to be > 100 µg a.s./larva, which corresponds to a 8-day LC<sub>50</sub> value of > 714.6 mg a.s./L diet.

At day 22, the adult emergence in the control was determined to be 70.2%, and 55.0% at the concentration of daminozide of 100 µg a.s./larva, and therefore no statistically significant difference could be observed when compared to the control. The no-observed-effect dose (NOED) was determined to be > 100 µg a.s./larva, whereas the lowest-observed-effect-dose (LOED) was determined to be ≥ 100 µg a.s./larva. The 22-day LD<sub>50</sub> was calculated to be > 100 µg a.s./larva, which corresponds to a 22-day LC<sub>50</sub> to > 714.6 mg a.s./L diet.

The mortality and the adult emergence in the reference item treatment was 100% and 0%, respectively. No behavioural abnormalities were recorded in the control or in the test item treatment throughout the study.

#### Material and methods:

##### A. MATERIALS

1. **Test material:** Daminozide  
**Batch number:** 101218026  
**Purity:** 99.8% (analysed)  
**Description:** White powder
2. **Test concentrations:** 100.0 µg a.s. per larva corresponding to 714.6 mg a.s./L diet
3. **Reference item:** Dimethoate (6.2 µg a.s. per larva corresponding to 0.044 g a.s./L diet or approximately 40 mg a.s./kg diet) diluted with acetone.
4. **Vehicle:** Aqueous sugar and yeast solution mixed with fresh royal jelly
5. **Test organism:**
  - Species:** Honey bee *Apis mellifera* L.
  - Age/life stage:** First instar larvae (L1)
  - Source:** Three healthy, disease-free and queen-right colonies, obtained from IES Ltd, Witterswil, Switzerland
  - Diet:** Daily feeding, except on day 2. Three different artificial diets (A, B and C), adapted to the needs of each larval stage, were prepared during the test.
    - Diet A (D1): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 2% weight of yeast extract, 12% weight of glucose and 12% weight of fructose.

- Diet B (D3): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 3% weight of yeast extract, 15% weight of glucose and 15% weight of fructose.
- Diet C (D4 to D6): 50% weight of fresh royal jelly + 50% weight of an aqueous solution containing 4% weight of yeast extract, 18% weight of glucose and 18% weight of fructose.

**Test units:** Crystal polystyrene grafting cells with a diameter of 9 mm and a depth of 8 mm, which were sterilised by immersion in Milton® sterilizing fluid (1% v/v, a.s. sodium hypochlorite 2% w/w) and then dried. Hereafter, the cells were placed into a 48-well plate, which was previously half-filled with a piece of dental roll wetted with 15% (w/v) glycerol. The plates were placed into a hermetically sealed Plexiglass desiccator, which was placed into a climate cabinet.

## **B. STUDY DESIGN**

### **1. Environmental conditions:**

**Temperature:** 34.6, 35.2 and 33.2°C for larval, pupal and adult emergence conditions, respectively

**Relative humidity:** 95.0, 86.8 and 52.5% for the larval, pupal and adult emergence conditions, respectively

**Photoperiod:** Continuous darkness

### **2. Assignment and treatment:**

First instar (L1) honey bee larvae were grafted from combs from three different colonies. Three replicates for the control and reference item, and five replicates for the test item limit dose were established from three different colonies consisting of sixteen larvae each. All larvae from the same treatment were placed in one well, i.e., four wells consisting of 48 larvae each in total. At day one the larvae were fed 20 µL of untreated diet A pipetted into each prepared grafting cell. At day three the larvae were fed with a defined quantity of diet B on a warming plate, which contained the nominal daminozide concentration of 714.6 mg a.s./L diet (nominal; equivalent to a cumulative dose of 100 µg a.s./larva). The treatment continued with administration of Diet C on day four, five and six. After the final larval mortality assessment on day 8, pupal mortality on day 15 and the adult emergence on day 22 were assessed.

A control (diet only), and a reference product (dimethoate; at 40 mg dimethoate/kg diet (nominal), equivalent to a cumulative dose of 6.2 µg a.s./larva) were concurrently tested.

### **3. Dose preparation:**

On day 3, the larval food was freshly prepared by adding accordingly 200 µL of the test item or control solution to 1980 mg of Diet B (corresponding to 1800 µL) to a final volume of 2000 µL. On day 4, the larval food was freshly prepared, and used for day 5 and 6, with 1170 µL of the test item or control solution added to 11583 mg of Diet C (corresponding to 10530 µL) to a final volume of 11700 µL.

### **4. Measurements and observations:**

Mortality was assessed at the time of feeding from day four to six and at test termination on day eight, which occurred at 24, 48, 72 and 120 h after the first feeding with treated diet. On day eight, the presence of uneaten food was also qualitatively assessed. Pupal mortality on day 15 and emerged adults on day 22 were counted.

Duplicate samples were taken from all freshly prepared and well dispersed application solutions on day 3 and 6 and analysed for the concentration of the test item using HPLC coupled to an UV/VIS detector.

Temperature and humidity were recorded continuously.

## 5. Statistics:

Mortality data were corrected according to Abbott (1925). Fisher's exact Binomial test with ( $\alpha = 0.05$ ) was used to assess whether there was a significant difference in cumulative larval mortality on day 8 and adult emergence on day 22 between the treated group and the control group. The LOED, NOED and LD<sub>50</sub> values with 95 % were determined manually due to the low toxicity of the test item. All statistics were performed with ToxRat Professional 2.10.

## Results:

### A. MORTALITY AND FOOD UPTAKE

At day 8 mortality of honey bee larvae was determined to be 2.2% in the control. The corrected mortality for the test item limit dose, i.e. 100 µg a.s./larva was 4.1% and was not significantly different when compared to the control. The mortality in the reference item treatment was 100%. The mean number of cells with uneaten food was 0.0% and 2.6% in the control and test item limit dose treatments respectively. The table below shows the larval mortality and food rejection at day eight.

