Document Title

Summary of the ecotoxicological studies for Aclonifen

Data Requirement(s)

Regulation (EC) No 1107/2009 & Regulation (EU) No 283/2013

Document MCA

Section 8: Ecotoxicological studies

According to the Guidance Document SANCO/10181/2013 for applicants on preparing dossiers for the approval of a chemical active substance

Date

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Version history

Date [yyyy-mm-dd]	Data points containing amendments or additions[1] and brief description	Document identifier and version number
2020-01-17	Original document	M-676898-01-1
2020-01-21	Addition of data on ED properties, paragraph 8.1.5	M-676898-02-1
2020-03-12	Update following admissibility check	M-676898-03-1

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4, 'How to revise an Assessment Report'.

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CA 8 ECOTOXICOLOGICAL STUDIES ON THE ACTIVE SUBSTANCE

Aclonifen was included in Annex I to Council Directive 91/414/EEC in 2008 (Directive 2008/116/EC, Entry into Force on 01 August 2009). This present dossier in support of approval renewal includes all the data submitted at the time of the Annex I inclusion, in summaries updated and re-evaluated as necessary to take account of current validity criteria and data requirements.

CA 8.1 Effects on birds and other terrestrial vertebrates

CA 8.1.1 Effects on birds

Table 8.1-1: Summary of the effects of Aclonifen on birds

Test species	Test item	Endpoint	Reference
Acute, oral			
Bobwhite quail (*Colinus virginianus*)	Aclonifen	**LD_{50} > 2000 mg/kg**	KCA 8.1.1.1/01 M-172009-01-1 [redacted], 1999
Japanese quail (*Coturnix japonica*)	Aclonifen	LD_{50} > 15000 mg/kg	KCA 8.1.1.1/02 M-235374-01-2 [redacted], 1981
German canary	Aclonifen	LD_{50} > 15000 mg/kg	KCA 8.1.1.1/03 M-235294-01-2 [redacted], 1981
Dietary toxicity (short-term)			
Bobwhite quail (*Colinus virginianus*)	Aclonifen	LC_{50} > 5000 mg/kg	KCA 8.1.1.2/01 M-224527-01-1 [redacted], 2003
Reproductive toxicity (long-term)			
Japanese quail (*Coturnix japonica*)	Aclonifen	NOEC = 1000 ppm **NOEL = 141 mg/kg bw/d**	KCA 8.1.1.3/01 M-174897-01-1 [redacted], H., 1995

Endpoints in **bold** were used in the risk assessment

CA 8.1.1.1 Acute oral toxicity to birds

Data Point:	KCA 8.1.1.1/01
Report Author:	[redacted]
Report Year:	1999
Report Title:	Aclonifen technical acute oral toxicity (LD50) to bobwhite quail
Report No:	R006041
Document No:	M-172009-01-1
Guideline(s) followed in study:	US-EPA subdiv E series 71, § 71 -1 SETAC part 2 - 1.1.1
Deviations from current test guideline:	Current Guideline: OECD 223, 2016 Birds were observed twice during the first 2 hours following dosing and a further two times (as opposed to three) during the first 24 hours following dosing. This deviation had no effect on the results of the study
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

The objective of this study was to evaluate the acute toxicity of Aclonifen administered to the northern bobwhite as a single oral dose. The method followed was that described in the United States Environmental Protection Agency Pesticide Assessment Guidelines, Subdivision E, Hazard Evaluation: Wildlife and Aquatic Organisms, Series 71 -Avian and Mammalian Testing, § 71-1 Avian single-dose oral LD50 test, dated October 1982, and draft revised guideline dated March 1988. The study also included modifications described in the SETAC Publication "Procedures for assessing the environmental fate and ecotoxicity of pesticides', Part 2: Ecotoxicity, 1.1.1 Birds - acute toxicity.

Groups of five male and five female adult birds were given a single oral dose, by intubation, of either 500, 1000 or 2000 mg aclonifen technical/kg bodyweight. A similar sized control group was dosed in the same way receiving the vehicle only. Birds were observed for 14 days following dosing. Observations included mortality, clinical signs, bodyweight, food consumption and post mortem examination.

No mortalities occurred during the study and no clinical signs of toxicity were observed in any birds.

Bodyweight changes for males and females were slightly lower at 2000 mg/kg over Days 0 to 7. Female bodyweights were found to be significantly lower ($p<0.05$) at 2000 mg/kg on Day 7 compared to the controls.

Food consumption was slightly reduced at 2000 mg/kg over Days 1 to 3 for both males and females.

No abnormalities were detected in any bird at *post mortem* examination.

The acute oral LD_{50} value of aclonifen technical to the Bobwhite quail was found to be in excess of 2000 mg/kg.

The no observed effect level was considered to be 1000 mg/kg.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen technical
	Batch no.:	OP 9750062
	Active Ingredient / Purity:	994 g/kg
	Appearance:	Yellow powder
	Storage:	Room temperature
	Expiry date:	18 April 1999
2.	**Test Organism:**	Bobwhite quail (*Colinus virginianus*)
	Source:	[redacted]
	Age:	Young adults, approximately six months of age at the start of the treatment period and were all from the same hatch.
	Weight:	170 - 207 g
	Acclimatization:	15 days prior to dosing
	Feeding:	[redacted]

B. STUDY DESIGN AND METHODS

1. In-life phase: 11 August 1983 – 06 February 1984

2. Exposure conditions

Test cages:	Tiered cages measuring approximately 0.31 x 0.39 x 0.24 m. Each cage was made of plastic coated steel wire mesh and contained an automatic drinker and food hopper
Experimental design:	Three test concentrations (500, 1000 and 2000 mg/kg) plus one control
Temperature:	16 – 18°C
Relative humidity:	57%
Photoperiod:	10 hours light:14 hours dark

3. Administration of the test substance

Corn oil was used for the controls and as a vehicle for the test substance. All birds were dosed at a rate of 10 ml/kg bodyweight.

The birds were given a single dose of the test material or vehicle by oral intubation using a disposable syringe and a Ch 10 Nelaton plastic catheter. Care was taken to ensure that the bird had ingested all the dose material before being returned to its cage.

A single preparation in the vehicle was made at three dose concentrations so that all birds received the same dose volume per unit of bodyweight. The test substance was mixed with the vehicle and then gradually made up to volume and mixed using a high shear homogeniser.

4. Observations

Birds were observed daily during the study and at frequent intervals during the post treatment period. Mortalities, bird health and clinical signs were recorded at each observation.

The following were recorded.

- Individual bodyweights on Days -15, -7, 0 (immediately prior to dosing), 7 and 14.
- Group mean food consumption over Days -15 to -8, -7 to -1, 1 to 3, 4 to 7 and 8 to 14.

All sporadic mortalities were examined *post mortem*.

At termination of the study, all birds were sacrificed by cervical dislocation. *Post mortem* examination was carried out on all ten control birds and all ten birds from the highest dose group. All other birds were discarded. Tissues examined included: digestive tract, liver, kidneys, heart, spleen, muscle and subcutaneous fat.

5. Statistical calculations

The mean bodyweights recorded on Days 7 and 14 were analysed for each sex separately. The mean pre-dose bodyweights (Days -15, -7 and 0) were included as a covariate in these analyses as this improved precision (covariate efficiency > 100%).

Comparisons between the treated groups and the control were carried out using Williams' test (Williams 1971, 1972) for a dose-related trend.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

No analytical verification of the dose solutions was performed.

B. BIOLOGICAL DATA

There were no mortalities and no clinical signs of toxicity or regurgitation of dose were observed.

Bodyweight changes for males and females were slightly lower at 2000 mg/kg over Days 0 to 7. Female bodyweights were found to be significantly lower ($p<0.05$) at 2000 mg/kg on Day 7 compared to the controls.

Food consumption was slightly reduced at 2000 mg/kg over Days 1 to 3.

No abnormalities were detected in any bird examined during the macroscopic *post mortem* examination.

Table: Body weight and body weight change in bobwhite quail following exposure to Aclonifen

Group	Treatment (mg/kg)	Sex	Days of study								
			Body weight (g)					Body weight change (g)			
			-15	-7	0	7	14	-15 to -7	-7 to 0	0 to 7	7 to 14
1	Control	m	191	192	189	195	197	1	-3	6	2
	0	f	186	190	187	193	195	4	-3	6	2
2	Aclonifen	m	192	195	192	200	203	3	-3	8	3
	500	f	188	191	189	194	195	3	-2	5	1

3	Aclonifen	m	192	193	191	199	202	1	-2	8	3
	1000	f	188	188	186	192	194	0	-2	6	2
4	Aclonifen	m	192	193	192	193	198	1	-1	1	5
	2000	f	189	192	189	192	196	3	-3	3	4

The acute oral LD_{50} value of aclonifen technical to the Bobwhite quail was found to be in excess of 2000 mg/kg. The no observed effect level was considered to be 1000 mg/kg.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 223, 2016)	Achieved
Mortality in controls	≤10%	0%

The validity criterion was satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal Concentration (mg/kg)
LD_{50}	>2000
NOEC	1000

III. CONCLUSION

The acute oral LD_{50} value of aclonifen technical to the Bobwhite quail was found to be in excess of 2000 mg/kg. The no observed effect level was considered to be 1000 mg/kg.

[redacted] *(1999)*

Assessment and conclusion by applicant:
All validity criteria were satisfied and therefore this study can be considered to be valid.

The LD_{50} of Aclonifen to Bobwhite quail, was determined to be greater than 2000 mg/kg. The no observed effect level was considered to be 1000 mg/kg.

Assessment and conclusion by RMS:

Data Point:	KCA 8.1.1.1/02
Report Author:	[redacted]; [redacted]
Report Year:	1981
Report Title:	KUB 3359 Batch T 5/81 - Acute oral toxicity in Japanese quails (Coturnix coturnix japonica)
Report No:	R003366
Document No:	M-235374-01-2
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 223, 2016 No control group included in study
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

The objective of this study was to evaluate the acute toxicity of KUB 3359 Batch T 5/81 (Aclonifen) administered to the Japanese quail (*Coturnix japonica*).

Groups of ten male and ten female adult birds were given a single oral dose, by intubation, of 15 g aclonifen/kg bodyweight. Birds were observed for 14 days following dosing. Observations included mortality, clinical signs, bodyweight, food consumption and post mortem examination.

No mortalities occurred during the study and no clinical signs of toxicity were observed in any birds.

After 14 days the average body weights of all animals showed the normal increases as compared to the baseline values.

No abnormalities were detected in any bird at *post mortem* examination.

The acute oral LD_{50} value of KUB 3359 Batch T 5/81 to the Japanese quail was found to be >15000 mg aclonifen/kg.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Item:
   - Batch no.: KUB 3359 T 5/81
   - Active Ingredient / Purity: Aclonifen, purity not reported
   - Appearance: Yellow powder
   - Storage: Room temperature
   - Expiry date: Not reported

2. Test Organism:
   - Source: [redacted]
   - Age: approximately 8 weeks (full-grown).
   - Weight: 96 - 146 g
   - Acclimatization: Approximately 7 days

Feeding: Memo sole feed for chicks (Hemo-KLiken-Alleinfutter) - standard type

B. STUDY DESIGN AND METHODS

1. In-life phase: 24 September – 13 October 1981

2. Exposure conditions

Test cages:	Type III Makrolon cage/2 quails
Experimental design:	Single test concentration of 15 g/kg
Temperature:	21 ± 2 ºC
Relative humidity:	45 - 55%
Photoperiod:	12 hours light: 12 hours dark

3. Administration of the test substance

The test item was administered as a 60% suspension in 0.5% carboxymethyl cellulose (CMC). This test suspension had a pH value of 7.5.

The test suspension was administered once to the animals by the oral route using a rigid stomach tube.

4. Observations

The clinico-toxicological symptoms were assessed in each individual animal at intervals depending on the course of the symptoms (modified screening method adopted from Irvin). The procedure followed was such that in animals showing unchanging effects over a prolonged period of time a corresponding note was made on the record sheets. Reassessments took place only when changes in the symptoms were observed. The evaluations were made after periods of 20 minutes, 1, 3, 24 and 72 hours as well as 7 and 14 days following administration.

The body weights were determined on day 0 (beginning of study) and day 14 (final autopsy) in the surviving animals.

Animals dying from acute or delayed effects of dosing were autopsied immediately after discovery of their bodies and investigated for macroscopic organ changes in the cranial, thoracic and abdominal cavities. The final post-mortem was performed in all surviving animals from the individual study groups at the end of the follow-up observation period.

5. Statistical calculations

No statistical analysis of the generated data was performed.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

No analytical verification of the dose solutions was performed.

B. BIOLOGICAL DATA

No mortalities occurred throughout the 14-day observation period.

In the treated animals a yellowish discoloration of the faeces was observed approximately 1 hour following administration. Twenty-four hours following administration, however, this discoloration

could no longer be detected. Throughout the observation period there were no further clinical symptoms observed.

During the final autopsy performed 14 days following administration no macroscopically visible organ changes were revealed in the cranial, thoracic and abdominal cavities.

After 14 days the average body weights of all animals showed the normal increases as compared to the baseline values.

Table: Body weight in Japanese quail following exposure to Aclonifen

Group	Treatment (mg/kg)	Sex	Bodyweight (g)	
			Day 0	Day 14
1	Aclonifen (15000)	m	109.3	119.5
		f	129.2	143

The acute oral LD_{50} value of aclonifen to the Japanese quail was found to be in excess of 15000 mg/kg bodyweight.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 223, 2016)	Achieved
Mortality in controls	≤10%	n.d.

n.d.: not determined

No control group was included in the study and therefore the validity of the study according to current guideline requirements cannot be assessed.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal Concentration (mg/kg)
LD_{50}	>15000

III. CONCLUSION

The acute oral LD_{50} value of aclonifen to the Japanese quail was found to be in excess of 15000 mg/kg.

[redacted] *(1981)*

Assessment and conclusion by applicant:
No control group was included in the study and therefore the validity of the study according to current guideline requirements cannot be assessed. There were no known circumstances which may have affected the quality or integrity of the study and hence the study may be used as supporting evidence of the low acute toxicity of Aclonifen to birds.

The LD_{50} of Aclonifen to Japanese quail, was determined to be greater than 15000 mg/kg.

Assessment and conclusion by RMS:

Data Point:	KCA 8.1.1.1/03
Report Author:	[redacted]
Report Year:	1981
Report Title:	KUB 3359 Batch T5/81 - Acute oral toxicity in canaries
Report No:	R003647
Document No:	M-235294-01-2
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 223, 2016 No control group included in study
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

The objective of this study was to evaluate the acute toxicity of KUB 3359 Batch T 5/81 (Aclonifen) administered to German canaries.

Groups of ten male and ten female adult birds were given a single oral dose, by intubation, of 15 g aclonifen/kg bodyweight. Birds were observed for 14 days following dosing. Observations included mortality, clinical signs, bodyweight, food consumption and post mortem examination.

No mortalities occurred during the study and no clinical signs of toxicity were observed in any birds.

After 14 days the average body weights of all animals showed the normal increases as compared to the baseline values.

No abnormalities were detected in any bird at *post mortem* examination.

The acute oral LD_{50} value of KUB 3359 Batch T 5/81 to the Japanese quail was found to be >15000 mg aclonifen/kg.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** KUB 3359
   Batch no.: T 5/81
   Active Ingredient / Purity: Aclonifen, purity not reported
   Appearance: Yellow powder
   Storage: Room temperature
   Expiry date: Not reported

2. **Test Organism:** German canaries
   Source: [redacted]

Age:	approximately 9 months
Weight:	17 - 22 g
Acclimatization:	Approximately 7 days
Feeding:	[redacted]

B. STUDY DESIGN AND METHODS

1. In-life phase: 02 November – 16 November 1981

2. Exposure conditions

Test cages:	Volary (60 x 80 x 60 cm)/5 birds
Experimental design:	Single test concentration of 15 g/kg
Temperature:	21 ± 2 °C
Relative humidity:	45 - 55%
Photoperiod:	12 hours light/12 hours dark

3. Administration of the test substance

The test item was administered as a 60% suspension in 0.5% carboxymethyl cellulose (CMC). This test suspension had a pH value of 7.5.

The test suspension was administered once to the animals by the oral route using a rigid stomach tube.

4. Observations

The clinico-toxicological symptoms were assessed in each individual animal at intervals depending on the course of the symptoms (modified screening method adopted from Irwin). The procedure followed was such that in animals showing unchanging effects over a prolonged period of time a corresponding note was made on the record sheets. Reassessments took place only when changes in the symptoms were observed. The evaluations were made after periods of 20 minutes, 1, 3, 24 and 72 hours as well as 7 and 14 days following administration.

The body weights were determined on day 0 (beginning of study) and day 14 (final autopsy) in the surviving animals.

Animals dying from acute or delayed effects of dosing were autopsied immediately after discovery of their bodies and investigated for macroscopic organ changes in the cranial, thoracic and abdominal cavities. The final post-mortem was performed in all surviving animals from the individual study groups at the end of the follow-up observation period.

5. Statistical calculations

No statistical analysis of the generated data was performed.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

No analytical verification of the dose solutions was performed.

B. BIOLOGICAL DATA

No mortalities occurred throughout the 14-day observation period.

In the treated animals a yellowish discoloration of the faeces was observed approximately 1 hour following administration. Twenty-four hours following administration, however, this discoloration could no longer be detected. Throughout the observation period there were no further clinical symptoms observed.

During the final autopsy performed 14 days following administration no macroscopically visible organ changes were revealed in the cranial, thoracic and abdominal cavities.

After 14 days the average body weights of all animals showed the normal increases as compared to the baseline values.

Table: Body weight in Japanese quail following exposure to Aclonifen

Group	Treatment (mg/kg)	Sex	Bodyweight (g)	
			Day 0	Day 14
1	Aclonifen (15000)	m	19.3	19.84
		f	18.7	19.25

The acute oral LD_{50} value of aclonifen to German canaries was found to be in excess of 15000 mg/kg bodyweight.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 223, 2016)	Achieved
Mortality in controls	≤10%	n.d.

n.d.: not determined

No control group was included in the study and therefore the validity of the study according to current guideline requirements cannot be assessed.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal Concentration (mg/kg)
LD_{50}	>15000

III. CONCLUSION

The acute oral LD_{50} value of aclonifen to German canaries was found to be in excess of 15000 mg/kg.

[redacted] *(1981)*

Assessment and conclusion by applicant:
No control group was included in the study and therefore the validity of the study according to current guideline requirements cannot be assessed. There were no known circumstances which may have affected the quality or integrity of the study and hence the study may be used as supporting evidence of the low acute toxicity of Aclonifen to birds.

The LD_{50} of Aclonifen to German canaries, was determined to be greater than 15000 mg/kg.

Assessment and conclusion by RMS:

CA 8.1.1.2 Short-term dietary toxicity to birds

Data Point:	KCA 8.1.1.2/01
Report Author:	[redacted]
Report Year:	2003
Report Title:	5-day-dietary LC50 for Bobwhite Quail (Colinus virginianus) Aclonifen (tech. a.s.)
Report No:	C038216
Document No:	M-224527-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 205, 1984 None
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Bobwhite quail (*Colinus virginianus*) received Aclonifen mixed directly with their feed at nominal concentrations of 313, 625, 1250, 2500 and 5000 mg a.s/kg for five days. Following the five-day exposure period all groups were given untreated feed *ad libitum* for a further 3 days. Observations, including mortality, clinical signs, bodyweight and food consumption, were made during the study. At termination of the study, post-mortem examination was carried out on all surviving birds.

None of the birds showed any signs of intoxication at any time of the study. There were no difference in the body weight development between test groups and controls.

The dietary LC_{50} of Aclonifen to the bobwhite quail was found to be greater than 5000 mg a.s./kg food; equivalent to 1027 mg a.s./kg bw/day.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen (tech. a.s.)
	Batch no.:	OP2150250
	Active Ingredient / Purity:	98.6%
	Appearance:	Yellow crystalline powder
	Storage:	25°C ± 5 °C
	Expiry date:	07 April 2005
2.	**Test Organism:**	Bobwhite quail (*Colinus virginianus*)

Source: [redacted]

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Age: Three days old
Weight: 23 - 27 g
Acclimatization: 7 days
Feeding: A standard rearing diet for quails, type: "Wachtel und Entenhaltungsfutter (Quail and Mallard food), batch No. 14/03 (3958.9.25) of the company "Kliba GmbH"

B. STUDY DESIGN AND METHODS

1. In-life phase: 21 May 2002 – 26 June 2003

2. Exposure conditions

Test cages: Stainless steel wire cages measuring 100 cm x 55 cm with a height of 25 cm

Experimental design: Five test concentrations (313, 625, 1250, 2500 and 5000 mg a.s./kg) plus a control

Temperature: 22 ± 1 °C
Relative humidity: 49 - 53%
Photoperiod: 12 hours light:12 hours dark

3. Administration of the test item

Diet preparations were performed according to the following preparation scheme:

Date of Preparation	Nominal Dietary Treatment Level (mg a.s. / kg diet)	Total Batch Amount (kg)	Test Item (g)	Basal Diet (g)
May 21, 2003 (for homogeneity & stability analysis)	313	5.000	1.587	4998.0
	5000	5.000	25.355	4974.0
June 16, 2003 (for exposure)	0 (Control)	10.000	-	10000.0
	313	5.000	1.588	4998
	625	5.000	3.169	4996
	1250	5.000	6.340	4994
	2500	5.000	12.679	4987
	5000	5.000	25.356	4974

Immediately after preparation, the total prepared amount for each treatment level was partitioned into separate sub-samples corresponding to the amount required for one day and stored in a freezer until use.

The appropriate amounts of the test item were directly admixed to the basal diet. No vehicles were used for diet preparation. Accuracy and usefulness of the applied methods were proofed by the analytical verification of homogeneity, stability and a.s.-content of the prepared diets

4. Test organism assignment and treatment

Three days prior to exposure the chicks were allocated randomly to each of the five treatment levels and two control groups. Each group consisted of 10 chicks. Afterwards, the test units were arranged in a randomised order. The birds were individually identified by leg bands and were observed daily on health and compatibility until start of exposure.

Food was provided ad libitum throughout the study. At all times, birds had free access to food and fresh tap water. During the post exposure period, weighed amounts of untreated food were placed into the cage-feeders. During exposure period, the birds received pre-weighed quantities of frozen-stored test diet, which were thawed immediately before exposure. In daily intervals, all uneaten food was removed from the feeders and feeders were refilled with fresh food after cleaning. During exposure and post-exposure period, the remaining uneaten food / diet was re-weighed for determination of food consumption. After weighing, the uneaten amounts were disposed of.

5. Measurements and observations

Observations on signs of intoxication were made daily during acclimatisation, twice on the first exposure day, continued at least once daily throughout the following study days until terminal sacrifice.

At the end of the study the birds were sacrificed by CO_2 asphyxiation and gross necropsies were carried out on all survivors.

Body weights were determined at the beginning of exposure (day 0), at the end of exposure (day 5) and after terminal sacrifice (day 8).

6. Statistics/Data evaluation

Due to the clear results a statistical analysis of the data was not necessary.

II. RESULTS AND DISCUSSION

A. Analytical verification

The analytical data revealed that the test item feeding mixtures were homogenous. The content checks confirmed that during the study appropriate and equal mixture procedures were followed.

Based on the measured concentrations, the test concentrations were determined to be 322, 628, 1318, 2559 and 5072 mg a.s./kg food.

The validated method is summarised in Document M-CA4 (CA 4.1.2/55).

B. Mortalities and clinical observations

None of the birds showed any signs of intoxication at any time of the study. There were no difference in the body weight development between test groups and controls.

During the exposure days the food consumption varied from day to day at the control and at 625 and 1250 mg a.s./kg food. In the other test groups (313, 2500 and 5000 mg a.s./kg food) the differences between the daily food consumption was smaller. A trend in regard of test concentration in the food was not visible.

The only pathological finding at gross necropsy were two enlarged gall bladder at 5000 mg a.s/kg, which were of no toxicological concern.

Table: Mean daily food consumption and mean active substance (Aclonifen) intake

Measured Concentration (mg a.s./kg diet)	Mean body weight per bird	Mean daily food consumption (g food /bird/24h)		Mean a.s. uptake (Days 0 – 4)	
	Day 0-4 (g)	Days 0 -4	Days 5 -7	mg a.s./bird /24h	mg a.s./kg bw /24h
0	32.2	10.6	9.7	0.0	0.0
322	32.2	7.1	6.8	2.3	71.4
628	32.5	13.1	13.4	8.2	252.3
1318	32.1	16.9	14.2	22.3	694.7
2559	28.0	6.2	7.4	15.9	567.9
5072	30.1	6.1	8.3	30.9	1026.6

bw: body weight

The LC_{50} for juvenile bobwhite quails was higher than 5000 mg a.s./kg food.

C. VALIDITY CRITERIA

Mortality in the controls was less than 10% at the end of the test and the active substance was shown to be stable in the prepared diet over a period of 5 days. In addition, there was no mortality or toxic effects at the lowest test concentration. Therefore, the validity criteria specified in OECD 205 (1984) were satisfied and the test is considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	mg a.s./kg food	mg a.s./kg bw/day
LC_{50}	>5000	>1027
NOEC	5000	1027

III. CONCLUSION

The dietary LC_{50} of Aclonifen to the bobwhite quail was found to be greater than 5000 mg a.s./kg food; equivalent to 1027 mg a.s./kg bw/day.

[redacted] *(2003)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The dietary LC_{50} of Aclonifen to the bobwhite quail was found to be greater than 5000 mg a.s./kg food; equivalent to 1027 mg a.s./kg bw/day.

Assessment and conclusion by RMS:

CA 8.1.1.3 Sub-chronic and reproductive toxicity to birds

Data Point:	KCA 8.1.1.3/01
Report Author:	[redacted]
Report Year:	1995
Report Title:	Aclonifen - Avian subchronic toxicity test - Oral toxicity in japanese quail (including effects on reproduction following a 6-week administration in the diet)
Report No:	R007424
Document No:	M-174897-01-1
Guideline(s) followed in study:	OECD: Draft guideline for testing of chemicals
Deviations from current test guideline:	Current Guideline: OECD Draft,1991 None
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Japanese quail (*Coturnix japonica*) received Aclonifen mixed directly with their feed at nominal concentrations of 100, 300 or 1000 mg aclonifen/kg diet over a 6 week period.

Effects monitored included, adult survival, adult body weight, egg production, egg shell thickness, egg fertility, egg cracks, hatching success, 14 day post hatch survival of chicks, chick bodyweight and gross pathology.

Analytical verification of aclonifen concentrations in the diet showed that the actual levels were near nominal. Aclonifen was seen to be homogeneously mixed with the diet, and sufficiently stable over a 7 day period between diet renewals.

No treatment related mortalities occurred among adult birds and no adverse effects of aclonifen were seen on any of the other parameters tested. No treatment related effects were observed in clinical signs and behaviour of chicks.

The no-adverse effect level (NOEL) was determined to be 1000 ppm (equivalent to 141 mg/kg bw/d).

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen
   Batch no.: DA 900
   Active Ingredient / Purity: 995 g/kg
   Appearance: Yellow powder
   Storage: Room temperature in the dark
   Expiry date: 22 November 1995

2. **Test Organism:** Japanese quail (*Coturnix japonica*)
   Source: [redacted]

[redacted]

Age: Approximately eight weeks old at the start of the treatment period of the study and were approaching their first reproductive season.

Weight: 144 - 234 g

Feeding: Avian layer diet, manufactured by Special Diets Services, Witham, Essex, England

B. STUDY DESIGN AND METHODS

1. In-life phase: 03 March – 16 May 1994

2. Exposure conditions

Test cages: Each cage, which housed a replicate of one male and one female bird, was constructed of polythene coated steel wire and measured approximately 0.31 x 0.39 x 0.24 m.
The cages had sloping floors with 0.1 m egg-catchers, and had externally attached food hoppers and automatic drinkers

Experimental design: Three test concentrations (100, 300 and 1000 ppm) plus a control

Temperature: 21 - 24 °C

Relative humidity: 55%

Photoperiod: 16 hours light:8 hours dark

Light intensity: 50 – 75 lux

3. Administration of the test item

A premix of suitable strength was prepared weekly by mixing the required quantity of test substance with untreated basal diet. Blending of the premix was achieved by mixing in a Turbula mixer for a minimum period of 5 minutes. The test diet concentrations were prepared by direct dilution of the prepared premix. Blending of the inclusion levels for feeding was achieved by mixing in a Turbula mixer for a minimum period of 7 minutes.

Prior to the start of the main study, samples were taken from a trial mix to determine stability and homogeneity of aclonifen in SDS layer diet at 100 and 1000 ppm. Duplicate 500 g samples were taken from the top, middle and bottom of the mix for analysis of homogeneity. Additional samples were taken to determine the stability of aclonifen in avian diet over 0, 4, 8 and 15 days under animal room conditions. Day 0 and 4 samples were taken as contingency only, and were not subsequently analysed. Results from the trial mix indicated that aclonifen in avian diet was stable under normal animal room conditions for up to fifteen days, therefore no further analysis was undertaken.

4. Test organism assignment and treatment

Prior to the start of the treatment period, the birds were allocated to cages with one male and one female in each cage. There were three test groups plus a control group and 12 cages (replicates) for each treatment.

Test diets were fed to the birds weekly during the six week egg production period. Controls received untreated basal diet.

5. Measurements and observations

Adult birds and chicks were observed daily for mortalities and clinical signs. Individual adult bodyweights were recorded in Weeks -2, 0 (immediately prior to the introduction of test diets) and at termination, Week 6. Food consumption for each replicate was recorded weekly throughout the pre-treatment and treatment periods of the adult phase. Individual chick bodyweights were recorded within 24 hours of Hatching and again after 14 days.

All data relating to food consumption, eggs and chicks were collated over weekly intervals except for egg shell thickness which was recorded for Weeks 2 and 4.

All sporadic mortalities were subjected to a macroscopic external and internal examination. At termination, all adult birds were examined following sacrifice by cervical dislocation. Net weights of heart, liver, spleen, testis/oviduct (without developing eggs) were recorded and examined for gross pathological changes. Clicks were not examined *post mortem* at termination of 14 days observation.

6. Statistics/Data evaluation

Williams' test ([redacted] 1971, [redacted] 1972) for contrasting increasing dose levels of a compound with a zero dose control was used to compare the treated groups with the control.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The analytical data revealed that the test item feeding mixtures were homogenous and that aclonifen was stable in the avian diet over a period of 15 days.

The validated method is summarised in Document M-CA4 (CA 4.1.2/56).

B. MORTALITIES AND CLINICAL OBSERVATIONS

Mortalities

Mortalities which occurred during the treatment phase of the study are summarised below:

Table: Adult mortalities during the treatment phase

Group	Dose level	Replicate	Bird number	Day of death
1	Control, 0 ppm	3	6♀	29
2	Aclonifen, 100 ppm	14	28♀	13
		22	44♀	21

Bird 6♀ in Group 1 was subdued immediately prior to being found dead on Day 29. The only other observation was in bird 56♀, Group 3, where a swelling on the head was observed on Day 27 and remained until termination of the study. The bird was in good health otherwise.

Organ weights

At study termination, organ weights were determined. No differences between treatment groups and control were detected.

Table: Adult organ weights (g)

Treatment (ppm)	Sex	Heart	Liver	Spleen	Testes/Oviduct
Control	Male	1.70	2.90	0.06	4.34
	Female	1.81	5.99	0.07	0.57
100	Male	1.87	2.96	0.05	4.60
	Female	1.84	5.53	0.07	0.63
300	Male	1.85	2.50	0.08	3.95
	Female	1.80	6.09	0.07	0.71
1000	Male	1.69	2.61	0.05	4.29
	Female	1.73	5.48	0.07	0.63

Adult Bodyweight and Feed Consumption

Bodyweights over the study period were analysed for each sex separately. There were no treatment related effects in bodyweight gain observed during the 6-week treatment period.

Table: Group mean bodyweight – adult birds (g)

Treatment (ppm)	Sex	Week of study		
		-2	0	6
Control	Male	183	192	210
	Female	194	211	235
100	Male	180	184	207
	Female	197	216	241
300	Male	172	180	191
	Female	202	230	233
1000	Male	166	175	187
	Female	195	208	224

Food consumption was similar in all groups with no evidence of a treatment-related effect.

Table: Group mean weekly food consumption (g/bird/day)

Week	Treatment (ppm)			
	Control	100	300	1000
-2	27	27	28	26
-1	28	28	29	27
1	29	30	30	29
2	30	31	30	28
3	29	30	29	27
4	29	30	28	28
5	29	30	29	27
6	29	30	29	28
Mean, weeks 1 - 6	**29**	**30**	**29**	**28**

A summary of the reproductive results obtained is presented in the tables below.

Table: Summary of reproductive effects of Aclonifen on Japanese quails

Reproductive parameters	Control	100 ppm	300 ppm	1000 ppm
Eggs laid per female	37.9	37	34.4	38.6
Mean weight egg (g)	11.3	11.8	11.5	11.5
Group mean egg shell thickness (mm)	0.2	0.19	0.19	0.19
Cracked eggs of eggs laid (%)*	15.4	22.2	26.3	19
Non-cracked eggs of egges laid (%)	84.6	77.8	73.7	81
Candling results: fertile eggs of eggs set (%)	84	82	85	93
Candling results: viable embryos of eggs set (%)	83	80	84	91
Hatching				
- % hatchlings of eggs set	83	68	71	83
- % hatchlings of viable embryos	96	86	89	91
Number of dead in shell as a proportion of fertile eggs (%)	4	8	9	7
Number of surviving chicks as a proportion of hatchlings (%)	99	99	97	99
Chick bodyweights at hatching (g)	8.5	8.5	8.1	8.2
Chick bodyweights at 14 days (g)	57.3	59.3	57.7	57.2

*The data on cracked eggs were transferred in a more meaningful way (no. of uncracked eggs related to eggs laid). It is now the state of the art to perform the statistical analyses with these data.

The number of cracked eggs was amazingly high in all groups including the control. It was reported that problems with egg-pecking occurred in the study with different pairs which at least to some extent may explain the abnormal rate of cracked eggs.

No statistically significant differences were detected for any of the endpoints. Dose responses were never observed.

Dietary administration of up to 1000 ppm aclonifen to Japanese quail had no adverse effect on health, growth and reproduction performance of adult birds or on the chicks. The no-adverse effect level (NOEL) was determined to be 1000 ppm.

C. VALIDITY CRITERIA

The study was performed according to a draft test guideline and hence no specific validity criteria exist. The quality criteria of the Draft Guideline (1982) were fulfilled:

- The test item concentration in the diet was satisfactorily maintained throughout the 42-day exposure period. A premix of suitable strength was prepared weekly by mixing the required quantity of test substance with untreated basal diet. Stability of the test substance in the food under test conditions was verified. On day +15 the concentration at the highest level was +0.3 of the concentration on day 0.
- The hatching success for the incubated eggs of the control during the 5th and 6th week of the administration was higher than 50% (amounted to 83%).
- The viability of the quails amounted to 99% and was therefore significantly greater than the required 50%.
- 11 breeding pairs of the control group survived until the end of the test (quality criterion: at least 10 pairs).

Additionally, mortality in the controls was less than 10% at the end of the test and there was no mortality or toxic effects at the lowest test concentration. Therefore, as there were no known circumstances which may have affected the quality or integrity of the study, the test is considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	ppm
NOEL	1000

III. CONCLUSION

Dietary administration of up to 1000 ppm aclonifen to Japanese quail had no adverse effect on health, growth and reproduction performance of adult birds or on the chicks. The no-adverse effect level (NOEL) was determined to be 1000 ppm.

[redacted] *(1995)*

Assessment and conclusion by applicant:

The study was performed according to a draft test guideline and hence no specific validity criteria exist, however, as there were no known circumstances which may have affected the quality or integrity of the study, the test is considered to be valid.