**Table B 9.3.1-5 Mortality and food rejection of *Apis mellifera* larvae exposed to daminozide on day 8**

Treatment	Dose (µg a.s./larva)	Mean larval mortality (%)	Number of cells with uneaten food (%)
Control	0.0	2.2	0
Daminozide	100	4.1 <sup>1</sup>	2.6
Reference item Dimethoate	6.2	100 <sup>1</sup>	n.a.

<sup>1</sup> Corrected mortality compared to the control group, according to Abbott (1925)

n.a. = not applicable since all larvae died

At day 15, mortality of honey bee pupae was determined to be 15.3% in the control. The corrected mortality at 100.0 µg daminozide/larva was determined to be 20.3%. No statistical analysis was performed.

The table below shows the pupal mortality at day 15.

**Table B 9.3.1-6 Mortality of *Apis mellifera* pupae exposed to daminozide on day 15**

Treatment	Dose( $\mu$ g a.s./larva)	Mean pupal mortality (%)	Mean pupal mortality corrected (%) <sup>1</sup>	Pupation rate (%)
Control	0.0	15.3	-	84.7
Daminozide	100	32.5	20.3	67.5
Reference item Dimethoate	6.2	n.a.	-	0.0

<sup>1</sup> Corrected mortality compared to the control group, according to Abbott (1925)

n.a. = not applicable since all larvae died on day 8

At day 22, the emergence rate of honey bee adults was determined to be 70.2% in the control. The adult emergence at 100.0  $\mu$ g daminozide/larva was determined to be 55.0% and was not significantly different when compared to the control.

The table below shows the adult emergence at day 22.

**Table B 9.3.1-7 Emergence rate of *Apis mellifera* adults exposed to daminozide on day 22**

Treatment	Dose ( $\mu$ g a.s./larva)	Introduced larvae in individual replicates (D1)	Hatched adults in individual replicates (D22)	Total hatched adults (D22)	Mean adult mortality (%)	Mean adult mortality corrected (%) <sup>1</sup>	Adult emergence rate (%)
Control	0.0	16 15 16	11 11 11	33	29.8	-	70.2
Daminozide	100	16 16 16 16 16	9 9 9 9 9	44	45.0	21.7	55.0
Reference item Dimethoate	6.2	16 16 16	0 0 0	0	100	100	0.0

<sup>1</sup> Corrected mortality compared to the control group, according to Abbott (1925)

No behavioural abnormalities were recorded in the control or the test item treatment throughout the study.

The mean recovery for daminozide in the application solution on day 3 and day 6 was determined to be 101.3% and 103.8% of the nominal concentrations, respectively.

## B. DEFICIENCIES

None.

## VALIDITY CRITERIA OF THE STUDY

For judging the acceptance and quality of data obtained with the repeated exposure test, the following performance criteria apply:

- In the control, cumulative larval mortality from D3 to D8 should be 15 % across replicates;
- In the control, the adult emergence rate should be 70 % on D22;
- In the Dimethoate positive control, larval mortality should be 50 % on D8.

### Conclusion:

Based on larval mortality on day 8, the No Observed Effect Dose (NOED) for daminozide was determined to be  $> 100 \mu\text{g a.s./larva}$ , whereas the Lowest Observed Effect Dose (LOED) was determined to be  $\geq 100 \mu\text{g a.s./larva}$ . The  $\text{LD}_{50}$  for daminozide was calculated to be  $> 100 \mu\text{g a.s./larva}$  (corresponding to  $714.6 \text{ mg a.s./L diet}$ ).

Based on adult mortality on day 22, the overall No Observed Effect Dose (NOED) for daminozide was determined to be  $> 100 \mu\text{g a.s./larva}$ , whereas the overall Lowest Observed Effect Dose (LOED) was determined to be  $\geq 100 \mu\text{g a.s./larva}$ . The  $\text{LD}_{50}$  for daminozide was calculated to be  $> 100 \mu\text{g a.s./larva}$  (corresponding to  $714.6 \text{ mg a.s./L diet}$ ).

### RMS comments and conclusion:

The reported study is GLP compliant and was conducted according to OECD Draft Test Guideline on Honey bee (*Apis mellifera*) Larval Toxicity Test Repeated Exposure (2014). It is in line with the current OECD 239 Guidance Document on Honey bee Larval Toxicity Test following Repeated Exposure (2016). The test results are in compliance with the current guidance document's validity criteria. The study is acceptable for regulatory use. The 22-day No Observed Effect Dose (NOED) is  $100 \mu\text{g a.s./larva}$  (corresponding to  $714.6 \text{ mg a.s./L diet}$ ).

For a risk assessment of daminozide to bees, see Vol. 3 CP B.9 for the formulated product.

## B.9.3.2 Effects on non-target arthropods other than bees

### i) Effects on *Typhlodromus pyri* in laboratory test

Reference:	Harwood, R. W. J. (2000) A laboratory evaluation of the side effects of daminozide on the predatory mite <i>Typhlodromus pyri</i> .
Report No.:	18099
Guideline:	IOBC/WPRS Overmeer (1988), improvements by Louis & Uffer (1995)
GLP:	No
Previous evaluation:	In Addendum 1 (2002)
<b>Material and methods:</b>	
Test material:	Daminozide

Lot/Batch No: SW991  
Purity: Not stated

A laboratory test to determine the effects of Daminozide on the predatory mite, *Typhlodromus pyri* was performed. Test was based on methods of Overmeer (1988) and Louis and Ufer (1995). Daminozide was applied to glass test arenas (consisting of 2 microscope cover glasses, approx. 24 mm x 50 mm, laid parallel to each other with the cover slips glued together) at 7.225 kg a.s./ha. The test arena bases consisted of vinyl tile (10 cm x10 cm) on a piece of foam rubber (10 cm x10 cm x 5 cm). The sprayer was calibrated prior to application and treatments were applied to the upper surface of the test arenas and to both sides of the glass cover slips used for shelters. For each treatment twenty, 2- 3 days old mites were placed in each test arena and 5 replicates were used. A small amount of pollen (*Pinus nigra*) was provided for food. Mortality assessments were performed at ca. 24 h after application and again at 7 days after application. Surviving females were transferred to unused test arenas 7 days after application with males to give a sex ratio approx. one male to 3 females. After 7 days of transfer (i.e. 14 days after application) an assessment of egg production was carried out. A further assessment of egg laid and newly hatched nymphs was made 21 days after application as the number of eggs laid in each test arena at the first assessment were low. A control with water and a toxic reference treatment with Dimethoate (40 EC 0.17 L/ha) were used.

## Results

Mortality in the water control was 1% after 24 h. In the Daminozide and in the reference toxicant treatment, mean corrected mortality using Abbott's formula was 2% and 24%, respectively after 24 h. After 7 days of application mortality increased to 13% in the control and 100% in the reference toxicant. In the Daminozide control corrected mortality was 87.4%.

For the fecundity assessments, 38 live females were found in the water control and 7 live females in the Daminozide treatments after 7 days of application. After 14 days of applications 6 eggs were found in the water control and 0 eggs were found in the Daminozide treated arena. In the further assessment of 21 days, 75 eggs and newly hatched larvae were found in the control and 3 eggs in the Daminozide treated arena. The number of eggs (including hatched eggs) per female in water was 1.97 and 0.43 in the control and in Daminozide treatment, respectively. The effect of fecundity compared to control was -78%. The author states that the effect of Daminozide 85 may have due to the sticky nature of the formulation rather than to direct toxic effects caused by contact exposure.

The results of mortality and fecundity assessment are summarized in the table below.

**Table B 9.3.2-1 Mortality and fecundity results**

Treatment	Mortality after 7 days		Fecundity		
	Mortality	Corrected mortality	Total females transferred	Total eggs laid	No. off eggs per female
Control	13%	-	38	75	1.97

Daminozide, 7.2225 kg a.s./ha	89%	87.4%	7	3	0.43
Dimethoate 40	100%	-	-	-	-

Remark from previous review: With the mean number of 1.97 eggs per female in the control, the validity criteria for fecundity (i.e. 4 eggs per female) was not met. Therefore the results are not used for risk assessment.

#### **RMS comments and conclusion:**

The non-GLP study was conducted according to the IOBC/WPRS guideline Overmeer (1988). It is also in line with the current guideline Blümel *et al.* (2000).

The study results meet the guidelines' validity criteria (mortality in the control treatment over the initial 7 days should not exceed 20%: actual value was 3%; corrected mortality in the toxic reference treatment should be 50-100%: actual value was 100% at 7 DAT), except for the mean cumulative number of eggs produced between 7 and 14 days (should be greater than 4.0 per female in the control treatment: actual number of eggs per female was 1.97).

Therefore, the study is not considered valid.

For the other study summaries and risk assessment of daminozide to non-target arthropods, see Vol. 3 CP B.9 for the formulated product.

#### **B.9.4 Effects on non-target soil meso- and macrofauna**

##### **B.9.4.1 Earthworms – sub-lethal effects**

##### **B.9.4.1.1 Earthworms – sub-lethal effects of active substance**

<b>Reference:</b>	<b>Pavić, B. (2014)</b> Effects of daminozide technical on reproduction and growth of earthworms <i>Eisenia fetida</i> in artificial soil with 10% peat
Report No.:	87714022
Guideline:	OECD 222 (2004); ISO 11268-2 (2012)
GLP:	Yes
<b>Previous evaluation:</b>	Submitted for the purpose of renewal

#### **Executive Summary**

The purpose of this study was to determine the sublethal effects of daminozide technical on mortality, weight change and reproduction of the earthworm *Eisenia fetida* over a 56 day exposure. The earthworms were exposed to the test item incorporated into the soil (10% peat), at concentrations of 61.73, 111.1, 200, 360 and 648 mg a.s./kg soil dry weight. Per test item treatment, four replicates were tested, containing ten earthworms each. An untreated control with eight replicates was included and tested in parallel.

After four weeks, the adult worms were removed from the test vessels. No mortality and changes in the behaviour of adult earthworms were observed in the control and for all test item treatment groups. The earthworm weight change increased in all test groups without a statistically significant reduction compared to the control. The test

was then continued without the adult earthworms, for assessment of the reproductive output. After a further four weeks the reproduction rate (average number of juveniles) was 205 in the control and ranged between 164 and 245 for the test groups. The reproduction rate of all test item treatment groups did not statistically differ compared to the control.

The NOEC of daminozide concerning mortality, weight change and reproduction of *Eisenia fetida* earthworms was determined to be at the highest tested rate of 648 mg a.s./kg soil dry weight. The LOEC is > 648 mg a.s./kg soil dry weight.