Compared with the mallard and bobwhite reproduction according OECD 206, the exposure of Japanese quails is shorter in this study. When the bird study was performed, the Draft OECD Guideline for testing of chemicals – avian subchronic test - oral toxicity in Japanese quails, in version of November 1992 was state of the art and to some extent this is still the case (for the improved version of 1997). The non-adoption of this was not driven by science. But even OECD 206 recommends using proven breeders if the test is performed with Japanese Quails. This is only possible if exposure during the pre-laying phases is omitted (8 week of short days and first weeks after switching to long day before egg laying starts). Insofar the reduced exposure period in the submitted study was in compliance with the OECD 206 The existing study deviates from OECD 206 in three aspects, while it followed the Draft OECD Guideline of November 1992.

1. The reduced exposure period of 6 weeks was in line with that draft, while OECD 206 requires 10 egg-laying weeks (but be aware that the egg laying rate is very reduced in the first egg laying weeks according OECD 206). The OECD draft of November 1992 and all successive versions aimed to avoid the shortcomings of the OECD 206, mainly its poor statistical power. Reason for the deficiency is the high variability of the animals in starting egg-laying after switching to long day conditions – therefore the recommendation to use proven breeders resp. sexually mature birds.
2. The new draft design allows following the development of impacts over the exposure periods and by including observations of organ weights (e.g. testes, oviduct) may give hints on adverse effects on reproductive system. Compared with OECD 206, the study conduct according new draft gives additional valuable information about the potential toxicity of a test compound.

3. On behalf animal welfare reasons, the OECD Draft of 1992 only recommends incubating the eggs from exposure week 1 to 4 only for 10 days and discarding all the eggs after candling on day 10. Only the chicks of week 5 and 6 were incubated until hatch and and reared for 2 further weeks.

The NOAEL used in the avian long-term risk assessment derives from this reproduction study with Japanese quails. The NOAEC was determined to be at the highest test concentration. The findings at that dose were either equal or even slightly more favourable than in the control.

At that dose level no effects occurred with deviations of more than 10% in comparison to the control hence an EC_{10} or an EC_{20} cannot be calculated on the basis of this study. No dose-effect relationship between test concentrations and any reproductive parameter was found. The statistical tests included in the report, revealed no significant differences.

Since the study did not reveal any indication of treatment related effects, further statistical analyses are not necessary.

Dietary administration of up to 1000 ppm aclonifen to Japanese quail had no adverse effect on health, growth and reproduction performance of adult birds or on the chicks. The no-adverse effect level (NOEL) was determined to be 1000 ppm, equivalent to 141 mg/kg bw/d*.

*Calculated according to SANCO/4145/2000 and considering the mean body weight of adult birds at 1000 ppm at week 0 and week 6 of the study of 198.5 g and the mean food consumption of birds at 1000 ppm over the period of week 1-6 of 28 g/bird/day.

$$Daily\ dietary\ dose\ (mg/kg\ bw/d) = \frac{Conc.\ in\ food\ (ppm) x\ Daily\ food\ consumption\ (g/bird/d)}{Body\ weight\ (g)}$$

Accepted in EFSA scientific report (2008) 149, 1-80, Conclusion on the peer review of aclonifen

Assessment and conclusion by RMS:

CA 8.1.2 Effects on terrestrial vertebrates other than birds

Table 8.1-2: Summary of the effects of Aclonifen on mammals

Test species	Test item	Endpoint	Reference
Acute, oral			
Rat	Aclonifen	**LD_{50} > 5000 mg/kg**	KCA 5.2.1/01 M-174876-01-1 [redacted], 1981
Short-term dietary			
28-day Mouse	Aclonifen	NOEC = 780 ppm $NOEL_{males}$ = 121.2 mg/kg bw/d $NOEL_{females}$ = 143.1 mg/kg bw/d	KCA 5.3.1/01 M-174234-01-1 [redacted]1988

Test species	Test item	Endpoint	Reference
90-day Rat	Aclonifen	NOEC = 500 ppm $NOEL_{males}$ = 26.4 mg/kg bw/d $NOEL_{females}$ = 29.4 mg/kg bw/d	KCA 5.3.2/01 M-174843-01-2 [redacted], 1982
90-day Rat	Aclonifen	NOEC = 50 ppm $NOEL_{males}$ = 3.6 mg/kg bw/d $NOEL_{females}$ = 4.2 mg/kg bw/d	KCA 5.3.2/02 M-174924-01-1 [redacted], 1997
90-day Rat	Aclonifen	NOEC = 500 ppm $NOEL_{males}$ = 29.4 mg/kg bw/d $NOEL_{females}$ = 36.3 mg/kg bw/d	KCA 5.3.2/03 M-205288-01-1 [redacted], 2001
Reproductive toxicity (long-term)			
Rat Two-generation	Aclonifen	NOEC = 500 ppm **NOEL = 35 mg/kg bw/d**	KCA 5.6.1/01 M-174748-01-1 [redacted], 1985
Rat Embryotoxicity	Aclonifen	NOEL = 60 mg/kg bw/d	KCA 5.6.2/01 M-174846-01-1 [redacted], 1982
Rat Embryotoxicity	Aclonifen	NOEL ≥ 25 mg/kg bw/d*	KCA 5.6.2/02 M-174853-01-1 [redacted], 1984

Endpoints in **bold** were used in the risk assessment
*No effects at the highest dose tested

CA 8.1.2.1 Acute oral toxicity to mammals

Please refer to the mammalian toxicology section of this dossier; Document M-CA5, Section 5.2.1 for studies performed on the active substance, aclonifen.

CA 8.1.2.2 Long-term and reproduction toxicity to mammals

Please refer to the mammalian toxicology section of this dossier; Document M-CA5, Section 5.6.1 for studies performed on the active substance, aclonifen.

Data Point:	KCA 8.1.2.2/01
Report Author:	[redacted]; [redacted]; [redacted]
Report Year:	2019
Report Title:	Aclonifen: Endpoint selection for long-term risk assessment for mammals (updated)
Report No:	M-675718-01-1
Document No:	M-675718-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	--
Previous evaluation:	
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	

Executive Summary

This document provides an updated assessment of the relevant scientific dataset to define a single, ecotoxicologically relevant endpoint for the long-term risk assessment of mammals exposed to Aclonifen ($NOAEL_{ecotox}$). The identification of the ecotoxicologically relevant endpoint for mammals is based on the mammalian toxicology studies.

The appropriate information has been collated, analysed, tabulated and the relevant endpoint derived. The data available, the method used and the EFSA guidance applied are described. This updated endpoint selection includes consideration of newly available, pertinent data and guidance as well as the previous assessment for Aclonifen.

The selected endpoint is based on the consideration of eleven different mammalian toxicology studies. These studies showed no effect of repeated Aclonifen exposure on: fertility, reproduction, pup development, survival, carcinogenicity or neurotoxicity. In the absence of any overt systemic toxicity, a potentially ecotoxicologically relevant effect is a significant reduction (≥20%) of F1 and F2 pup bodyweight during lactation, at the highest dose level tested (2000 ppm). At lower dose levels, pup bodyweight changes were small (<10%), grew even smaller as the pups matured (≤6%) and were not associated with any adverse biological effects, these slight bodyweight changes were considered ecologically non-relevant. Pup exposure is represented by the F0 parent pre-mating dietary exposure, per dose level.

The derived $LOAEL_{ecotox}$ is at 2000 ppm (≥140/152 mg ai/kg bw/day, M/F) and the mid-dose level from that study is the basis for the $NOAEL_{ecotox}$ = 500 ppm (≥35 /40 mg Aclonifen/kg bw/day, M/F).

[redacted] *(2019)*

Assessment and conclusion by applicant:

The re-evaluation of the available study data is considered to be acceptable and hence the conclusions drawn are considered to be valid.

A $NOAEL_{ecotox}$ of 35 mg/kg bw/d (equivalent to a dietary concentration of 500 ppm) is considered a very conservative value for reproductive / long-term risk assessment in wild mammals.

Assessment and conclusion by RMS:

CA 8.1.3 Effects of active substance bioconcentration in prey of birds and mammals

This point is addressed in Document M-CP10, Sections CP 10.1.1 and CP 10.1.2.

CA 8.1.4 Effects on terrestrial vertebrate wildlife (birds, mammals, reptiles and amphibians)

No further data are presented. A literature review (Document M-CA9) did not reveal any relevant studies addressing the effects of aclonifen on terrestrial vertebrate wildlife.

CA 8.1.5 Endocrine disrupting properties

Data Point:	KCA 8.1.5/01
Report Author:	[redacted]
Report Year:	2020
Report Title:	Appendix I - Assessment of the endocrine disrupting properties of the active substance aclonifen in accordance with Commission Regulation (EU) 2018/605
Report No:	M-676736-01-1
Document No:	M-676736-01-1
Guideline(s) followed in study:	in Accordance with Commision Regulation (EU) 2018/605
Deviations from current test guideline:	--
Previous evaluation:	
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	

Executive Summary

The potential of aclonifen to interact with endocrine systems in birds and other terrestrial vertebrates has been reviewed to facilitate an assessment of whether aclonifen may be judged to be an endocrine disrupter (ED) within the framework of European legislation.

Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 has been published (EFSA/ECHA, JRC, 2018). This guidance document describes how to gather, evaluate and consider all relevant information for the assessment, conduct a

MoA analysis, and apply a WoE approach, in order to establish whether the ED criteria are fulfilled. The guidance states that a substance shall be considered as having endocrine disruption properties if it meets all of the following criteria:

i. It shows an adverse effect in an intact organism or its progeny, which is a change in the morphology, physiology, growth, development, reproduction, or, life span of an organism,
ii. of the capacity to compensate for additional stress, or an increase in susceptibility to other influences.
iii. It has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system.
iv. The adverse effect is a consequence of the endocrine mode of action.

Standard toxicology and ecotoxicology studies conducted to meet to the data requirements under Regulation (EU) 283/2013 have been submitted in this renewal dossier. A literature search was conducted to find relevant studies in the open literature conducted in the last 10 years. Further in vitro studies have been conducted to investigate EATS-mediated endocrine activity.

A summary of all relevant studies is provided in the excel spreadsheet Appendix E.

Overall conclusion on the ED assessment for birds and other terrestrial vertebrates

EAS and T modalities in mammals have been sufficiently investigated.

Aclonifen caused adversity and changes in thyroid hormones. The MoA analysis provided sufficient evidence to demonstrate that the most plausible MoA was via enhanced hepatic clearance of thyroid hormones.

Aclonifen is not an ED via the EAS-modality in mammals as there was no evidence of EAS adversity in *in vivo* studies. *In vitro* findings of endocrine activity via the A-modality were not replicated *in vivo*.

All parameters that were investigated in the Avian Reproduction test are either not assignable to any endocrine modality, or sensitive to, but not diagnostic of, EATS modalities.

According to the ED Guidance, investigation of ED properties in birds is currently hampered by a lack of test methods investigating endocrine specific endpoints. Once such methods become available, they should be considered in the ED assessment strategy with regard to non-target organisms. Information on birds is therefore given as supportive information only.

Assessment and conclusion by applicant: The review of the available data is considered to be acceptable and hence the conclusions drawn are considered to be valid.

Assessment and conclusion by RMS:

CA 8.2 Effects on aquatic organisms

Table 8.2-1: Summary of the effects of Aclonifen on aquatic organisms

Test species	Test item	Endpoint	Reference
Acute toxicity to fish			
Rainbow trout (*Oncorhynchus mykiss)*	Aclonifen	**96-Hour LC_{50} = 0.67 mg/L (nom)**	KCA 8.2.1/01 M-174317-01-1 [redacted], M.T., 1991
Common carp (*Cyprinus carpio*)	Aclonifen	96-Hour LC_{50} = 1.7 mg/L (nom)	KCA 8.2.1/02 M-174326-01-1 [redacted] 1991
Long-term and chronic toxicity to fish			
Rainbow trout (*Oncorhynchus mykiss)*	Aclonifen	21-Day NOEC = 0.10 mg/L (nom) 21-Day LC_{50} = 0.132 mg/L (nom)[1]	KCA 8.2.2/01 M-174328-01-1 [redacted] 1991
Rainbow trout (*Oncorhynchus mykiss)*	Aclonifen	21-Day NOEC = 0.0924 mg/L (mm) 21-Day LC_{50} = 0.200 mg/L (nom)[1]	KCA 8.2.2/02 M-174871-01-1 [redacted], C.A., 1993
Fathead minnow (*Pimephales promelas*)	Aclonifen	37-Day $NOEC_{hatchability}$ = 0.0091 mg/L (mm) 37-Day $NOEC_{survival}$ = 0.044 mg/L (mm) 37-Day $NOEC_{growth}$ = 0.004 mg/L (mm) 37-Day $EC_{10,hatchability}$ = ND 37-Day $EC_{10,survival}$ = ND 37-Day $EC_{10,growth}$ = ND[2]	KCA 8.2.2.1/01 M-174931-01-1 [redacted]. A., 1997
Fathead minnow (*Pimephales promelas*)	Aclonifen	4-Day $NOEC_{hatchability}$ = 0.117 mg/L (nom) 4-Day $EC_{10,hatchability}$ = ND	KCA 8.2.2.1/02 M-408628-01-1 [redacted], 2011
Fathead minnow (*Pimephales promelas*)	Aclonifen	**35-Day $NOEC_{survival}$ = 0.0425 mg/L (mm)** 35-Day $NOEC_{growth}$ = 0.106 mg/L (mm) 35-Day $EC_{10,survival}$ = ND 35-Day $EC_{10,growth}$ = ND	KCA 8.2.2.1/03 M-626723-01-1 [redacted], 2018
Bioconcentration in fish			
Rainbow trout (*Oncorhynchus mykiss)*	Aclonifen	BCF_K = 2248 L/kg[3]	KCA 8.2.2.3/01 M-174910-01-1 [redacted], L.E., 1995
Rainbow trout (*Oncorhynchus mykiss)*	Aclonifen	BCF_K = 1301 L/kg[3]	KCA 8.2.2.3/02 M-235029-01-1 [redacted] 1995
Rainbow trout (*Oncorhynchus mykiss)*	Aclonifen	BCF_K = 1169 L/kg[3]	KCA 8.2.2.3/03 M-235556-01-2 [redacted], 1992
Rainbow trout (*Oncorhynchus mykiss)*	Aclonifen	**BCF_{KgL} = 1349 L/kg**	KCA 8.2.2.3/04 M-667576-02-1 [redacted], H.S., 2019

Test species	Test item	Endpoint	Reference
Acute toxicity to aquatic invertebrates			
Daphnia magna	Aclonifen	**48-Hour EC_{50} = 1.2 mg/L (nom)**	[redacted] 1991 M-174313-01-1 KCA 8.2.4.1/01
Long-term and chronic toxicity to aquatic invertebrates			
Daphnia magna	Aclonifen	21-Day $NOEC_{reproduction}$ = 0.016 mg/L (mm) 21-Day $EC_{10,reproduction}$ = ND[4]	KCA 8.2.5.1/01 M-174321-01-1 [redacted] 1991
Daphnia magna	Aclonifen	**21-Day $NOEC_{body\ length}$ = 0.0042 mg/L (twa)** 21-Day $EC_{10,body\ length}$ = 0.0193 mg/L (twa)	KCA 8.2.5.1/02 M-573305-02-1 [redacted] 2017
Daphnia magna	Aclonifen	Pulse exposure (Days 0-2 and 7-9) 21-day $NOEC_{mortality,\ reproduction,\ body\ length}$ = 0.213 mg/L (gmm) 21-day $EC_{10,mortality,\ reproduction,\ body\ length}$ = ND	KCA 8.2.5.1/03 M-670399-01-1 [redacted], 2019
Daphnia magna	Aclonifen	Pulse exposure (Days 0-2 and 14-16) 21-day $NOEC_{mortality,\ reproduction,\ body\ length}$ < 0.237 mg/L (nom) 21-day $EC_{10,mortality,\ reproduction,\ body\ length}$ = ND	KCA 8.2.5.1/04 M-670403-01-1 [redacted], 2019
Development and emergence in *Chironomus riparius*			
Chironomus riparius	Aclonifen	**21-Day spiked water $NOEC_{emergence}$ = 0.472 mg/L (im)** 21-Day spiked water $EC_{10,emergence}$ = ND	KCA 8.2.5.3/01 M-174918-01-1 [redacted], 1996
Sediment dwelling organisms			
Chironomus riparius	Aclonifen	**28-Day spiked sediment $NOEC_{emergence}$ = 32 mg/kg (nom)** 28-Day spiked sediment $EC_{10,\ emergence}$ = 36 mg/kg (nom)	KCA 8.2.5.4/01 M-227300-01-1 [redacted] 2004 & KCA 8.2.5.4/02 M-674905-01-1 [redacted], 2019
Effects on growth of green algae			
Scenedesmus subspicatus	Aclonifen	$NOEC_{growth\ rate}$ (0 – 24h) = 0.0025 mg/L (nom) E_rC_{10} (0 – 24h) = ND E_rC_{50} (0 – 24h) = 0.0069 mg/L (nom)[5] $NOEC_{AUC}$ (0 – 96h) = 0.0025 mg/L (nom) E_bC_{10} (0 – 96h) = ND E_bC_{50} (0 – 96h) = 0.0067 mg/L (nom)	KCA 8.2.6.1/01 M-174303-01-1 [redacted] 1990
Scenedesmus subspicatus	Aclonifen	$NOEC_{growth\ rate}$ (0 – 96h) = 0.0055 mg/L (nom) E_rC_{10} (0 – 96h) = ND	KCA 8.2.6.1/02 M-201114-01-1