#### Material and methods:

##### A. MATERIALS

1. **Test material:** Daminozide technical  
**Batch:** 101218026  
**Purity:** 99.8% (analysed)  
**Description:** White solid
2. **Reference material:** Luxan Carbendazim 500 FC  
(Tested in a separate study)
3. **Vehicle:** Demineralised water

##### B. STUDY DESIGN AND METHODS

1. **Test substrate:** Artificial soil: 10% peat, 20% kaolin clay, 69.6% quartz sand and 0.4% calcium carbonate  
**Test units:** Plastic boxes (18.3 x 13.6 x 6 cm, bottom surface area of 189.8 cm<sup>2</sup>) containing 500 g dry weight soil. Transparent perforated lids were placed on top.
2. **Test organisms:**  
**Species:** Earthworm, *Eisenia fetida* (Savigny 1826)  
**Age:** Adult worms, with well-developed clitellum (approximately 10 months old at test initiation)  
**Source:** In-house culture  
**Weight:** 307 to 595 mg at test initiation  
**Acclimation:** One day, under test conditions  
**Diet:** Finely ground cattle manure. Adults were fed weekly and offspring were only fed at the start (after removal of the adults)
3. **Treatment groups:** 61.73, 111.1, 200, 360 and 648 mg a.s./kg soil dry weight



**4. Environmental conditions:**

**Temperature:** 18 - 22°C

**Soil pH:** 5.7 - 6.1

**Soil water content:** Test start: 31.7% (corresponding to 57.6% of the maximum water holding capacity (MWHC), test end: 33.2% (which equals 60.3% of MWHC)

**Photoperiod:** 16 hours light: 8 hours darkness (400 ± 800 lux)

**5. Animal assignment and treatment:**

The earthworms were washed with tap water, dried, weighed individually and then randomly assigned to the test containers. For each test item treatment group four test containers (replicates) were prepared and the control consisted of eight replicates. Each replicate contained ten earthworms. The test comprised five test item treatments and an untreated control. After 28 days adult earthworms were removed and the remaining cocoons were incubated for a further 28 days.

**6. Dose preparation:**

First a stock solution was prepared by adding daminozide technical to deionised water. This suspension was stirred using a magnetic stirrer and thereafter treated for 1 minute in an ultrasonic bath to obtain a homogenous dispersion. Different amounts of the stock solution were then added to dry artificial soil to prepare the target nominal concentrations of the test item in the soil. While mixing the stock solution through the soil, in a laboratory mixer, the soil was moistened with deionised water to obtain soil moisture of 57-58% of the maximum water holding capacity.

**7. Measurements and observations:**

Mortality and behavioural abnormalities were recorded for the adult earthworms, 28 days after application. Missing earthworms and earthworms that failed to respond to gentle stimulation were assumed to be dead. Adults were removed and the individual weight of the earthworms was recorded. After the assessment the artificial soil, containing the produced offspring, was returned to the test containers.

After 56 days the test was terminated and the number of offspring was determined. The extraction of juvenile worms from the soil substrate was performed by placing the test containers in a water bath at 50 to 60°C, as well as checking the soil (emptied out on a tray) visually for any remaining earthworms.

Soil water content was determined once a week. pH was measured at test start and test end.

**8. Statistics:**

Body weight change and reproduction data were tested for normal distribution and homogeneity of variance ( $\alpha=0.05$ ) using the Shapiro-Wilk test and the Levene's test, respectively. The Williams' test was used to compare the test item treatments to the control. ToxRat Professional, Version 2.10.05 (ToxRat Solutions GmbH) was used to perform the statistical analysis.

**Results:****A. Mortality / behaviour:**

After 28 days, no mortality of adult earthworms was observed in any of the control or test item treatment groups (see Table 8.4.1/01-1).

No behavioural abnormalities were observed for the control and the test item treatment groups.

**B. Body weight:**

The body weights of the adult earthworms increased in the control and at in the test item treatment groups throughout the 28 days without any statistical differences compared to the control (see Table 8.4.1/01-1).

**C. Reproduction capacity:**

For the control group, the mean number of juveniles ranged between 125 and 241, and the coefficient of variation of reproduction was 18.5%. The average number of juveniles in the treated groups was between 164 and 245 (Table 8.4.1/01-1). The reproduction of the earthworms in all test item concentrations did not statistically significantly differ compared to the control.

A summary of the results is presented in the table below.

**Table B 9.4.1-1 Summary of mortality, body weight change and reproduction of *Eisenia fetida* exposed to daminozide technical**

Endpoint	Test concentration (mg a.s./kg soil dw)					
	Control	61.73	111.1	200	360	648
Mortality (%)	0.0	0.0	0.0	0.0	0.0	0.0
Mean weight change (%)	31.4 ± 4.3	28.8 ± 6.3	38.6 ± 2.8	37.6 ± 2.6	33.9 ± 5.6	36.3 ± 1.4
Juveniles/replicate (mean)	205 ± 38	213 ± 29	206 ± 23	183 ± 21	216 ± 36	199 ± 18
Reproduction as % of control	-	103.7	100.7	89.3	105.4	97.1

The reference item Luxan Carbendazim 500 FC was tested in a separate study, at concentrations of 0.57, 0.87, 1.30, 1.96 and 2.91 mg carbendazim/kg soil dry weight. The most sensitive endpoint was reproduction, with a NOEC of 0.87 mg a.s./kg soil d.w. and EC<sub>50</sub> value of 1.32 mg a.s./kg soil d.w. These results are as expected and in the range of the historical data.

**D. Toxicity endpoints:**

No statistically significant effects of daminozide technical on mortality, weight change and reproduction were observed. The toxicity endpoints are presented in the table below.

**Table B 9.4.1-2 Toxicity endpoints for *Eisenia fetida* exposed to daminozide technical**

Endpoint	Endpoints (mg a.s./kg soil dw)
NOEC (mortality, weight change, reproduction)	648
LOEC (mortality, weight change, reproduction)	>648
EC <sub>50</sub> (reproduction)	>648

**Validity Criteria of the Study**

- Control Mortality: Should not exceed 10% over initial 4-week test period (actual value was 0%).
- Reproduction of Control: Should be  $\geq 30$  worms per replicate container ((actual value were 125 – 241).
- Coefficient of Variation of Reproduction in Control: Should not exceed 30% (actual value was 18.5%).

All the validity criteria were met.