Test species	Test item	Endpoint	Reference
		E_rC_{50} (0 – 96h) > 0.046 mg/L (nom)[5] $NOEC_{AUC}$ (0 – 96h) = 0.0055 mg/L (nom) E_bC_{10} (0 – 96h) = ND E_bC_{50} (0 – 96h) = 0.0215 mg/L (nom)	[redacted], 2001
Desmodesmus subspicatus	Aclonifen	$NOEC_{growth\ rate}$ (0 – 96h) = 0.0000811 mg/L (mm) E_rC_{10} (0 – 96h) = 0.0104 mg/L (mm) **E_rC_{50} (0 – 96h) = 0.0203 mg/L (mm)** $NOEC_{yield}$ (0 – 96h) = 0.0000811 mg/L (mm) E_yC_{10} (0 – 96h) = 0.0244 mg/L (mm) E_yC_{50} (0 – 96h) = 0.0107 mg/L (mm)	KCA 8.2.6.1/03 M-574872-02-1 [redacted], 2016
Effects on growth of an additional algal species			
Navicula pelliculosa	Aclonifen	$NOEC_{growth\ rate}$ (0 – 72h) = 0.23 mg/L (mm) E_rC_{10} (0 – 72h) = ND E_rC_{50} (0 – 72h) = 1.2 mg/L (mm)[5] $NOEC_{AUC}$ (0 – 72h) = 0.068 mg/L (mm) E_bC_{10} (0 – 72h) = ND E_bC_{50} (0 – 72h) = 0.47 mg/L (nom)	KCA 8.2.6.2/01 M-171422-01-1 [redacted], J.R., 1998
Chlorella vulgaris	Aclonifen	$NOEC_{growth\ rate}$ (0 – 72h) = 0.085 mg/L (mm) E_rC_{10} (0 – 72h) = 0.129 mg/L (mm) E_rC_{50} (0 – 72h) = 0.45 mg/L (mm)[5] $NOEC_{AUC}$ (0 – 72h) < 0.0375 mg/L (mm) E_bC_{10} (0 – 72h) = 0.0162 mg/L (mm) E_bC_{50} (0 – 72h) = 0.0868 mg/L (mm)	KCA 8.2.6.2/02 M-278578-02-1 [redacted], 2019
Chlamydomonas reinhardtii	Aclonifen	$NOEC_{growth\ rate}$ (0 – 72h) = 0.00342 mg/L (mm) E_rC_{10} (0 – 72h) = 0.0051 mg/L (mm) E_rC_{50} (0 – 72h) = 0.0753 mg/L (mm)[5] $NOEC_{AUC}$ (0 – 72h) = 0.00342 mg/L (mm) E_bC_{10} (0 – 72h) = 0.00243 mg/L (mm) E_bC_{50} (0 – 72h) = 0.0158 mg/L (mm)	
Xanthonema debile	Aclonifen	$NOEC_{growth\ rate}$ (0 – 72h) = 0.0456 mg/L (mm) E_rC_{10} (0 – 72h) = 0.108 mg/L (mm) E_rC_{50} (0 – 72h) = 0.319 mg/L (mm) $NOEC_{AUC}$ (0 – 72h) = 0.0066 mg/L (mm) E_bC_{10} (0 – 72h) = 0.0215 mg/L (mm) E_bC_{50} (0 – 72h) = 0.0.0987 mg/L (mm)	
Closterium cornu	Aclonifen	$NOEC_{growth\ rate}$ (0 – 72h) = 0.111 mg/L (mm)	

Test species	Test item	Endpoint	Reference
		E_rC_{10} (0 – 72h) = 0.0478 mg/L (mm) E_rC_{50} (0 – 72h) = 0.112 mg/L (mm) $NOEC_{AUC}$ (0 – 72h) < 0.0163 mg/L (mm) E_bC_{10} (0 – 72h) = 0.0195 mg/L (mm) E_bC_{50} (0 – 72h) = 0.0682 mg/L (mm)	
Synechococcus leopoliensis	Aclonifen	$NOEC_{growth\ rate}$ (0 – 72h) = 0.0193 mg/L (mm) E_rC_{10} (0 – 72h) = 0.0344 mg/L (mm) E_rC_{50} (0 – 72h) = 0.0749 mg/L (mm)[5] $NOEC_{AUC}$ (0 – 72h) = 0.0193 mg/L (mm) E_bC_{10} (0 – 72h) = 0.0201 mg/L (mm) E_bC_{50} (0 – 72h) = 0.037 mg/L (mm)	
Nannochloropsis limnetica	Aclonifen	$NOEC_{growth\ rate}$ (0 – 72h) = 0.263 mg/L (mm) E_rC_{10} (0 – 72h) = 0.389 mg/L (mm) E_rC_{50} (0 – 72h) = 0.513 mg/L (mm) $NOEC_{AUC}$ (0 – 72h) = 0.263 mg/L (mm) E_bC_{10} (0 – 72h) = 0.303 mg/L (mm) E_bC_{50} (0 – 72h) = 0.461 mg/L (mm)	
Synechococcus leopoliensis	Aclonifen	$NOEC_{growth\ rate}$ (0 – 96h) = 0.008 mg/L (gmm) E_rC_{10} (0 – 96h) = 0.0136 mg/L (gmm) E_rC_{50} (0 – 96h) = 0.644 mg/L (gmm) $NOEC_{yield}$ (0 – 96h) = 0.008 mg/L (gmm) E_yC_{10} (0 – 96h) = 0.0145 mg/L (gmm) E_yC_{50} (0 – 96h) = 0.0376 mg/L (gmm)	KCA 8.2.6.2/03 M-649614-01-1 [redacted], 2018
Navicula pelliculosa	Aclonifen	$NOEC_{growth\ rate}$ (0 – 96h) = 0.132 mg/L (gmm) E_rC_{10} (0 – 96h) = 0.231 mg/L (gmm) E_rC_{50} (0 – 96h) = 0.672 mg/L (gmm) $NOEC_{yield}$ (0 – 96h) = 0.132 mg/L (gmm) E_yC_{10} (0 – 96h) = 0.157 mg/L (gmm) E_yC_{50} (0 – 96h) = 0.305 mg/L (gmm)	KCA 8.2.6.2/04 M-648378-01-1 [redacted], 2018
Chlorella vulgaris	Aclonifen	$NOEC_{growth\ rate}$ (0 – 96h) = 0.0935 mg/L (gmm) E_rC_{10} (0 – 96h) = 0.132 mg/L (gmm) E_rC_{50} (0 – 96h) >1.583 mg/L (gmm) $NOEC_{yield}$ (0 – 96h) < 0.0935 mg/L (gmm) E_yC_{10} (0 – 96h) = 0.0563 mg/L (gmm) E_yC_{50} (0 – 96h) = 0.190mg/L (gmm)	KCA 8.2.6.2/05 M-646486-01-1 [redacted], 2018

Test species	Test item	Endpoint	Reference
Effects on aquatic macrophytes			
Lemna gibba	Aclonifen	$NOEC_{growth\ rate,\ dry\ weight}$ = 0.00200 mg/L (mm) E_rC_{10} (0 – 14d)$_{dry\ weight}$ = 0.000265 mg/L (mm) **E_rC_{50} (0 – 14d)$_{dry\ weight}$ = 0.0136 mg/L (mm)**	KCA 8.2.7/01 M-171423-01-1 [redacted] 1998 KCA 8.2.7/02 M-255537-01-1 [redacted], 2005
Ceratophyllum demersum	Aclonifen	Water-sediment system $NOEC_{growth\ rate,\ fresh\ weight}$ = 0.00056 mg/L (gmm) E_rC_{10} (0 – 14d)$_{fresh\ weight}$ = 0.00046 mg/L (gmm) E_rC_{50} (0 – 14d)$_{fresh\ weight}$ = 0.0108 µg/L (gmm)	KCA 8.2.7/03 M-408091-02-1 [redacted], 2018
Elodea canadensis	Aclonifen	Water-sediment system $NOEC_{growth\ rate,\ shoot\ length}$ ≥ 0.306 mg/L (gmm) E_rC_{10} (0 – 14d)$_{shoot\ length}$ = ND E_rC_{50} (0 – 14d)$_{shoot\ length}$ > 0.306 mg/L (gmm)	KCA 8.2.7/04 M-408117-02-1 [redacted], 2019
Cabomba caroliniana	Aclonifen	Water-sediment system $NOEC_{growth\ rate,\ shoot\ length}$ ≥ 0.0795 mg/L (gmm) E_rC_{10} (0 – 14d)$_{shoot\ length}$ = ND E_rC_{50} (0 – 14d)$_{shoot\ length}$ > 0.0795 mg/L (gmm)	KCA 8.2.7/05 M-408124-01-1 [redacted], 2011
Limnophila heterophylla	Aclonifen	Water-sediment system $NOEC_{growth\ rate,\ shoot\ length}$ = 0.089 mg/L (gmm) E_rC_{10} (0 – 14d)$_{shoot\ length}$ = 0.064 mg/L (gmm) E_rC_{50} (0 – 14d)$_{shoot\ length}$ = 0.122 mg/L (gmm)	KCA 8.2.7/06 M-408152-01-1 [redacted], 2011
Heteranthera zosterifolia	Aclonifen	Water-sediment system $NOEC_{growth\ rate,\ shoot\ length}$ = 0.0938 mg/L (gmm) E_rC_{10} (0 – 14d)$_{shoot\ length}$ = ND E_rC_{50} (0 – 14d)$_{shoot\ length}$ > 0.0985 mg/L (gmm)	KCA 8.2.7/07 M-408168-01-1 [redacted], 2011
Egeria densa	Aclonifen	Water-sediment system $NOEC_{growth\ rate,\ shoot\ length}$ ≥ 0.221 mg/L (gmm) E_rC_{10} (0 – 14d)$_{shoot\ length}$ = ND E_rC_{50} (0 – 14d)$_{shoot\ length}$ > 0.221 mg/L (gmm)	KCA 8.2.7/08 M-408189-01-1 [redacted], 2011
Myriophyllum spicatum	Aclonifen	Water-sediment system $NOEC_{growth\ rate,\ dry\ weight}$ = 0.00015 mg/L (gmm)	KCA 8.2.7/09 M-398530-01-1 [redacted] 2011

Test species	Test item	Endpoint	Reference
		E_rC_{10} (0 – 14d)$_{dry\ weight}$ = ND E_rC_{50} (0 – 14d)$_{dry\ weight}$ = 0.0421 mg/L (gmm)	KCA 8.2.7/10 M-543492-01-1 [redacted], 2016
Lemna gibba	Aclonifen	Water-sediment system $NOEC_{growth\ rate,\ frond\ number}$ = 0.024 mg/L (nom) E_rC_{10} (0 – 14d)$_{frond\ number}$ = 0.0388 mg/L (nom) E_rC_{50} (0 – 14d)$_{frond\ number}$ = 0.116 mg/L (nom)	KCA 8.2.7/11 M-263844-01-1 [redacted], 2006
Lemna gibba	Aclonifen	Peak exposure (Days 0, 3 and 6) $NOEC_{growth\ rate,\ frond\ number}$ = 0.0007 mg/L (nom) E_rC_{10} (0 – 14d)$_{frond\ number}$ = 0.0101 mg/L (nom) E_rC_{50} (0 – 14d)$_{frond\ number}$ = 0.104 mg/L (nom) Peak exposure (Days 0 and 7) $NOEC_{growth\ rate,\ frond\ area}$ = 0.0007 mg/L (nom) E_rC_{10} (0 – 14d)$_{frond\ area}$ = 0.0119 mg/L (nom) E_rC_{50} (0 – 14d)$_{frond\ area}$ = 0.127 mg/L (nom)	KCA 8.2.7/12 M-612847-01-1 [redacted], 2018
Lemna gibba	Aclonifen	Peak exposure (Days 0 and 3) $NOEC_{growth\ rate,\ frond\ number}$ = 0.0007 mg/L (nom) E_rC_{10} (0 – 14d)$_{frond\ number}$ = 0.0622 mg/L (nom) E_rC_{50} (0 – 14d)$_{frond\ number}$ = 0.600 mg/L (nom)	KCA 8.2.7/13 M-612732-01-1 [redacted], 2018

[1]: Study design and endpoint no longer required for the registration of active ingredients in the EU
[2]: Study does not meet the validity criteria of OECD 210 (2013)
[3]: Study does not meet the validity criteria of OECD 305-1 (2012)
[4]: Study does not meet the validity criteria of OECD 211 (2012)
[5]: Study does not meet the validity criteria of OECD 201 (2011)

Endpoints in **bold** were used in the risk assessment
ND: not determined
nom: nominal test concentrations
mm: mean measured test concentrations
twa: time-weighted average measured test concentrations
im: initial measured test concentrations
gmm: geometric mean measured test concentrations

CA 8.2.1 Acute toxicity to fish

Data Point:	KCA 8.2.1/01
Report Author:	[redacted]; [redacted]; [redacted]
Report Year:	1991
Report Title:	The acute toxicity of ACLONIFEN to Rainbow trout (Oncorhynchus mykiss)
Report No:	R007151
Document No:	M-174317-01-1
Guideline(s) followed in study:	OECD no. 203
Deviations from current test guideline:	Current Guideline: OECD 203, 2019 None
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The acute toxicity of aclonifen to rainbow trout, *Oncorhynchus mykiss*, was determined in a 96-hour, flow-through exposure. Test solutions were prepared using stock solutions prepared in Tween 80 acetone. Ten rainbow trout per test group were exposed to an untreated control, solvent control and nominal Aclonifen concentrations of 0.25, 0.44, 0.79, 1.4 and 2.5 mg/L. The total test period was96 hours. Samples for analytical confirmation of actual exposure concentrations were taken at the start and after 24 and 96 hours of exposure.9

Dissolved oxygen, pH, and temperature were measured in the controls and each test concentration at the beginning of the test and end of the test. Daily observations were made of mortality and symptoms of toxicity.

Analysis of test samples resulted in measured concentrations remaining within the range 89 to 110% throughout the study, with the exception of the highest tested concentration (2.5 mg/L). Settlement of undissolved test material was considered to have accounted for the low measured value (75%) at 24 hours. The results of the study were based on the nominal test concentrations.

The 96-Hour LC_{50} of Aclonifen to rainbow trout was determined to be 0.67 mg/L (confidence limits 0.52 – 0.84 mg/L). The NOEC was 0.25 mg/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Item: Aclonifen technical
2-chloro-6-nitro-3-phenoxyaniline

Batch no.: DA 618

Active Ingredient / Purity: 91.3%

Appearance: Green yellow powder

Date received: 20 June 1990

Storage: Room temperature, in the dark

Expiry date: December 1990

2.	**Test Organism:**	*Oncorhynchus mykiss*
	Mean length:	4.9 cm (± 0.6 cm)
	Mean weight:	1.75g (± 0.57g)
	Source:	[redacted]
	Feeding:	Commercial trout pellets daily. Discontinued 24 hours prior to study start
3.	**Test water:**	Laboratory tap water, dechlorinated by addition of sodium thiosulphate
	Total hardness:	350 mg/L as $CaCO_3$ Value was slightly higher than recommended but was not considered to have affected study results

B. STUDY DESIGN AND METHODS

1. In-life phase: 9 to 13 July 1990

2. Exposure conditions

Test vessels:	Glass aquaria containing 20 L test solution
Experimental design:	Five test concentrations (0.25, 0.44, 0.79, 1.4 and 2.5 mg/L) plus one control and one solvent control
Loading:	0.88 g bodyweight/L (static volume) 0.10 g bodyweight/L (volume in 24 hours)
Temperature:	14 ± 1°C
pH:	8.2
Dissolved oxygen:	>10.1 mg O_2/L
Aeration:	Continuous flow
Photoperiod:	16 h light: 8 h dark

3. Administration of the test item

Stock solution prepared in 10% Tween 80-acetone. Continuous flow apparatus set up 24 hours prior to study start to allow equilibration of test concentrations. Solutions supplied continuously to test aquaria at 148 mL/min by a Watson-Marlow ® multi-channel variable speed peristaltic pump with solvent stock solutions dosed by 2 Braun Perfusor ® triple channel syringe pumps at 0.3553 mL/h.

4. Measurements and observations

Observations for mortality were undertaken at 24, 48, 72 and 96 hours. Mortality was defined as absence of respiratory movement and absence of response to physical stimulation.

Dissolved oxygen concentrations and pH values were measured in all the test groups and the control and solvent control vessels at the beginning and at the end of the test. The temperature was recorded at 0, 24 and 48 hours.

Samples were taken from the solvent control and each test concentration for analysis at 0, 24 and 96 hours (end of the test).

5. Statistics/Data evaluation

The LC_{50} and associated 95% confidence limits were calculated following the method described by [redacted] (1952). The No Observed Effect Concentration (NOEC) was determined by visual inspection of the data.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Analysis of nominal test treatment concentrations 0.25, 0.44, 0.79 and 1.4 mg/L remained within 89 to 110% of nominal throughout the study. At the highest nominal test treatment concentration (2.5 mg/L) measured values were 83 and 75% of nominal at 0 and 24 hours, respectively, indicating some settlement of undissolved test substance. This slight reduction in measured concentration was not considered to affect the test results. Test results were calculated using nominal test concentrations since analysis had shown test substance stability over the period of the test.

Table: Measured concentrations of Aclonifen

Nominal concentration (mg/L)	Mean measured concn (mg/L)	Mean % of nominal	No. samples
Solvent control	<0.01	-	3
0.25	0.247	99	3
0.44	0.445	101	3
0.79	0.797	101	3
1.4	1.252	89	2
2.5	1.981	79	2

The validated method is summarised in Document M-CA4 (CA 4.1.2/57).

B. BIOLOGICAL DATA

The cumulative mortality of rainbow trout after 3, 6, 24, 48, 72 and 96 hours are presented in the following table.

Table: Cumulative mortality for rainbow trout from the exposure to Aclonifen

Nominal concentration (mg/L)	Cumulative mortality					
	3h	6h	24h	48h	72h	96h
Control	0	0	0	0	0	0
Solvent control	0	0	0	0	0	0
0.25	0	0	0	0	0	0
0.44	0	0	0	0	0	1
0.79	0	0	0	0	0	7
1.4	0	0	4	8	10	10
2.5	0	0	8	10	10	10

Symptoms of toxicity, other than death, were swimming at the surface, increased pigmentation, lethargy, slight and total loss of equilibrium, lying on the bottom and moribundity.