**Conclusion:**

The NOEC and LOEC values for mortality, weight change and reproduction for *Eisenia fetida* earthworms was determined to be 648 and higher than 648 mg daminozide/kg soil dry weight, respectively.

**RMS comments and conclusion:**

The reported study is GLP compliant and was conducted according to OECD 222 guideline (2004) without significant deviations. The test results are in compliance with the guideline's validity criteria. The study is acceptable for regulatory use.

The 56-day no-observed-effect concentration (NOEC) is 648 mg daminozide /kg dry weight soil.

**B.9.4.1.2 Earthworms – sub-lethal effects of metabolites**

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For a risk assessment of daminozide to earthworms, see Vol. 3 CP B.9 for the formulated product.

**B.9.4.2 Effects on non-target soil meso- and macrofauna (other than earthworms)**

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**B.9.5 Effects on soil nitrogen transformation**

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**B.9.6 Effects on terrestrial non-target higher plants****B.9.6.1 Summary of screening data**

-

**B.9.6.2 Testing on non-target plants**

For study summaries and risk assessment of daminozide to non-target plants, see Vol. 3 CP B.9 for the formulated product.

**B.9.7 Effects on other non-target organisms (flora and fauna)**

i)

<b>Reference:</b>	<b>Lengen (2001)</b> Review of the effects of daminozide on non-target fauna and flora
Report No.:	Report Uniroyal Chemical Co., Inc., Middlebury, CT, U.S.A., December 2001
Guideline:	Not applicable
GLP:	No
<b>Previous evaluation:</b>	In DAR (1999)

A literature review on the effects of daminozide on non-target fauna and flora has been provided. A preliminary screening on the effects on insects and fungi and information on the effects on mammalian species, insects, plants and micro-organisms are available.

From the preliminary screening, daminozide did not show any significant effects on insects (mosquito larvae) and fungi (tomato blight and bean rust).

From the study on mammalian species, daminozide showed low toxicity to small mammals ( $LC_{50} > 4500$  ppm).

From the study on non-target insects, daminozide showed no effects on the foraging habits of bees. Daminozide was also found not be toxic to predatory mites with  $LC_{50}$  values of 350 mg/L (*Neoseiulus fallacis*), >1011 mg/L (*Amyseius fallacis*) and 6306 mg/L (*Tetranychus urticae* Koch).

From the study on non-target plants, daminozide did not affect the growth of the algal species *Chlorella sorokiniana* at concentrations up to 16 mg/L. Daminozide increased heterocyst production in blue green algae but inhibitory effects at high concentrations were reversible. In studies with over 80 species of wild seeds exposed to up to 1000 mg/L daminozide, germination was affected in only two species. Some effects were noted in spruce seedlings grown directly in nutrient media containing daminozide. Since daminozide is used on monocultures of ornamentals, these effects were not considered to have any practical relevance.

From the study on micro-organisms, daminozide at a concentration of 500 ppm did not have a strong effect on microbial activity.

**RMS comments and conclusion:**

The reported study is a literature review. The study focused on biological activity of daminozide and on effects of daminozide on non-target fauna and flora. It is not a standard regulatory study. The results cannot be used in the regulatory assessment.

**B.9.8 Effects on biological methods for sewage treatment**

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**B.9.9 Monitoring data**

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**B.9.10 Biological activity of metabolites potentially occurring in groundwater**

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**B.9.11 References relied on****Literature search**

A literature search was carried out by the notifier in accordance with Article 7, Paragraph 1(m) of Commission Implementing Regulation (EU) No. 844/2012.

The search was performed for active substance daminozide and daminozide metabolites and relevant impurities:

- Daminozide (common name)
- 1596-84-5 (CAS Number)
- UDMH (unsymmetrical dimethyl hydrazine)
- 57-14-7 (CAS Number)
- NDMA (N,N-dimethylnitrosamine)
- 62-75-9 (CAS Number)
- Methanol
- 67-56-1 (CAS number)
- 200-659-6 (EC number)

A series of searches were carried out using the STN and the Dialog platforms:

- an initial search covering studies published from 2004 to 2011;
- additional searches to cover the time periods 2012 to 2013, 2013 to 2014 and February to December 2014;
- a separate search for all in vitro/in vivo metabolism studies excluding hits preceding 2004 (to address the requirement under Regulation (EU) No. 283/2013 for comparative in vitro metabolism studies);
- a separate search covering the metabolite methanol from 2005 to 2015.

These literature searches were performed to cover the 10 years prior to the expected submission of the AIR 3 dossier for daminozide which was submitted for review in April 2015.

**Table B.9.9-1 Summary of the literature review**

<b>Summary of the review</b>	<b>n</b>
Total number of summary records retrieved after removing duplicates from all database searches	2134
Number of summary records excluded after rapid assessment for relevance (by title/abstract)	2074
Number of summary records of potential/unclear relevance assessed in further detail (by abstract/full-text)	60
Number of studies excluded from further consideration after detailed assessment for relevance (by abstract/full-text)	48

Number of studies not excluded for relevance after detailed assessment (i.e. relevant studies and studies of unclear relevance)	12
Number of relevant and reliable studies (Klimisch criteria 1-2) identified by the literature search and appraisal process	8
Number of studies considered relevant and reliable relate to ecotox	1

As part of the determination of relevancy, the following criteria are considered to be fundamental when considering the relevance of an open-literature study:

- Studies should address the data requirements detailed in Commission Regulations (EU) No. 283/2013 and 284/2013.
- Studies need to be performed with defined test material which is the appropriate active substance, metabolite or plant protection product.
- The test species in laboratory studies should be relevant to the EU i.e. studies which contain test species which are not found in the EU are not considered to be relevant.
- Both the route of exposure and length of exposure of the test material should be appropriate. Studies which have exposure which is either too long or too short or via an inappropriate route are not considered to be relevant. Studies with in vivo or ex vivo exposure are considered relevant; in vitro tests may potentially be relevant and should be considered appropriately.
- Toxicity modelling (e.g. QSAR), literature review papers, meta-analysis papers, risk analysis papers and environmental monitoring papers are generally considered to not be relevant.
- Apart from mixture toxicity, other multi-stressor studies e.g. active substance and physico-chemical stress, are not considered to be relevant.
- Field studies should be performed to conditions which are relevant to the EU e.g. climate, crop species, test species.

The criteria considered for relevancy of studies relating to individual ecotoxicology data requirements are detailed in the table below:

Table B.9.9-2 Relevancy criteria considered

Data requirement (data point)	Relevancy criteria considered
<b>Active substance</b>	
Effects on birds and other terrestrial vertebrates (KCA 8.1)	<ol style="list-style-type: none"> <li>1. Study is appropriate to data requirements detailed in Regulations 283/2013 and 284/2013.</li> <li>2. Well-defined test material applied as the appropriate active substance, metabolite or plant protection product.</li> <li>3. Test species is relevant to the EU. Studies on mammals are covered by the toxicology section except for non-target vertebrate species.</li> <li>4. Route and length of exposure should be appropriate.</li> </ol>
Effects on aquatic organisms (KCA 8.2)	<ol style="list-style-type: none"> <li>1. Study is appropriate to data requirements detailed in Regulations 283/2013 and 284/2013.</li> <li>2. Well-defined test material applied as the appropriate active substance, metabolite or plant protection product.</li> <li>3. Test species is relevant to the EU.</li> <li>4. Route and length of exposure should be appropriate.</li> </ol>
Effect on arthropods (KCA 8.3)	<ol style="list-style-type: none"> <li>1. Study is appropriate to data requirements detailed in Regulations 283/2013 and 284/2013.</li> <li>2. Well-defined test material applied as the appropriate active substance, metabolite or plant protection product.</li> <li>3. Test species is relevant to the EU.</li> <li>4. Route and length of exposure should be appropriate.</li> </ol>
Effects on non-target soil meso- and macrofauna (KCA 8.4)	<ol style="list-style-type: none"> <li>1. Study is appropriate to data requirements detailed in Regulations 283/2013 and 284/2013.</li> <li>2. Well-defined test material applied as the appropriate active substance, metabolite or plant protection product.</li> <li>3. Test species is relevant to the EU.</li> <li>4. Length of exposure should be appropriate.</li> </ol>
Effects on soil nitrogen transformation (KCA 8.5)	<ol style="list-style-type: none"> <li>1. Study is appropriate to data requirements detailed in Regulations 283/2013 and 284/2013.</li> <li>2. Well-defined test material applied as the appropriate active substance, metabolite or plant protection product.</li> <li>3. Length of exposure should be appropriate.</li> <li>4. Substrate used should be appropriate.</li> </ol>
Effects on terrestrial non-target higher plants (KCA 8.6)	<ol style="list-style-type: none"> <li>1. Study is appropriate to data requirements detailed in Regulations 283/2013 and 284/2013.</li> <li>2. Well-defined test material applied as the appropriate active substance, metabolite or plant protection product.</li> <li>3. Test species is relevant to the EU.</li> <li>4. Route and length of exposure should be appropriate.</li> </ol>
Effects on other terrestrial organisms (flora and fauna) (KCA 8.7)	<ol style="list-style-type: none"> <li>1. Study is appropriate to data requirements detailed in Regulations 283/2013 and 284/2013.</li> <li>2. Well-defined test material applied as the appropriate active substance, metabolite or plant protection product.</li> <li>3. Test species is relevant to the EU.</li> <li>4. Route and length of exposure should be appropriate.</li> </ol>
Effects on biological methods for sewage treatment (KCA 8.8)	<ol style="list-style-type: none"> <li>1. Study is appropriate to data requirements detailed in Regulations 283/2013 and 284/2013.</li> <li>2. Well-defined test material applied as the appropriate active substance, metabolite or plant protection product.</li> <li>3. Route and length of exposure should be appropriate.</li> <li>4. Substrate used should be appropriate.</li> </ol>

**RMS comments and conclusion:**

A literature search was performed according to EFSA guidance on submission of scientific literature (EFSA Journal 2011;9(2):2092. The RMS agrees with the the literature search performed by the notifier. No relevant and reliable study has been found in the literature search.

As a result of the literature search, one study was found to be relevant and reliable relate to ecotoxicological assessment of daminozide. The study is summarized and evaluated below.