All chemical and physical parameters in the definitive test were within expected ranges.

Based on the observed mortality, the LC_{50} values at each observation point were determined to be:

Table: LC_{50} values from the exposure of rainbow trout *Oncorhynchus mykiss* to Aclonifen

Time (Hours)	LC_{50} (mg/L)	95% confidence limits (mg/L)
24	1.6	1.2 – 2.2
48	1.2	0.98 – 1.4
72	1.1	0.79 – 1.4
96	0.67	0.52 – 0.84
No Observed Effect Concentration (96 hours) = 0.25 mg/L		

C. VALIDITY CRITERIA

Validity criteria	Required (OECD 203, 2019)	Achieved
Mortality in controls	≤10%	0%
Dissolved oxygen concentration at the end of the test	>60% ASV	>9.8 mg/L >95% ASV
Analytical measurement of test concentrations	Compulsory	Performed

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal concentration (mg/L)
LC_{50} (96 hours)	0.67
95% confidence limits	0.52 – 0.84
NOEC	0.25

III. CONCLUSION

The 96-Hour LC_{50} of Aclonifen to rainbow trout, *Oncorhynchus mykiss*, was determined to be 0.67 mg/L (confidence limits 0.52 – 0.84 mg/L). The NOEC was 0.25 mg/L.

[redacted] *(1991)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The 96-Hour LC_{50} of Aclonifen to rainbow trout, *Oncorhynchus mykiss*, was determined to be 0.67 mg/L (confidence limits 0.52 – 0.84 mg/L). The NOEC was 0.25 mg/L.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.1/02
Report Author:	[redacted]; [redacted]; [redacted]
Report Year:	1991
Report Title:	The acute toxicity of ACLONIFEN to Common Carp (Cyprinus carpio)
Report No:	R007155
Document No:	M-174326-01-1
Guideline(s) followed in study:	OECD: 203
Deviations from current test guideline:	Current Guideline: OECD 203, 2019 None
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The acute toxicity of aclonifen to common carp, *Cyprinus carpio*, was determined in a 96-hour, flow-through exposure. Test solutions were prepared using stock solutions prepared in Tween 80 acetone. Ten common carp per test group were exposed to an untreated control, solvent control and nominal Aclonifen concentrations of 0.25, 0.44, 0.79, 1.4 and 2.5 mg/L. The total test period was 96 hours. Samples for analytical confirmation of actual exposure concentrations were taken at the start and after 24 and 96 hours of exposure.

Dissolved oxygen, pH, and temperature were measured in the controls and each test concentration at the beginning of the test and end of the test. Daily observations were made of mortality and symptoms of toxicity.

Analysis of test samples resulted in measured concentrations remaining within the range 82 to 99% throughout the study, with the exception of the highest tested concentration (2.5 mg/L). Undissolved test material in the analytical sample was considered to have accounted for the high measured value (183%) at 0 hours. The results of the study were based on the nominal test concentrations.

The 96-Hour LC_{50} of Aclonifen to common carp was determined to be 1.7 mg/L (confidence limits 1.2 – 2.5 mg/L). The NOEC was 0.44 mg/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen technical
   2-chloro-6-nitro-3-phenoxyaniline
   Batch no.: DA 618
   Active Ingredient / Purity: 91.3%
   Appearance: Green yellow powder
   Date received: 20 June 1990
   Storage: Room temperature, in the dark
   Expiry date: December 1990

2.	**Test Organism:**	*Cyprinus carpio*
	Mean length:	4.0 cm (± 0.3 cm)
	Mean weight:	2.64g (± 0.29g)
	Source:	[redacted]
	Feeding:	Commercial carp pellets daily. Discontinued 24 hours prior to study start
3.	**Test water:**	Laboratory tap water, dechlorinated by addition of sodium thiosulphate
	Total hardness:	350 mg/L as $CaCO_3$ Value was slightly higher than recommended but was not considered to have affected study results

B. STUDY DESIGN AND METHODS

1. In-life phase: 30 August to 3 September 1990

2. Exposure conditions:

Test vessels:	Glass aquaria, containing 20 L test solution
Experimental design:	Five test concentrations (0.25, 0.44, 0.79, 1.4 and 2.5 mg/L) plus one control and one solvent control
Loading:	0.16 g bodyweight/L (volume in 24 hours)
Temperature:	20 ± 1°C
pH:	8.0 – 8.2
Dissolved oxygen:	8.9 mg O_2/L
Aeration:	Continuous flow and via narrow bore glass tubes
Photoperiod:	16 h light: 8 h dark

3. Administration of the test item

Stock solution prepared in 10% Tween 80-acetone. Continuous flow apparatus set up 24 hours prior to study start to allow equilibration of test concentrations. Solutions supplied continuously to test aquaria at 118 mL/min by a Watson-Marlow ® multi=channel variable speed peristaltic pump with solvent stock solutions dosed by 2 Braun Perfusor ® triple channel syringe pumps at 0.3553 mL/h.

4. Measurements and observations

Observations for mortality were undertaken at 24, 48, 72 and 96 hours. Mortality was defined as absence of respiratory movement and absence of response to physical stimulation.

Dissolved oxygen concentrations and pH values were measured in all the test groups and the control and solvent control vessels at the beginning and at the end of the test. The temperature was recorded at 0, 24 and 48 hours.

Samples were taken from the solvent control and each test concentration for analysis at 0, 24 and 96 hours (end of the test).

5. Statistics/Data evaluation

The LC_{50} and associated 95% confidence limits were calculated following the method described by [redacted] (1952). The No Observed Effect Concentration (NOEC) was determined by visual inspection of the data.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Analysis of nominal test treatment concentrations 0.25, 0.44, 0.79 and 1.4 mg/L remained within 82 to 99% of nominal throughout the study. At the highest nominal test treatment concentration (2.5 mg/L) measured values were 183 and 85% of nominal at 0 and 24 hours, respectively. It was considered that the high value determined at 0 hours was attributable to undissolved test substance in the water sample. This increase in measured concentration was not considered to affect the test results. Test results were calculated using nominal test concentrations since analysis had shown test substance stability over the period of the test.

Table: Measured concentrations of aclonifen

Nominal concentration (mg/L)	Mean measured concn (mg/L)	Mean % of nominal	No. samples
Solvent control	Not detected		3
0.25	0.231	93	3
0.44	0.397	90	3
0.79	0.764	97	3
1.4	1.212	87	3
2.5	3.353	134	2

The validated method is summarised in Document M-CA4 (CA 4.1.2/58).

B. BIOLOGICAL DATA

The cumulative mortality of common carp are presented in the following table:

Table: Cumulative mortality for common carp from the exposure to Aclonifen

Nominal concentration (mg/L)	Cumulative mortality					
	3h	6h	24h	48h	72h	96h
Control	0	0	0	0	0	0
Solvent control	0	0	0	0	0	0
0.25	0	0	0	0	0	0
0.44	0	0	0	0	0	0
0.79	0	0	0	0	0	0
1.4	0	0	0	4	5	7
2.5	0	0	9	10	10	10

Symptoms of toxicity, other than death, were lethargy, slight and total loss of equilibrium and moribundity.

All chemical and physical parameters in the definitive test were within expected ranges.

Based on the observed mortality, the LC_{50} values at each observation point were determined to be:

Table: LC_{50} values from the exposure of common carp *Cyprinus carpio* to Aclonifen

Time (Hours)	LC_{50} (mg/L)	95% confidence limits (mg/L)
24	2.1	1.6 – 2.8
48	2.3	1.6 – 3.4
72	2.1	1.5 – 3.1
96	1.7	1.2 – 2.5
No Observed Effect Concentration (96 hours) = 0.44 mg/L		

C. VALIDITY CRITERIA

Validity criteria	Required (OECD 203, 2019)	Achieved
Mortality in controls	<10%	0%
Dissolved oxygen concentration at the end of the test	>60% ASV	>8.9 mg/L >98% ASV
Analytical measurement of test concentrations	Compulsory	Performed

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal concentration (mg/L)
LC_{50} (96 hours)	1.7
95% confidence limits	1.2 – 2.5
NOEC	0.44

III. CONCLUSION

The 96-Hour LC_{50} of Aclonifen to common carp, *Cyprinus carpio*, was determined to be 1.7 mg/L (confidence limits 1.2 – 2.5 mg/L). The NOEC was 0.44 mg/L.

Douglas, M.T. (1991)

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The 96-Hour LC_{50} of Aclonifen to common carp, *Cyprinus carpio*, was determined to be 1.7 mg/L (confidence limits 1.2 – 2.5 mg/L). The NOEC was 0.44 mg/L.

Assessment and conclusion by RMS:

CA 8.2.2 Long-term and chronic toxicity to fish

Data Point:	KCA 8.2.2/01
Report Author:	[redacted]; [redacted]; [redacted]
Report Year:	1991
Report Title:	The prolonged toxicity of ACLONIFEN to Rainbow trout (Oncorhynchus mykiss)
Report No:	R007156
Document No:	M-174328-01-1
Guideline(s) followed in study:	OECD: 204
Deviations from current test guideline:	Not applicable as OECD 204 guideline has been deleted and there is no equivalent current test guideline
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

In the previous submission (DAR, 2006), this study was evaluated and accepted as valid for risk assessment purposes. This study design and endpoint is no longer required for the registration of active ingredients in the EU and hence a summary of this study is not presented in this dossier.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.2/02
Report Author:	[redacted]
Report Year:	1993
Report Title:	Aclonifen: 21-day rainbow trout toxicity study under flow-through conditions Final report
Report No:	R007413
Document No:	M-174871-01-1
Guideline(s) followed in study:	OECD: 204
Deviations from current test guideline:	Not applicable as OECD 204 guideline has been deleted and there is no equivalent current test guideline
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

In the previous submission (DAR, 2006), this study was evaluated and accepted as valid for risk assessment purposes. This study design and endpoint is no longer required for the registration of active ingredients in the EU and hence a summary of this study is not presented in this dossier.

Assessment and conclusion by RMS:

CA 8.2.2.1 Fish early life stage toxicity test

Data Point:	KCA 8.2.2.1/01
Report Author:	[redacted]
Report Year:	1997
Report Title:	Aclonifen - Early life stage toxicity test to fathead minnow (pimephales promelas)
Report No:	R007440
Document No:	M-174931-01-1
Guideline(s) followed in study:	OECD: 210; USEPA (=EPA): 72-4
Deviations from current test guideline:	Current Guideline: OECD 210 2013 Only two replicate vessels per treatment group were used, variation in measured concentrations exceeded the validity criterion of ±20% and dissolved oxygen was not maintained at >60% throughout the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

The purpose of the study was to evaluate the effects of aclonifen to fathead minnow *(Pimephales promelas)* under flow-through test conditions, during an exposure period of 35 days.

160 embryo (2 replicates of 80) were exposed to each of five concentrations of the test substance, a solvent (DMF) control and a dilution water control. Following hatching of the embryo (completed on day 4 of the test), 80 newly hatched fry (2 replicates of 40) were exposed to the same concentrations of the test substance for a further 31 days.

Analytical verification of the nominal concentrations of test substance at test initiation showed the measured concentrations were close to the nominal values (70 - 96% recovery). Further analytical verifications once a week during the test period and at test termination, showed that the nominal concentrations of test substance were generally maintained by the flow-through test system (58- 101% recovery). The majority of recoveries were within the range 71 - 101% of nominal values, with three lower values of 58, 62 and 64% observed at the lowest concentration of 4.0 µg/L.

The results of the test are reported in terms of the measured concentrations recorded during the test period. These concentrations were as follows; 4.0, 9.1, 19.7 44.0 and 103.6 µg/L.

Following completion of hatch; 95.0, 93.8, 91.3, 90.6 and 95.7% live normal fry were recorded at the concentrations of 4.0, 9.1, 19.7, 44.0 and 103.6 µg/L. In the control and solvent control groups 93.2 and 97.5% hatch was recorded respectively. At test termination the percentage survival of fry recorded at these same concentrations was 100, 98.8, 96.3, 98.8 and 91.3%. In the control and solvent control groups 98.8 and 100% survival was recorded.

The lengths and weights of all live normal fry in the controls and at each test concentration at test termination were 205.3, 216.8, 225.5, 178.2, 193.8, 198.4 and 163.3 mg in the control, solvent control and at the concentrations of: 4.0, 9.1, 19.7, 44.0 and 103.6 µg/L, respectively. The mean total length of the test fish at test termination was 28.1, 28.0, 28.2, 26.6, 26.3, 26.5 and 25.8 mm respectively in these same test groups.

No statistically significant difference was observed between the control and solvent control groups for any of the biological variables, therefore, the exposed groups were compared to the solvent control for subsequent comparisons. No statistically significant differences were observed between the replicates of any test group, replicates were pooled for subsequent analysis. Statistical analysis of the hatch and fry survival data showed that the percent hatch was significantly reduced compared to the solvent control at the concentrations of 19.7 and 44.0 µg/L, no significant difference was observed at the lower concentrations of 4.0 and 9.1 ug/1 or at the higher concentration of 103.6 µg/L. Percentage survival of fry at test termination was significantly reduced (α=0.01) compared to the solvent control at the highest concentration of 103.6 µg/L no significant difference was observed at the lower test concentrations.

Statistical analysis of the length and weight data showed significant differences occurred between the solvent control and the concentrations of 9.1, 19.7 and 44.0 µg/L. No significant difference occurred between the solvent control and the lowest concentration of 4.0 µg/L. The highest concentration of 103.6 µg/L was excluded from the analysis of length and weight variables.

Based on all of the statistical analyses of the test data, the no observed effect concentration (NOEC) was estimated to be 4.0 µg/L, and the lowest effect concentration (LOEC) was estimated to be 9.1 µg/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen
   Batch no.: 9536203
   Purity: 1000 g/kg
   Appearance: Yellow powder
   Date received: 15 June 2016
   Storage: Room temperature (ca. 20°C) in the dark
   Expiry date: 7 May 1998 (re-analysis date)

2. **Test Organism:** Fathead minnow (*Pimephales promelas*)
   Age: Freshly fertilised eggs, less than 24 hours old at the start of the study
   Source: [redacted]

3. **Test water:** Tap water filtered through activate charcoal and diluted with reverse osmosis deionised water
   Hardness: 40 – 48 mg $CaCO_3$/L

B. STUDY DESIGN AND METHODS

1. **In-life phase:** 24 July – 28 August 1997

2. Exposure conditions

Test vessels: Glass aquaria with a total volume of 20 L filled with approximately 15 L of test medium. Test aquaria were positioned in a water bath containing circulating water.
The embryos were incubated in egg incubation cups constructed from glass cylinder of 44 mm internal diameter. A nylon mesh (pore size 450 µm) support was attached using silicone sealant to the lower end. The egg cups were gently oscillated in the test solutions by means of a rocker arm apparatus driven by a 2 rpm electric motor.
During approximately 10 days post hatch, the fry were contained in an enclosure (inside the relevant aquarium) made of a nylon screen attached to a glass petri dish. The purpose of the fry enclosure is to facilitate feeding and biological observations when the fish are very small. A single fry enclosure was suspended in each test aquarium

Experimental design: Five nominal test concentrations of 5.0, 11, 24.2, 53.2 and 117.1 µg aclonifen/L plus a control and a solvent control

Replicates: Two replicate vessels were prepared for each treatment

Loading: 80 embryos per egg incubation cup
40 healthy fry released into fry enclosure (any remaining fry discarded)

Temperature: 25 ± 2°C

pH: 7.0 – 7.8

Dissolved oxygen: 4.8 – 8.0 mg/L (>60% oxygen saturation)

Aeration: No aeration

Photoperiod: 16 h light, 8 h dark with 30-minute transition periods

Light intensity: 364 – 560 lux

3. Administration of the test item

Dose preparation

Replicate test vessels (A and B) were employed for all concentrations and controls. For each test concentration, one stock solution was prepared approximately every 15 days by dissolution of the test substance in the solvent, dimethylformamide (DMF). At the nominal concentrations of 5.0, 11.0, 24.2, 53.2 and 117.1 µg/L, the concentrations of the stock solutions of test substance were 0.1, 0.22, 0.48, 1.06 and 2.34 mg of aclonifen per mL of solvent (DMF) respectively.

For each test solution (each replicate), a 25 ml plastic syringe in conjunction with a Harvard syringe pump was calibrated to deliver approximately 2.5 µL/min of the relevant stock solution into a chemical mixing chamber which also received approximately 50 mL/min of dilution water via a peristaltic pump.

The final concentration of solvent (DMF) in the solvent control and at each of the test substance concentrations was 0.05 mL/L.

Dosing system

The test was performed using an exposure system consisting of a continuous flow of fresh test solution, a temperature-controlled water bath and a set of 14 exposure aquaria (two replicate aquaria for each test group).

The test system was designed to provide five concentrations of the test substance, a solvent control and a dilution water control.

Test concentrations were maintained by introducing approximately 5.0 aquarium volumes per day, of newly prepared test solution via a constant flow system consisting of peristaltic pumps providing fixed flow rates of dilution water (approximately 50 mL/min.) and syringe injectors providing fixed volumes of the test substance stock solutions (approximately 2.5 µL/min). The test solutions homogenized in a pre-mixing chamber prior to delivery to the test aquaria. The test solutions in the mixing chamber were maintained in suspension by magnetic stirring. The flow through system was started at least 24 hours prior to test initiation.

Calibration by measuring delivery volumes of dilution water was performed the day before test initiation at test termination and once a week during the test period. The function of the system was visually inspected at least every 24 hours during the test period.

Analysis of the exposure solutions for the test substance concentration was also used to verify proper operation of the flow through system.

4. Test organism assignment and treatment

The test was initiated when the fertilized embryos (in the egg cups) were randomly placed in the test aquaria. Embryos which were not eyed, were opaque or showed any signs of coagulation and/or precipitation of protein were not used. The embryos were pooled in a clean glass dish with dilution water at 25°C ± 2°C. The viability of eggs used for test initiation was verified using a microscope. Viable embryos were randomly selected and assigned to egg cups at a rate of 5 at a time until each egg cup contained 80 embryos. The egg cups were immersed in dilution water at test temperature while they received embryos.

When the percentage embryo hatch for any individual test level was at least 90% complete or 48 hours after first hatch, 40 live healthy fry were released into the fry enclosure in the same aquarium and any remaining fry were discarded. The fry were fed live brine shrimp nauplii *(Anemia salind).* Feeding began on the same day the fry were transferred from the egg cups. The fish were fed three times per day on weekdays and twice daily on weekends. When the fry were approximately 10 days post hatch they were released into the main aquaria. At each feeding, fry were fed an excess of live brine shrimp *ad libitum* such that all fry were afforded equal access to food.

5. Measurements and observations

From the test initiation until hatching began (Day 3) the embryos in each egg cup were examined daily and the number of dead eggs was recorded. Dead eggs were discarded after counting. Once hatching began the eggs were not handled until about 90% of the embryos had hatched or 48 hours after first hatch was observed.

When hatching was complete the number of live normal, deformed, dead and unaccounted for fry were recorded. Following transfer of 40 fry into each aquarium (fry enclosure), observations of mortality, abnormal, behaviour and physical appearance were recorded daily. Fry enclosures and test aquaria were

cleaned when required. Uneaten food was removed by siphoning at least once every day from enclosures and aquaria.

After 31 days of post hatch exposure, the number of surviving fry in each test vessel was recorded. Surviving fry were anesthetized and the total length and wet weight of each individual were recorded.

Temperature, pH and dissolved oxygen were recorded in each test aquarium at test initiation, termination and three times a week during the test. The water temperature in one test aquarium was continuously recorded throughout the test period. Total hardness, alkalinity and specific conductance were measured from one replicate of the highest and lowest test substance concentrations, the solvent control and the dilution water control, at test initiation, termination and once a week during the test period. Light intensity (immediately above the test aquaria) was measured at test initiation and termination and once per week during the test period.

Samples (100 mL each) of each replicate at each treatment level were collected at test initiation (before the introduction of test embryos), at test termination and once per week during the duration of the test. All samples were collected in duplicate from the approximate midpoint of the test solutions. One sample was prepared immediately after sampling and analyzed within 24 hours after preparation. The second sample was appropriately preserved and stored in case required for verification purposes.

5. Statistics/Data evaluation

Statistical analysis was performed in three steps: comparison between the two replicates of each treatment level; comparison (when relevant) between the control and solvent control groups and comparison between the control (or solvent control) and test concentrations.

For each parameters and for each concentration, replicates A and B were compared using a t-test for length and weight data or Fisher's exact test (2 tails) for percentage embryo hatch and percentage of hatched eggs that produce normal live fry at test termination.

If statistical comparisons of the percentage embryo hatch, percentage of hatched eggs that produce normal live fry at test termination, weight or length of the dilution water control and solvent control groups establish that no significant differences existed (α=0.01) between the two replicates, the replicate A and the replicate B were pooled for subsequent comparisons.

For each parameter, the dilution water control group was compared to the solvent control group using Fisher's exact test (two tail) for percentage embryo hatch, percentage of hatched eggs that produce normal live fry at test termination and using F-test for results of length and weight variables. If the F-test was not significant, a t test was performed. If the F-test was significant, a modified t test was performed.

If no significant difference is observed between the dilution water control group and the solvent control group, the treatment group will be compared to the solvent control group for subsequent comparison.

Percentage embryo hatch, percentage of hatched eggs that produce normal live fry at test termination were analyzed by comparing each exposed group to the solvent control group using Fisher's exact test (1 tail).

Results of the length and weight variables of individually fish were intercompared for the exposed groups and the control solvent group by use of [redacted] test for homogeneity of variances, analysis of

variance (ANOVA). If Bartlett test indicated homogeneous variances and the ANOVA was significant, the exposed group means were intercompared to the solvent control group using the Dunnett test.

If Bartlett's test indicated heterogeneous variances, non-parametric statistical procedure was performed using the Kruskal-Wallis non-parametric one-way analysis of variance by ranks. If the Kruskal-Wallis test was significant, Mann-Whitney test was used to compare each group to the solvent control group mean.

Percentage embryo hatch data were analyzed before percentage of hatched eggs that produce normal live fry at test termination; if there was a concentration(s) that caused significant effects, then that treatment level was excluded from the statistical analysis of the survival, length and weight data unless there was a higher concentration without significant effects.

For comparative purposes, an additional comparison between the dilution water control group and each of the exposed groups was also performed for each parameter.

The alpha levels for each statistical comparison were 0.05 and 0.01.

Statistical analyses were performed using SAS programs.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Analytical verification of the nominal concentrations of test substance at test initiation showed the measured concentrations were close to the nominal values (70 - 96% recovery). Further analytical verifications once a week during the test period and at test termination, showed that the nominal concentrations of test substance were generally maintained by the flow-through test system (58 - 101% recovery). The majority of recoveries were between 71 - 101% of nominals, with three lower recoveries of 58, 62 and 64% observed at the lowest concentration of 4.0 µg/L. The majority of the recoveries were within 20% of the nominal test concentrations.

The results of the test are reported in terms of the measured concentrations recorded during the test period (4.0, 9.1, 19.7, 44.0 and 103.6 µg/L).

The flow-through system provided approximately 4.8 complete test solution renewals per 24-hour exposure period under the conditions of the test. All of the control samples were below the quantification limit (LoQ) for the test substance in the dilution water (0.5 µg/L).

Table: Mean measured test concentrations from the continuous exposure of Fathead minnows to Aclonifen

Nominal Concentration (µg/L)	Measured concentration (µg/L)						Mean measured concn (µg/L)	SD (µg/L)
	Day							
	0	7	14	21	28	35		
Control	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	-	-
	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ		
Solvent control	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	-	-
	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ		
5.0	4.8	4.4	4.3	3.7	3.8	3.2	4.0	0.6
	4.3	4.5	4.4	3.1	4.6	2.9		
11.0	10.6	9.3	9.1	8.8	8.8	9.2	9.1	0.7

	9.7	9.4	9.8	8.0	7.9	9.0		
24.2	19.7	21.9	20.2	19.2	17.2	17.4	19.7	1.6
	22	20.7	21.0	20.7	17.1	19.5		
52.3	45.3	44.1	47.8	45.9	40.5	43.5	44.0	2.9
	41.8	39.3	48.6	45.2	40.2	45.2		
117.1	112.1	118.7	92.9	101.1	111.2	114.6	103.6	11.2
	82.3	113.1	90.0	92.5	104.3	109.9		
Stability of 500 mg/L stock solution (DMF) prior to definitive test was 499 mg/L (day 0), 424 mg/L (day 6) and 477 mg/L (day 14)								

LOQ = Limit of Quantification = 0.5 µg/L

The validated method is summarised in Document M-CA4 (CA 4.1.2/82).

B. BIOLOGICAL DATA

Following completion of hatch the % survival was 95.0, 93.8, 91.3, 90.6 and 95.7% live normal fry at the concentrations of 4.0,9.1, 19.7, 44.0 and 103.6 µg/L. In the control and solvent control groups 93.2% and 97.5% hatch was recorded, respectively. At test termination the percentage survival of fry recorded at these same concentrations was 100, 98.8, 96.3, 98.8 and 91.3%. In the control and solvent control groups 98.8% and 100% survival was recorded, respectively.

Some embryo and fry remained unaccounted for on completion of hatch and also at test termination. The number of unaccounted embryo was most notable at the concentrations of 19.7 µg/L (replicate B) and 44.0 µg/L (replicate A). The number of unaccounted fry was most notable at the highest concentration of 103.6 µg/L (replicate B). Unaccounted for organisms were included with the mortality data for calculation of % hatch and % survival and for subsequent statistical analysis of these parameters. The statistical results for the % hatch and survival data should therefore be considered as worst-case conclusions.

The lengths and weights of all live normal fry in the controls and at each test concentration were recorded at test termination. The mean wet weights of the test fish were 205.3, 216.8, 225.5, 178.2, 193.8, 198.4 and 163.3 mg in the control, solvent control and at the concentrations of: 4.0, 9.1, 19.7, 44.0 and 103.6 µg/L respectively. The mean total length of the test fish at test termination was 28.1, 28.0, 28.2, 26.6, 26.3, 26.5 and 25.8 mm respectively in these same test groups. The length and weight data at test termination, showed that the test fish were somewhat smaller at the concentrations of 9.1, 19.7, 44.0 and 103.6 µg/L. However, no clear dose response was observed at the three intermediate concentrations of 9.1, 19.7 and 44.0 µg/L.

Table: Summary of effects from the continuous exposure of fathead minnow to Aclonifen

Mean measured concentration (µg/L)	Hatching success (%)	Post hatch survival (day 35) (%)	Total length, (cm)	Wet weight (mg)
Control	93.2	98.8	28.1	205.3
Solvent control	97.5	100	28.0	216.8
4.0	95.0	100	28.2	225.5
9.1	93.8	98.8	26.6**	178.2**
19.7	91.3*	96.3	26.3**	193.8**
44.0	90.6**	98.8	26.5**	198.4**
103.6	95.7	91.3**	25.8 [a]	163.3[a]

*	Significant compared to solvent control (α = 0.05)
**	Significant compared to solvent control (α = 0.01)
a	Excluded from statistical analyses

Statistical analysis

There was no significant difference between replicates of the control, solvent control or the test concentrations for any of the biological parameters (% hatch, % survival and length and weight data), therefore, the replicate data (A and B) of each of the treatment levels were pooled for further statistical comparisons.

Statistical analysis also determined that no statistically significant difference existed for % hatch, survival of fry, total length or wet weight data between the control and solvent control groups. The exposed groups were therefore compared to the solvent control group for subsequent comparisons.

Statistical analysis of the % hatch and fry survival data showed that the hatch was significantly reduced compared to the solvent control at the concentrations of 19.7 (α = 0.05) and 44.0 µg/L (α = 0.01). No significant difference was observed at the higher concentration of 103.6 µg/L or at the lower concentrations of 4.0 and 9.1 µg/L. Because no significant effect was observed at the highest concentration of 103.6 µg/L, it was decided not to exclude the lower concentrations of 19.7 and 44.0 µg/L from the subsequent analysis of survival, length and weight data.

Percentage survival of fry at test termination was significantly reduced (α = 0.01) compared to the solvent control at the highest concentration of 103.6 µg/L. No significant difference was observed at any of the lower test concentrations. This concentration was excluded from subsequent analysis of the length and weight data.

Statistical analysis of the total fish length data showed significant differences (α = 0.01) occurred between the solvent control and the concentrations of 9.1, 19.7 and 44.0 µg/L. No significant difference occurred between the solvent control and the lowest concentration of 4.0 µg/L. The highest concentration of 103.6 µg/L was excluded from the analysis.

Statistical analysis of the wet fish weight data showed significant differences occurred between the solvent control and the concentrations of 9.1, 19.7 (α = 0.01) and 44.0 (α = 0.05) µg/L. No significant difference occurred between the solvent control and the lowest concentration of 4.0 µg/L. The highest concentration of 103.6 µg/L was excluded from the analysis. For both the length and weight parameters, Bartlett's test was significant, therefore the non-parametric Kruskal-Wallis test and the Mann-Whitney test were used to perform the analyses.

Statistical analysis of the hatch and fry survival data showed no significant difference between the control and any of the test concentrations. Percentage survival at test termination was significantly reduced (α = 0.05) compared to the control at the highest concentration of 103.6 µg/L. No significant difference was observed at the lower test concentrations.

The weight of surviving fish was significantly lower (α = 0.01) than the control group at the concentration of 9.1 µg/L, no significant reduction was observed at any of the other test concentrations analyzed. The mean weight of the surviving fish at the concentration of 4.0 µg/L was significantly greater (α = 0.01) than the control group. This was not considered to be an adverse effect of the test substance.

The length of surviving fry was significantly different (α = 0.01) from the control group at the concentrations of 9.1, 19.7 and 44.0 µg/L. No significant difference occurred between the control and the lowest concentration of 4.0 µg/L. The highest concentration of 103.6 µg/L was excluded from the analysis.

In conclusion, although no significant differences were observed for the % hatch data and the length parameter was inconclusive compared to the dilution water control, the estimation of the NOEC and LOEC values are the same whether the exposed groups are compared to the dilution water control or solvent control groups.

C. VALIDITY CRITERIA

Validity criteria	Required (OECD 210, 2013)	Achieved
Dissolved oxygen concentration (% ASV)	>60%	≥84%
Water temperature between test chambers or between successive days at any time during the test	±1.5°C	Not recorded
Temperature range for test species	25±1.5°C	25 ± 0.4°C
Analytical verification of test concentrations	Compulsory	Yes
Overall survival of fertilised eggs (control)	≥70%	93.2%
Post-hatch success (control)	≥75%	98.8%

Validity criteria set out in OECD 210 (1992) and EPA/FIRA 72-4 (1986) relevant to percent hatch and survival were met. Validity criteria relevant to the current test guideline (OECD 210, 2013) for the control treatment percent hatch and survival were also satisfied. The dissolved oxygen concentration was not maintained at greater than 60% ASV at nominal exposure concentrations 4.0, 9.1, 19.7 and 44.0 µg a.s./L. Therefore, according to the current test guideline, this study is not valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Mean measured concentration (µg/L)	Hatching success	Post hatch survival (day 35)	Total length,	Wet weight
NOEC	9.1	44.0	4.0	4.0
LOEC	19.7	103.6	9.1	9.1

III. CONCLUSION

The early life stages of fathead minnow (*Pimephales promelas*) were examined under flow through conditions and exposure to aclonifen.

The overall NOEC was determined to be 4.0 µg/L, based on hatching success, post-hatch survival, total length and wet weight.

[redacted] *(1997)*

Assessment and conclusion by applicant:

Validity criteria set out in OECD 210 (1992) and EPA/FIRA 72-4 (1986) and in OECD 210, 2013 relevant to percent hatch and survival were met. However, the dissolved oxygen concentration was not maintained at greater than 60% ASV at all test concentrations throughout the study.

Analytical verification of the nominal concentrations of test substance at test initiation showed the measured concentrations were outside 100 ± 20% (70 - 96% recovery) and further analytical verifications during the test period and at test termination, showed that the nominal concentrations of test substance were maintained by the flow-through test system between 58 - 101% recovery. The majority of recoveries were between 71 - 101% of nominal, however, three lower recoveries of 58, 62 and 64% observed at the lowest nominal exposure concentration of 4.0 µg/L.

In addition to the above validity criteria deviations the study design also did not meet current guideline requirements. In this study only 2 replicates of 40 embryos were used, instead of 4 replicates of 20 embryos.

During statistical analysis of the study it was assumed that any unaccounted for embryos and fry should be considered mortalities. The number of unaccounted for embryos was most notable at nominal exposure concentrations of 19.7, 44.0 and 103.6 µg/L where this study also found statistically significant differences from the control for hatching success and post-hatch survival.

Therefore, this study does not meet current OECD guideline validity criteria and should be considered as being supportive only. A full assessment of the validity of this study is provided in KCA 8.2.2.1/04 (M-676414-01-1).

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.2.1/02
Report Author:	[redacted]
Report Year:	2011
Report Title:	Toxicity of aclonifen (techn.) to embryo and egg hatch life stages of fish (Pimephales promelas)
Report No:	EBCLX030
Document No:	M-408628-01-1
Guideline(s) followed in study:	EPA-FIFRA § 72-4a/SEP-EPA-560/6-82-002 (1982) ASTM E 1241-92 (1992) OPPTS 850.1400 (1996) OECD No. 210 (1992)
Deviations from current test guideline:	Current Guideline: OECD 210, 2013 None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to determine the toxicity of the test item to the embryo and egg hatching life stages of fathead minnow (*Pimephales promelas*) under semi-static conditions, expressed as NOEC and LOEC. The study was performed to investigate possible effects of Aclonifen on the hatching success of fathead minnows. The study was necessary to verify findings of an existing Fish Early-life Stage Test with fathead minnow (*Pimephales promelas*).

The earliest life stages of fathead minnow (embryos until egg hatching) were exposed to various test item concentrations, a control and a solvent control under semi-static conditions with four replicates per test level for 5 days (post hatch day 1). The definitive test was conducted at nominal test concentrations of 5.00, 11.0, 24.2, 53.2 and 117 µg a.s./L under semi-static conditions.

Recoveries of Aclonifen were measured in one alternating replicate in all newly prepared test media (study day 0 and 3) and in addition in all aged test media (study day 3 and 5).

Based on analytical measurements of the newly prepared test media recoveries between 111 and 116% of nominal were found. Regarding aged test media measured after 3 days or 2 days of use, respectively, the mean measured values still reflecting well the nominal values, the recoveries ranged between 106 and 115% of nominal. Therefore, all reported results refer to the nominal concentrations of Aclonifen.

Egg hatching began on study day 3 (post hatch day -1) and was completed on study day 5 (post hatch day 1) in all test levels, when all fertilised and living embryos successfully hatched. Start and end of hatching showed no significant difference compared to the pooled control data.

Post hatch day 0 was reached on study day 4, when 99% of all fertilised and living embryos in the pooled controls had hatched. On study day 4 (post hatch day 0) mean embryo survival / hatching success (based on the total number of inserted eggs) ranged overall between 87 and 93% and showed no significant difference in any test level compared to the pooled control data.

Based on statistical analysis of time to hatch and embryo survival / hatching success the NOEC was determined to be 117 µg a.s./L, the highest concentration tested.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen (tech.)
	Batch no.:	AE F068300
	Purity:	99.6% w/w
	Appearance:	Yellow powder
	Date received:	Not available
	Storage:	5°C ± 5°C
	Expiry date:	02 April 2018
2.	**Test Organism:**	Fathead minnow (*Pimephales promelas*)
	Age:	Freshly fertilised eggs, less than 24 hours old at the start of the study

Source: [redacted]

3. Test water: Reconstituted water (according to ISO)
Hardness: 40 – 60 mg/L (as $CaCO_3$)

B. STUDY DESIGN AND METHODS

1. In-life phase: 22 February – 05 May 2011

2. Exposure conditions

Test vessels: Glass crystallizing dish with a diameter of 140 mm (w) x 74 mm (h), filled up to about 35 mm. The test volumes amounted to 500 mL each. Each test vessel was covered with a glass petri dish plate (with a diameter of 145 mm) during exposure to avoid evaporation

Experimental design: Five nominal test concentrations of 5.0, 11.0, 24.2, 53.2 and 117 µg a.s./L plus one control and one solvent control (100 µL/L).