<b>Zebrafish and development screening of the ToxCast Phase I chemical library</b>	
<b>Data point: CA 8.2.2.1</b>	
Author(s)	Padilla, S., Corum, D., Padnos, B., Hunter, D.L., Beam, A., Houck, K.A., Sipes, N., Kleinstreuer, N., Knudsen, T., Dix, D.J., Reif, D.M.
Year	2012
Journal	Reprod Toxicol (2012) Vol. 33(2), pp. 174-87
Relevance check	Relevant
Reliability check	Klimisch score: 2
Reasons for no reliability	Not relevant
Summary	Daminozide was tested in a zebra fish embryonic developmental assay. Embryos were exposed for five days under semi-static conditions (daily renewal). Lethality, hatching, and malformation were assessed at the end of the exposure. Zebra fish exposed to a single concentration of daminozide resulted in a negative effect. The half-maximal activity-concentration (AC <sub>50</sub> ) for daminozide was 66.51 µM.
<b>Reliability check: study details</b>	
<b>Parameter</b>	<b>Information available</b>
<b>Test protocol</b> GLP, GEP, Guidelines (US EPA, OECD, ...)	<ul style="list-style-type: none"> <li>EPA zebra fish embryonic developmental assay for ToxCast</li> </ul>
<b>Test substance</b> Identification of test substance, source, purity, stability	<ul style="list-style-type: none"> <li>Daminozide (a.s.); no other information provided</li> </ul>
<b>Test conditions</b> Temperature, pH, oxygen concentration, water hardness, conductivity, photoperiod, light intensity, number of animals, food availability	<ul style="list-style-type: none"> <li>Temperature: 26 ± 0.1 °C</li> <li>Photoperiod: 14:10 light: dark cycle</li> <li>Water: freshwater</li> </ul>
<b>Controls</b> Positive control, negative control	<ul style="list-style-type: none"> <li>Positive control: chlorpyrifos ethyl</li> <li>Vehicle control: DMSO (0.4% v/v)</li> </ul>
<b>Dosing system</b> Exposure (dose, duration, frequency)	<ul style="list-style-type: none"> <li>Test concentrations: Range from 0.001356 to 80 µM (24 hr water-renewal for 5 days)</li> <li>Complete solution change with chemical renewal every 24 h</li> </ul>
<b>Test species</b> Body weight or length, gender, age/life stage, source	<ul style="list-style-type: none"> <li>Zebrafish (<i>Danio rerio</i>)</li> <li>Source: Aquatic Research Organisms, New Hampshire, USA</li> <li>Age: embryos (6-8 hrs after fertilization)</li> </ul>
<b>Statistical analyses</b> Sample size/replicates, statistical analysis of data (significance level, variability)	<ul style="list-style-type: none"> <li>4 embryo / test concentration for 5 days</li> <li>Statistics: Standard sigmoidal curves were fit using a 4-parameter Hill model to determine the half-maximal activity concentrations (AC<sub>50</sub>).</li> </ul>
<b>Biological effects</b> Determined effect concentration, dose response observed	<ul style="list-style-type: none"> <li>Lethality, hatching, and malformation were assessed at the end of the exposure.</li> </ul>



	<ul style="list-style-type: none"><li>• Daminozide had a negative effect on zebra embryos; calculated <math>AC_{50} = 66.5075 \mu\text{M}</math> (chemicals are considered toxic to developing embryo when <math>AC_{50}</math> is below <math>80 \mu\text{M}</math>).</li><li>• Toxicity score: 0.00 (score below 2.24 indicates the chemical to be “inactive”)</li></ul>
<b>Overall assessment</b>	<ul style="list-style-type: none"><li>• Methodology, results and discussion are documented.</li><li>• The statistical analysis used was described.</li><li>• The study is considered reliable.</li></ul>

**RMS comments and conclusion:**

As part of the Computational Toxicology Research Program of the U.S. EPA, the toxicity of the 309 ToxCast<sup>TM</sup> Phase I chemicals was assessed using a zebrafish screen for developmental toxicity in the study. The study generally follows OECD 236 guideline Fish Embryo Acute Toxicity (FET) Test but with many deviations, e.g. the endpoint derived is  $AC_{50}$  instead of standard  $LC_{50}$ . No full study report was available and, therefore, a lot of information is missing: batch number was not reported, no raw data were available, no chemical analysis was carried out to verify test concentrations etc. Due to the lack of information the validity criteria could not be checked.

The study is not considered suitable for regulatory use.

## New studies

Data point	Author(s)	Year	Title Company Report 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 8.1.1.1/02	██████ ██████ ██████	2006	Daminozide technical: An acute oral toxicity study with the Japanese quail ████████████████████ Report No. 429-104 GLP Unpublished	Y	Y	New data for AIR 3 renewal	Fine Agrochemicals Ltd.
CA 8.1.1.3/01	██████ ██████ ██████ ██████ ██████ ██████ ██████ ██████	2012	Daminozide: a reproduction study with the Northern bobwhite ████████████████████ Report. No. 616-104 GLP Unpublished	Y	Y	New data for AIR 3 renewal	Arysta LifeScience Great Britain Limited
CA 8.2.1/04	Poirier, S.H., Knuth, M.L., Anderson- Buchou, C.D., Brooke, L.T., Lima, A.R., Shubat, P.J.	1986	Comparative toxicity of methanol and N,N-dimethylformamide to freshwater fish and invertebrates Bulletin of Environmental Contamination and Toxicology (1986) Vol. 37, pp. 615-621 Non-GLP Published	Y	N	New data for AIR 3 renewal	Public domain
CA 8.2.2.1/01	██████████████████ ██████████████████ ██████████████████ ██████████████████	2015	Daminozide: an early life-stage toxicity test with the fathead minnow ( <i>Pimephales promelas</i> ) ████████████████████ Report No. 616A-123 GLP Unpublished	Y	Y	New data for AIR 3 renewal	EU Daminozide Task Force
CA 8.2.4.1/04	Dom, N., Pennick, M., Knapen, D., Blust, R.	2012	Discrepancies in the acute versus chronic toxicity of compounds with a designated narcotic mechanism Chemosphere (2012) Vol. 87, pp. 742-749 Non-GLP Published	Y	N	New data for AIR 3 renewal	Public domain
CA 8.2.5.1/01	Last, G.	2011	Chronic effects to <i>Daphnia magna</i> from exposure to daminozide and formaldehyde Covance Laboratories Ltd. Report No. 8252736 Non-GLP Unpublished	N	Y	New data for AIR 3 renewal	EU Daminozide Task Force