Replicates: Four replicate vessels were prepared for each treatment
Loading: 30 embryos per replicate vessel (120 embryos per treatment)
Temperature: 25 ± 2°C
pH: 6.0 – 8.0
Dissolved oxygen: > 60% oxygen saturation
Aeration: No aeration
Photoperiod: 16 h light: 8 h dark
Light intensity: 165 – 315 lux

3. Administration of the test item

Dose preparation and dosing

For the entire study, one series of stock solutions of the test item Aclonifen was prepared. The stock solutions were prepared by weighing the adequate amount of test item into the solvent dimethylformamide (DMF). Afterwards they were intensely stirred over 1 hour at room temperature. As solvent control a stock solution with pure dimethylformamide was used.

On study Day 0 and 3, respectively, new test solutions were prepared by adding 0.05 mL of each stock solution into each of the four replicates per test level with 500 mL test water, resulting in a solvent concentration of 100 µL dimethylformamide per litre test water and in a dilution factor of 10 000, except for the dilution water control group. Each test medium was mixed as homogeneously as possible after addition of the stock solution, with magnetic stir bars for approximately 10 minutes. In case of test medium renewal on day 3 old medium was decanted from each test vessel after all living eggs were carefully removed with a plastic pipette in another small vessel containing old medium. Then the new test medium was prepared as described above in the same test vessel as used before with the following exception that the test water used for all test vessels was tempered to 25°C before use on a temperature

controlled heating plate. Afterwards the eggs were carefully placed again in the corresponding test vessel with newly prepared test medium.

4. Test organism assignment and treatment

Fertilized eggs were distributed among the test vessels by adding groups of 5 eggs via a glass pipette, and this procedure was repeated until each test vessel contained the desired number of eggs.

5. Measurements and observations

Every day all test vessels were observed for embryo mortality, as discerned by a distinct change in coloration (white opaque appearance). Dead embryos were recorded and discarded. Hatched larvae were recorded and sacrificed. In this study the post-hatch period began on study Day 4 when 99% of all fertilised and living embryos in the control(s) had hatched. The study was terminated when all fertilised and living embryos in the controls had hatched, on study Day 5.

Dissolved oxygen (in percent saturation), the water temperature and the pH-value was measured in two alternating replicates of all test levels on study Days 0, 1, 2, 3, 4 and 5. On study Day 3, when the test medium was changed in all test vessels, water quality parameters were measured twice in the aged and the newly prepared test medium.

Samples of test solutions, including the control and the solvent control, were taken from alternating replicate test chambers on study Days 0 (new medium), 3 (aged and new medium) and 5 (aged medium) in order to measure actual test concentrations of Aclonifen.

Samples of newly prepared stock solutions were taken once during the test on study Day 0 and samples of aged stock solutions were taken on study Day 6 (corresponding to study Day 5 for all replicates C and D, because they were started with one day delay).

5. Statistics/Data evaluation

Biological data (embryo survival and hatching success) for the replicate test vessels of each concentration were grouped together for analysis. Replicate means were used for statistical analysis. For each parameter analysed the following statistical tests were conducted:

-Student t-test to determine if replicates A-D of the diluent control and the solvent control could be pooled

-Shapiro Wilk-test procedure in order to test the correspondence with normal distribution

-Levene's-test to check homogeneity of variances

Control data (control and solvent control) were pooled if the t-test criteria were met.

The percent data were arcsine transformed before analysis.

The William's test on multiple pair-wise comparisons was used subsequently to determine a significant difference between the treatment groups and the control.

Statistical analyses were conducted using a PC-based computer program (TOXRAT® Professional Version 2.10) developed by ToxRat Solutions GmbH, 52477 Alsdorf, Germany) with conclusions of statistical significance based on a 95% confidence level ($\alpha = 0.05$).

II. RESULTS AND DISCUSSION

A: ANALYTICAL VERIFICATION

Based on analytical measurements of the newly prepared test media recoveries between 111 and 116% of nominal were found. Regarding aged test media measured after 3 days or 2 days of use, respectively, the mean measured values still reflecting well the nominal values, the recoveries ranged between 106 and 115% of nominal. Therefore, all reported results refer to the nominal concentrations of Aclonifen.

Table: Measured test concentrations from the exposure of Fathead minnows to Aclonifen

Nominal Concentration (µg/L)	Study Day									
	0 (fresh)		3 (old)		3 (fresh)		5 (old)		Overall Mean	
	µg/L	%	µg/L	%	µg/L	%	µg/L	%	µg/L	%
Control	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
Solvent Control	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
5.00	5.91	118	5.76	115	5.72	114	5.71	114	5.78	116
11.0	12.3	112	12.0	109	12.9	117	12.3	112	12.4	113
24.2	26.9	111	27.1	112	27.7	114	27.1	112	27.2	112
53.2	59.3	111	57.7	108	60.7	114	55.9	105	58.4	110
117	129.0	110	121.0	103	130.0	111	128.0	109	127.0	109

LOQ: Limit of Quantitation = 0.500 mg/L

The validated method is summarised in Document M-CA4 (CA 4.1.2/88).

B: BIOLOGICAL DATA

Time to Hatch

Egg hatching began on study Day 3 (post hatch Day -1) and was completed on study Day 5 (post hatch Day 1) in all test levels, when all fertilised and living embryos successfully hatched. Start and end of hatching showed no significant difference compared to the pooled control data.

Embryo Survival / Hatching Success

Post hatch Day 0 was reached on study Day 4, when 99% of all fertilised and living embryos in the pooled controls had hatched. On study Day 4 (post hatch Day 0) mean embryo survival / hatching success (based on the total number of inserted eggs) ranged overall between 87 and 93% and showed no significant difference in any test level compared to the pooled control data.

Table: Summary of embryo survival/hatching success of Fathead Minnows exposed to Aclonifen on study day 4 (post hatch day 0)

Nominal Concentration (µg/L)	Embryo Survival = Hatching Success (Egg Hatch[1] in %)	Hatching Success (Egg Hatch[2] in %)
Control	92	99
Solvent Control	90	98
Pooled control	91	99
5.00	87	97
11.0	93	97
24.2	87	99
53.2	90	100
117	88	98

Egg Hatch 1 = hatch data (cumulative) =(no. of larvae)/(no. of inserted eggs on study Day 0)*100
Egg Hatch 2 = hatch data (cumulative) =(no. of larvae)/(no. of living and fertilized eggs on Day 4)*100

Based on statistical analysis of time to hatch and embryo survival / hatching success the NOEC was determined to be 117 µg a.s./L, the highest concentration tested.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 210, 2013)	Achieved
Dissolved oxygen concentration (% ASV)	>60%	≥83%
Water temperature between test chambers or between successive days at any time during the test	±1.5°C	±2.9°C*
Temperature range for test species	25±1.5°C	25±1.5°C
Analytical verification of test concentrations	Compulsory	Yes
Overall survival of fertilised eggs (control)	≥70%	≥98%
Post-hatch success (control)	≥75%	≥90%

* The difference of water temperatures was in nearly all test levels higher than 1.5°C between the study day 0 and study day 1, since the preparation of test media on study day 0 has to be performed using slightly to cold diluent water due to time limitations. Water temperatures measured on study day 0 ranged between 22.3°C and 23.2°C for all test levels. After exposure in the temperature controlled room over night the water temperatures ranged between 24.3°C and 25.2°C for all test levels on study day 1. This level of temperature was kept over the following days of exposure until test termination without any deviations, including the test medium exchange on study day 3, where 25°C-temperated diluent water was used for the preparation of new test media. Since this deviation similarly affected all test levels and was only observed on one day, this deviation was regarded to have no relevance on the further outcome of this study, because overall the development of embryos was not negatively influenced.

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Parameter	Nominal Concentration (µg/L)	
	NOEC	LOEC
Time to Hatch (study Day 3-5)	117	> 117
Embryo Survival, Hatching Success (study Day 4)	117	> 117

III. CONCLUSION

Based on statistical analysis of time to hatch and embryo survival / hatching success, Aclonifen was shown to have no effect at concentrations up to and including 117 µg a.s./L, the highest concentration tested.

[redacted] *(2011)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

Aclonifen was shown to have no effect on time to hatch and embryo survival / hatching success at concentrations up to and including 117 µg a.s./L, the highest concentration tested. Correspondingly, the NOEC was determined to be 117 µg a.s./L.

Due to the lack of significant effects, EC_{10} and EC_{20} values were not able to be calculated.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.2.1/03
Report Author:	[redacted]
Report Year:	2018
Report Title:	Early life stage toxicity test with fathead minnow (Pimephales promelas) under continuous flow through conditions and pulsed exposure - Aclonifen
Report No:	EBCL0003
Document No:	M-626723-01-1
Guideline(s) followed in study:	Test conditions following OECD 210 (2013). Guideline for Testing of Chemicals – Fish early life stage toxicity test
Deviations from current test guideline:	Current Guideline: OECD 210, 2013 None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A Fish Early Life Stage (FELS) Toxicity Test was performed to identify potential adverse effects of aclonifen to Fathead minnow (*Pimephales promelas*) following a continuous and a pulsed exposure to the test item.

The first approach was applied as continuous exposure with the goal to record and assess the effects of aclonifen to fish early life stages compared to existing data, including survival and growth. Five test concentrations of the test item were applied: 2.56, 6.40, 16, 40 and 100 µg aclonifen/L. In this continuous exposure setup, the eggs and larvae remained constantly exposed to the test item throughout the test period.

In the second part #2 of the study, a pulsed exposure scenario was applied at three concentration levels of the test item, 125, 250 and 500 µg aclonifen/L. The pulses were set as precisely as possible by transferring the fry chambers holding the larvae from the vessels containing the test substance to dilution water only and vice versa. Four dilution water control vessels were run in parallel to the treatment vessels. Within the controls, the same transfer procedures were applied as set for the treatment concentrations.

The first pulse was initiated at the day of fertilization (day 0) and was applied until 24 hours after hatch was completed (>90%) in controls. The first pulse was followed by a recovery period of 7 days where only dilution water was applied. The second peak was applied for 24 hours only. After completion of the second peak, the larvae were kept unexposed in dilution water under flow through conditions until test end.

The concentrations of the test item aclonifen were assessed by chemical analysis using LC/MSMS. The LOQ was set to 1.5 µg aclonifen/L. At test start, samples were taken from all test vessels in order to confirm correct dosing of the flow through device.

Continuous exposure

Mean measured concentrations in the test vessels of aclonifen treatments were calculated to be between 85.3% and 142.6% of the nominal concentrations. The mean measured concentrations of the single treatment steps were determined to be between 92.7 and 122.0% of the nominal concentrations. The mean measured concentrations were calculated to be 2.37, 7.22, 19.5, 42.5 and 106 µg aclonifen/L. As the mean measured test concentrations deviated by more than 20% of the nominal test concentrations, the evaluation of biological effects was based on the arithmetic mean measured concentrations.

Hatch of larvae was total in controls. No dose related impact could be observed. Post hatch survival was determined to be ≥ 90% in controls. Post hatch survival after 35 days, i.e. at test end, was significantly reduced at 105.8 µg aclonifen/L (NOEC: 42.5 µg aclonifen/L).

No substance related impact on individual length as well as wet and dry weight could be detected. Thus, the NOEC for growth was determined to be ≥ 105.8 µg aclonifen/L. Due to the lack of a clear dose response relationship, it was not possible to calculate an EC_{10} and an EC_{20}.

Pulsed exposure

The pulsed mean measured concentrations were 103, 210 and 508 µg aclonifen/L at nominal concentration levels 125, 250 and 500 µg/L, respectively.

No dose related impact on hatch could be observed. A significant decrease of post hatch survival rates after 35 days pf could be observed at 103, 210 and 508 µg aclonifen/L (NOEC: < 103 µg aclonifen/L). Furthermore, the post hatch survival rates decreased in a dose dependent manner. The maximum of mortality occurred within the first 14 days of in-life phase, thus, was clearly related to the aclonifen exposure. After 21 days, no further mortality of fish occurred.

Sufficient growth of larvae and juvenile fish could be confirmed for control fish. Although fish growth was impacted within the pulsed exposure period, finally, a recovery of growth performance could be observed for treated fish groups kept in dilution water until the end of the test period.

Finally, no substance related impact on individual length as well as wet and dry weights could be detected. Thus, the NOEC for fish growth was determined to be ≥ 508 µg aclonifen/L. Due to an effect size for fry mortality of already 47.9% compared to control at the lowest test concentration, it was not possible to derive an EC_{10} and EC_{20} for this parameter.

Conclusion

All results regarding biological effects following continuous and pulsed exposure to the test item are summarized in the following table:

Table: Summary of effects during the time course of the study (based on mean measured concentrations of aclonifen)

Parameter	Continuous exposure NOEC	Pulsed exposure NOEC
Hatching success	≥106 µg/L	>508 µg/L
Post-hatch survival at test end	42.5 µg/L	<103 µg/L

Individual length at test end	≥106 µg/L	>508 µg/L
Individual weight at test end	≥106 µg/L	>508 µg/L

It was not possible to determine EC_{10} or EC_{20} values from the generated data.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Item: Aclonifen
Batch no.: AE F068300-01-15
Purity: 99.5% w/w
Appearance: Yellow crystalline solid
Date received: 15 June 2016
Storage: 25°C ± 5°C
Expiry date: 15 February 2018

2. Test Organism: Fathead minnow (*Pimephales promelas*)
Age: Freshly fertilised eggs, less than 24 hours old at the start of the study
Source: [redacted]

3. Test water: Purified tap water was used according to the OECD-Guideline 210
Hardness: 1.1 – 1.2 mmol/L

B. STUDY DESIGN AND METHODS

1. In-life phase: 01 September – 30 October 2017

2. Exposure conditions

Test vessels: Glass aquaria with a total volume of 12 L filled with approximately 10 L of test medium. At test start, each test vessel was equipped with a fry cage, being an analytical sieve of stainless steel with a diameter of 10 cm and a brim height of 4.5 cm. The sieve net at the bottom had a mesh width of 355 µm. Each replicate group kept in an individual fry cage. The fry cages in the pulse setup was equipped with a flat petri dish. Placing the cage in a dish prevented dry fall of larvae during the transfer procedure.

Experimental design: *Continuous exposure:* Five nominal test concentrations of 2.56, 6.40, 16, 40 and 100 µg aclonifen/L plus a control.

	Pulsed exposure: Five nominal test concentrations of 125, 250 and 500 µg aclonifen/L plus a control
Replicates:	Four replicate vessels were prepared for each treatment
Loading:	20 embryos per replicate vessel (80 embryos per treatment)
Temperature:	25 ± 1.5°C
pH:	7.8 – 8.3
Dissolved oxygen:	> 60% oxygen saturation
Aeration:	No aeration
Photoperiod:	16 h light: 8 h dark
Light intensity:	Approximately 1000 lux

3. Administration of the test item

Dose preparation

For preparation of the test media, a primary stock solution was prepared. An appropriate amount of test item was weighed out and was dissolved in dilution water. A primary stock of small volume, i.e. 500 mL, was prepared and was treated by ultrasonification overnight. Ultrasonification of 1 hour duration was applied, followed by 2 hours with no treatment, followed by 1 hour treatment, and so forth. The primary stock solution was acidified before ultrasonic treatment in order to increase test item stability.

After overnight treatment, a secondary stock was prepared by transferring the primary stock to a 20 L glass bottle. The bottles were pre-filled with 10 L of dilution water, before the primary stock solution was added. Afterwards, the bottle was filled with dilution water to the final volume of 20 L.

The bottles were placed on a magnetic stirrer and were stirred overnight. In a final step, the aqueous secondary stock solutions were transferred to stainless steel tanks and were filled to their final volume. These solutions served as application solutions in the flow through device.

To achieve the final concentration in the test vessels, the application solutions were mixed with dilution water in adequate volumes via dosing pumps.

Dosing system

Controls and all test concentrations were run in 4 replicate aquaria, each. For each treatment plot, an individual dosage system consisting of two dosage pumps was used. Dilution water was pumped by a water dosage pump (membrane pump, Prominent, Heidelberg, Germany) into a mixing chamber, placed on a magnetic stirrer. An adequate amount of the stock solution was added into the magnetic stirrer via a stock solution dosage pump (membrane pump with a stainless steel head, Prominent, Heidelberg, Germany). The prepared test solution flows into the test vessels via flexible tubes, distributed to the four vessels by an electronically regulated distributor driven by compressed air. The dilution water control was served by dilution water only. For every test vessel an appropriate water flow rate was adjusted. A daily exchange rate of 5 volumes per vessel and day was applied.

Pulsed exposure

For the pulsed exposure, the pulses were set as precisely as possible by transferring the fry chambers holding the larvae from the vessels containing the test substance to dilution water only and vice versa.

The first pulse was initiated at the day of fertilization (day 0) and was applied until 24 hours after hatch was completed in controls. Hatch completion of ≥ 90% of living eggs was achieved at 6 dpf. Thus, the

first peak was finished after 7 days. The first peak set was followed by a recovery period of 7 days where only dilution water was applied. The second peak was applied at 14 dpf for 24 hours only. After completion of the second peak (15 dpf), the larvae were kept unexposed until test end.

4. Test organism assignment and treatment

At test start, 20 fertilized and randomized eggs were placed on stainless steel nets forming the bottom of fry cages fixed at the water surface of each test vessel. Each aquarium was equipped with one cage. 80 eggs (i.e. 4 x 20) were used for each test concentration.

5. Measurements and observations

One day after hatch of first larvae (e.g. from 7 dpf on (dpf = days post fertilization)), larvae were fed once daily with ground breeding food (TetraMin® Baby, Tetra Werke, Melle, Germany) and 24 h old brine shrimp nauplii (Artemia salina).