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 8.2.6.1/02	Manson, P.S., Scholey, A.	2006	Daminozide Technical: Inhibition of growth to the alga <i>Pseudokirchneriella subcapitata</i> Covance Laboratories Ltd. Report No. 2242/049-D2149 GLP Unpublished	N	Y	New data for AIR 3 renewal	Fine Agrochemicals Ltd.
CA 8.2.6.1/03	Cho, C-W., Heon, Y-C., Pham, T.P.T., Vijayaraghavan, K., Yun, Y-S.	2008	The ecotoxicity of ionic liquids and traditional organic solvents on microalga <i>Selenastrum capricornutum</i> Ecotoxicology and Environmental Safety (2008) Vol. 71, pp. 166-171 Non-GLP Published	Y	N	New data for AIR 3 renewal	Public domain
CA 8.2.6.2/01	Seeland-Fremer, A., Mosch, W.	2014	Toxicity of daminozide technical to <i>Anabaena flos-aquae</i> in an Algal Growth Inhibition Test IBACON. Report No. 87711210 GLP Unpublished	N	Y	New data for AIR 3 renewal	EU Daminozide Task Force
CA 8.3.1.2/01	Haupt, S.	2014	Chronic oral toxicity of daminozide technical on the honey bee ( <i>Apis mellifera</i> L.) in the laboratory IBACON. Report No. 87715136 GLP Unpublished	N	Y	New data for AIR 3 renewal	EU Daminozide Task Force
CA 8.3.1.3/01	Odemer, R.	2015	Daminozide: toxicity to honey bee ( <i>Apis mellifera</i> L.) larvae after repeated exposure under in vitro laboratory conditions IES. Report No. 20150038 GLP Unpublished	N	Y	New data for AIR 3 renewal	EU Daminozide Task Force
CA 8.4.1/01	Pavić, B.	2014	Effects of daminozide technical on reproduction and growth of earthworms <i>Eisenia fetida</i> in artificial soil with 10% peat IBACON. Report No. 87714022 GLP Unpublished	N	Y	New data for AIR 3 renewal	EU Daminozide Task Force

## Studies relied upon for the first inclusion of daminozide in Annex I to Directive 91/414/EEC and for renewal of approval under Regulation (EC) No 1107/2009

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	██████	1992	Alar technical: an acute oral toxicity study with the mallard ██████████. Report No. A.7.4.2.9 GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Limited
CA 8.1.1.2/01	██████████	1977	Eight-day dietary LC50 – bobwhite quail: technical Alar ██████████ Report No. A.7.4.2.4 Non-GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Limited
CA 8.1.1.2/02	██████	1974	Eight-day dietary toxicity – mallard ducks: technical Alar ██████████ Report No. A.7.4.2.5 Non-GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Limited
CA 8.2.1/01	██████	1987	The toxicity of daminozide to rainbow trout ██████████. Report No. FAL 0020 Non-GLP Unpublished	Y	N	Not applicable	Fine Agrochemicals Ltd.
CA 8.2.1/02	██████	1977	Acute toxicity of Alar technical, Lot BL 8190 to the rainbow trout ( <i>Salmo gairdneri</i> ) Report No. A.7.4.1.5 Non-GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Limited
CA 8.2.1/03	██████	1972	Acute toxicity of Alar to bluegill ( <i>Lepomis macrochirus</i> ) Report No. A.7.4.1.4 Non-GLP Unpublished	Y	N	Not applicable	Arysta LifeScience Great Britain Limited
CA 8.2.4.1/01	Lintott, D.	1992	Alar technical: Acute toxicity to the water flea, <i>Daphnia magna</i> , under flow-through test conditions Toxikon Environmental Sciences. Report No. A.7.4.1.8 GLP Unpublished	N	N	Not applicable	Arysta LifeScience Great Britain Limited
CA 8.2.4.1/02	LeBlanc, G.	1976	Acute toxicity of Alar standard to <i>Daphnia magna</i> E G & G Bionomics, Report No. A.7.4.1.3 Non-GLP Unpublished	N	N	Not applicable	Arysta LifeScience Great Britain Limited

Data point	Author(s)	Year	Title Company Report 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 8.2.4.1/03	Abram, F.	1987	The toxicity of daminozide to <i>Daphnia magna</i> Report No. FAL 3 Non-GLP Unpublished	N	N	Not applicable	Fine Agrochemicals Ltd.
CA 8.2.6.1/01	Douglas, M., Pell, I.	1986	The algistatic activity of Alar Technical Huntingdon Research Centre, Ltd. Report No. A.7.4.1.7 GLP Unpublished	N	N	Not applicable	Arysta LifeScience Great Britain Limited
CA 8.2.6.1/02	Abram, F.	1987	The Toxicity of Daminozide to a Green Alga Report No. FAL 4 Non-GLP Unpublished	N	N	Not applicable	Fine Agrochemicals Ltd.
CA 8.2.7/01	Palmer, S.J., Kendall, T.Z., Krueger, H.O.	2001	A 7-day toxicity test with duckweed ( <i>Lemna gibba</i> G3) Wildlife International Ltd. Report No. 117A-1197 GLP Unpublished	N	N	Not applicable	Arysta LifeScience Great Britain Limited
CA 8.3.1.1.1/01	Davies, L.	1987	Report on a laboratory investigation into the toxicity of daminozide to honey bees ( <i>Apis mellifera</i> ) Report No. FAL 5 Non-GLP Unpublished	N	N	Not applicable	Fine Agrochemicals Ltd.
CA 8.3.1.1.2/01	Davies, L.	1987	Report on a laboratory investigation into the toxicity of daminozide to honey bees ( <i>Apis mellifera</i> ) Report No. FAL 5 Non-GLP Unpublished	N	N	Not applicable	Fine Agrochemicals Ltd.
CA 8.3.2.1/01	Harwood et al.	2000	A laboratory evaluation of the side effects of daminozide on the predatory mite <i>Typhlodromus pyri</i> . Inveresk Research Scotland Inveresk Report Number 18099 GLP, Unpublished.	N	N	Not applicable	Fine Agrochemicals Ltd.
CA 8.7/01	Lengen	2001	Review of the effects of daminozide on non-target fauna and flora Uniroyal Chemical Co. Inc. Non-GLP Unpublished	N	N	Not applicable	Arysta LifeScience Great Britain Limited