From day 14 (dpf) on, ground TetraMin® flakes were added once daily to the fish feed. Visual assessment of feeding (qualitative, and quantitative estimate of feed uptake during the in-life phase) was performed on each working day. After two weeks of exposure, the fish were transferred from the fry cages to the main water body of the test aquaria. In the continuous exposure conditions, fish larvae were held in fry cages until day 16 pf. For the pulsed exposure, the fish larvae were transferred on day 15 dpf, i.e. after the end of the second peak exposure. The fish larvae were transferred to the main vessel to ensure undisturbed growth of the animals up to test end.

Qualitative observations on hatching and survival were made daily. Dead embryos, larvae and juvenile fish were removed as soon as observed. Observations on abnormal appearance of behaviour were made daily, too.

After 16, 21, 28 and 35 dpf (continuous exposure), and after 15, 21, 28 and 35 dpf (pulsed exposure), larvae/juvenile fish were photographed and the survival rates as well as the individual lengths of the animals were determined. The pictures were assessed using digital image processing software (UTHSCSA ImageTool Version 3.0; University of Texas Health Science Center at San Antonio, USA).

At test end, the remaining fish of each test vessel were blotted dry and the individual wet weight of each fish were measured using an analytical balance. Afterwards, they were dried overnight in a cabinet dryer. The group dry weight was measured using an analytical balance. The single dry weight per fish was calculated by dividing the group dry weight by the number of surviving fish at test end.

5. Statistics/Data evaluation

For each endpoint, the NOEC was determined. All statistics were calculated using ToxRat® Professional 3.2.1.

For NOEC / LOEC determination, quantal data were arcsine-transformed prior to analysis. No Observed Effect Concentrations (NOEC) were calculated, using ANOVA, followed by Dunnett's, or Williams test or respective non-parametric approaches (e.g. Jonckheere- Terpsta test).

In the continuous exposure, the observed dose response relationship did not allow a suitable regression analysis, e.g. probit analysis. In the pulsed exposure, the maximum effect size of the most prominent effect, i.e. reduction of post hatch survival, was already about 40% compared to control. Thus, EC_{10} and EC_{20} could not be derived.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

In the continuous exposure, mean measured concentrations in the test vessels of aclonifen treatments were calculated to be between 85.3% and 142.6% of the nominal concentrations. The mean measured concentrations of the single treatment steps were determined to be between 92.7 and 122.0% of the nominal concentrations. The mean measured concentrations were calculated to be 2.37, 7.22, 19.5, 42.5 and 106 µg aclonifen/L.

The measured test concentrations deviate by more than 20% of the nominal test concentrations. Thus, the evaluation of biological effects was based on the arithmetic mean measured concentrations.

Table: Mean measured test concentrations from the continuous exposure of Fathead minnows to Aclonifen

Nominal Concentration (µg/L)	Mean Measured Concentration		% Nominal Concentration
	µg/L	SD	
Control	<LOQ	-	-
2.56	2.37	0.2	92.7
6.40	7.22	0.5	112.8
16.0	19.5	3.6	122.0
40.0	42.5	4.6	106.2
100	106	11.3	105.8

LOQ = Limit of Quantitation = 1.5 µg/L

For the pulsed exposure, samples from all test vessels were taken at start and end of each peak. Additionally, samples of water-only vessels were taken after the transfer in order to confirm the absence of the test item.

A final sampling of the water-only vessels was performed at test end. After transfer to the water only vessels minor amounts of aclonifen were detected for the nominal concentrations 250 and 500 µg/L. The amounts did not exceed 1% of nominal values and thus should have only minor impact. The pulse mean measured concentrations were 103, 210 and 508 µg aclonifen/L at nominal concentration levels of 125, 250 and 500 µg/L, respectively.

No aclonifen was found after the second transfer and at the end of in-life phase.

Table: Mean measured test concentrations from the pulsed exposure of Fathead minnows to Aclonifen

Nominal Concentration (µg/L)	Mean Measured Concentration		% Nominal Concentration
	µg/L	SD	
Control	<LOQ	-	-
125	103	6.8	82.4
250	210	19.8	84.0
500	508	28.5	101.5

LOQ = Limit of Quantitation = 1.5 µg/L

The validated method is summarised in Document M-CA4 (CA 4.1.2/77).

B. BIOLOGICAL DATA

Continuous exposure

Hatch of larvae started after day 4 post fertilization (pf). Mean hatching rates >90% in controls and in treatment groups were achieved after 6 days pf.

In the treatment concentrations, a slight decrease of survival rates could be observed. However a clear dose-relationship could not be detected.

Post hatch survival after 35 days, i.e. test end, were significantly reduced at a mean measured concentration of 106 µg aclonifen/L (NOEC = 42.5 µg aclonifen/L).

Due to the lack of a clear dose response relationship, it was not possible to calculate an EC_{10} and EC_{20} for any of the biological parameters.

No substance related impact on fish length could be observed (NOEC ≥ 106 µg aclonifen/L). No impact on individual wet and dry weight could be detected (NOEC ≥ 106 µg aclonifen/L).

Table: Summary of effects from the continuous exposure of Fathead minnows to Aclonifen

Mean Measured Concentration (µg/L)	Hatching success (%)	Post hatch survival (%)	Total length, (cm)	Wet weight (mg)	Dry weight (mg)
Control	100.0	90.0	1.95	76.1	17.2
2.37	100.0	67.5	2.04	91.0	20.5
7.22	100.0	73.8	2.04	89.5	20.0
19.5	92.5	75.4	1.97	87.7	19.5
42.5	98.8	78.6	1.96	79.4	17.3
106	100.0	62.5	1.88	70.2	15.5

[1]: Significant reduction compared to control. Jonckheere-Terpstra test, p>0.05, one-sided smaller

Pulsed exposure

Hatch of larvae started after day 4 post fertilization (pf). Mean hatching rates of ≥85% in controls and in treatment groups were achieved after 6 days pf. As 90% hatch in controls was observed on day 6 pf, the first exposure pulse was finished on day 7 pf. Following a 7-day recovery where all larvae were placed in dilution water, the second pulse was set on day 14 pf and was finished at day 15 pf.

Post hatch survival was first recorded on day 15 pf, before the fish were transferred to the main water body of the test vessels. Post hatch survival in controls was determined to be 87.4% for day 15 pf and decreased to 84.8% at test end.

In the treatment concentrations, a clear decrease of survival rates could be observed. Furthermore, post hatch survival decreased in a dose dependent manner.

The maximum of mortality occurred within the first 14 days of in life phase, thus, was clearly related to aclonifen exposure. After 21 days, no mortality of fish was observed.

Post hatch survival after 35 days, i.e. test end, were significantly reduced to 51.1, 32.6 and 16.3% at mean measured concentrations of 103, 210 and 508 µg aclonifen/L, respectively, (NOEC < 103 µg aclonifen/L).

Due to an effect size for fry mortality of already 47.9% compared to control at the lowest test concentration, it was not possible to derive an EC_{10} and EC_{20} for this parameter.

Length measurements on day 15 revealed a significant decrease at 508 µg aclonifen/L (NOEC: 210 µg aclonifen/L). The following length measurements on day 21 pf revealed a significant decrease at 210 and 508 µg aclonifen/L (NOEC: 103 µg aclonifen/L). On the following dates of measurement, no impact on fish length could be found. A NOEC for fish length at ≥ 508 µg aclonifen/L was determined. It can be postulated, that the remaining fish recovered from aclonifen exposure in the non-exposure phase, following day 15 pf.

No substance related impact on individual wet and dry weight could be detected. The NOEC for fish growth was determined to be ≥ 508 µg aclonifen/L.

Table: Summary of effects from the pulsed exposure of Fathead minnows to Aclonifen

Mean Measured Concentration (µg/L)	Hatching success (%)	Post hatch survival (%)	Total length, (cm)	Wet weight (mg)	Dry weight (mg)
Control	97.5	84.8	2.15	103.7	23.6
103	88.8	51.1[1]	2.22	108.5	26.4
210	95.0	32.6[1]	2.17	112.4	26.4
508	85.0	16.3[1]	2.44	154.4	38.4

[1]: Significant reduction compared to control. Williams test, p>0.05, one-sided smaller

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 210, 2013)	Achieved	
		Continuous exposure	Pulsed exposure
Dissolved oxygen concentration (% ASV)	≥60%	≥86%	≥77%
Water temperature between test chambers or between successive days at any time during the test	±1.5°C	<1.0°C	<1.0°C
Temperature range for test species	25±1.5°C	25±1.2°C	25±1.3°C
Analytical verification of test concentrations	Compulsory	Yes	Yes
Overall survival of fertilised eggs (control)	≥70%	90%	97.5%
Post-hatch success (control)	≥75%	90%	84.8%

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Parameter	Continuous exposure NOEC	Pulsed exposure NOEC
Hatching success	≥106 µg/L	>508 µg/L
Post-hatch survival at test end	42.5 µg/L	<103 µg/L
Individual length at test end	≥106 µg/L	>508 µg/L
Individual weight at test end	≥106 µg/L	>508 µg/L

It was not possible to determine EC_{10} or EC_{20} values from the generated data.

III. CONCLUSION

The early life stages of fathead minnow (*Pimephales promelas*) were examined under flow through conditions and exposure to aclonifen. The study was performed at five test concentrations under continuous exposure and at three concentrations applied in a pulsed exposure design with two pulses (1st pulse from test start to 24 hours post hatch, 2nd pulse starting at approximately 15 (i.e. 7 days after the end of the 1st pulse) days post fertilization (dpf) for 24 hours).

Continuous exposure

Hatch of larvae was total in controls. No dose related impact could be observed. Post hatch survival was determined to be ≥ 90% in controls.

Post hatch survival after 35 days, i.e. at test end, was significantly reduced at a mean measured concentration of 106 µg aclonifen/L (NOEC: 42.5 µg aclonifen/L).

No substance related impact on individual length as well as wet and dry weight could be detected. Thus, the NOEC for growth was determined to be ≥ 106 µg aclonifen/L.

Due to the lack of a clear dose response relationship, it was not possible to calculate an EC_{10}, EC_{20} and EC_{50} for any of the biological parameters.

Pulsed exposure

No dose related impact on hatch could be observed.

A significant decrease of survival rates could be observed at mean measured concentrations of 103, 210 and 508 µg aclonifen/L (NOEC: < 103 µg aclonifen/L). Furthermore, the post hatch survival rates decreased in a dose dependent manner.

The maximum of mortality occurred within the first 14 days of in-life phase, thus, was clearly related to the aclonifen exposure. After 21 days no further mortality of fish occurred.

Sufficient growth of larvae and juvenile fish could be confirmed for control fish. Although fish growth was impacted within the pulsed exposure period, finally, a recovery of growth performance could be observed for treated fish groups kept in dilution water until the end of the test period.

Finally, no substance related impact on individual length as well as wet and dry weights could be detected. Thus, the NOEC for fish growth was determined to be ≥ 508 µg aclonifen/L.

For post hatch survival on day 35 pf, due to an effect size for fry mortality of already 47.9% compared to control at the lowest test concentration, it was not possible to derive an EC_{10} and EC_{20} for this parameter.

[redacted] *(2018)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

Mean measured concentrations in the continuous exposure scenario ranged from 92.7 to 122.0% of nominal. Guideline recommendations are that measured concentrations should be used where measured test concentrations deviate by more than 20% of nominal concentrations. However, in this

instance statistical analyses were conducted using nominal concentrations. As measured concentrations were typically higher than nominal then basing statistical analyses on nominal concentrations represents a 'worst-case' outcome.

In the continuous exposure scenario, aclonifen was shown to have no effect on hatching success or growth at concentrations up to and including 106 µg a.s./L, the highest concentration tested. Correspondingly, the NOEC for these parameters was determined to be 106 µg a.s./L. Effects on post-hatch survival were observed and the NOEC for this parameter was determined to be 42.5 µg a.s./L.

In the pulsed exposure scenario, aclonifen was shown to have no effect on hatching success or growth at concentrations up to and including 508 µg a.s./L, the highest concentration tested. Correspondingly, the NOEC for these parameters was determined to be 508 µg a.s./L. Significant effects on post-hatch survival were observed and the NOEC for this parameter was determined to be less than 103 µg a.s./L, the lowest concentration tested.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.2.1/04
Report Author:	[redacted]
Report Year:	2020
Report Title:	Aclonifen: Re-evaluation of early life-stage (ELS) toxicity studies with aclonifen and fathead minnow, Pimephales promelas
Report No:	VC/19/016/01
Document No:	M-676414-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	--
Previous evaluation:	
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	

Executive Summary

Two fish early-life stage toxicity tests with aclonifen have been conducted, together with an additional bespoke study designed to determine the toxicity of aclonifen to the embryo and egg hatching life stage of fathead minnow, *Pimephales promelas*. The first study ([redacted] 1997, M-174931-01-1) was conducted according to OECD test guideline 210 (1992) and was completed in 1997. The second study ([redacted], 2018, M-626723-01-1) was conducted according to OECD test guideline 210 (2013) and was completed in 2018. An additional bespoke study ([redacted], 2011 M-408628-01-1) was designed to

follow as closely as possible several internationally accepted guidelines including OECD test guideline 210 (1992).

The data generated in these studies have been re-evaluated to define an appropriate regulatory endpoint for the risk assessment of aclonifen in aquatic organisms.

The study by [redacted] (2018) followed the current OECD test guideline, adopted 2013, and all validity criteria laid out in the current guideline were satisfied. Additionally, the study design permitted adequate statistical analysis be conducted in order to detect changes of biological importance. The endpoints determined in this study are therefore suitable for risk assessment purposes.

Based on the current re-evaluation, this study should be considered the key study for aclonifen and the endpoint for risk assessment should be 42.5 µg aclonifen/L, derived from post-hatch survival.

Sufficient deviations were identified in the earlier study ([redacted] 1997) for the study to be considered unreliable. This study is therefore considered to be a supplementary study.

(2020)

Assessment and conclusion by applicant:
The re-evaluation of the available study data is considered to be acceptable and hence the conclusions drawn are considered to be valid. The No Observed Effect Concentration of 42.5 µg aclonifen/L, derived from post-hatch survival is therefore considered the relevant chronic fish endpoint to be used for risk assessment purposes.

Assessment and conclusion by RMS:

CA 8.2.2.2 Fish full life cycle test

No data submitted. Whilst the bioconcentration factor (BCF) of aclonifen was determined to be > 1000, the time required to reach 95% depuration was determined to be less than 14 days (BCF = 1349 L/kg, t_{95D} = 7.38 days, see section CA 8.2.2.3/03). In addition, the LC_{50} for aclonifen was determined to be > 0.1 mg/L (LC_{50} = 0.67 mg/L, see section CA 8.2.1/01). A fish life cycle test with aclonifen is not therefore required.

CA 8.2.2.3 Bioconcentration in fish

Data Point:	KCA 8.2.2.3/01
Report Author:	[redacted]
Report Year:	1995
Report Title:	Aclonifen: Bioconcentration of (14C)-Residues in Rainbow Trout
Report No:	R007430
Document No:	M-174910-01-1
Guideline(s) followed in study:	OECD: 305E
Deviations from current test guideline:	Current Guideline: OECD 305-I, 2012 BCFk was not corrected for fish growth. Lipid content of fish not determined; no lipid correction of BCF. Variation in measured concentrations exceeded ±20%
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

The bioconcentration and depuration of (^{14}C)-aclonifen was determined in edible, nonedible and whole rainbow trout tissues using a flow-through test system.

The fish were continuously exposed to (^{14}C)-aclonifen at a mean measured concentration of 26.9 µg/L for a period of 28 days. Thereafter, the fish were transferred to clean tanks containing dilution water only for a depuration period of 20 days.

The range of (^{14}C)-residues in fish tissues from Day 10 to Day 28 of uptake were 31.35 to 46.0, 72.7 to 102 and 49.1 to 77.9 µg/g for edible, non-edible and whole fish tissues respectively.

After two days of depuration (since transfer of fish to clean tanks and dilution water only), approximately 45% (14.2 µg/g), 50% (36.6 µg/g) and 46% (22.8 µg/g) of the (^{14}C)-residues, relative to those residues at the end of the uptake phase, were detected in edible, non-edible and total fish tissues.

By the end of the 20-Day depuration period, 0.9% (0.271 µg/g), 1.2% (0.840 µg/g) and 1.0% (0.479 µg/g) of the (^{14}C)-residues remained in edible, non-edible and total fish tissues, relative to those residues at the end of the uptake phase.

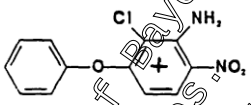
The time taken for 50% of the (^{14}C)-residues to be eliminated from fish tissues were 1.9, 2.3 and 2.0 days for edible, non-edible and whole fish tissues. The corresponding 95% elimination values were 8.3, 9.7 and 8.8 days.

Static bioconcentration factors (BCF's) for the uptake period of 10 to 28 days ranged from 1165 to 1710, 2703 to 3792 and 1825 to 2896 for edible non-edible and whole fish tissues respectively.

The kinetic bioconcentration factors (BCF_k) were 1369, 3344 and 2248 for edible, nonedible and whole fish tissues respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Non-radiolabelled test item:**	Aclonifen
	Batch no.:	MOY 1521
	Purity:	> 98%
	Appearance:	Yellow powder
	Date received:	Not provided
	Storage:	Approximately 4°C, in the dark
	Expiry date:	February 1998
2.	**Radiolabelled test item:**	(^{14}C)-Aclonifen
	Structure:	 + Position of ^{14}C-label
	Batch no.:	GHS 854
	Radiochemical purity:	99.1% (08-06-95)
	Specific activity:	728 MBq/mmol
	Date received:	Not provided
	Storage:	Glass bottle, 0 to 4°C
	Expiry date:	Not provided
2.	**Test Organism:**	Rainbow trout (*Oncorhynchus mykiss*)
	Age:	Juvenile
	Source:	[redacted]
	Feeding:	Proprietary food ([redacted] No. 4 Crumb)
	Mean fork length:	5.6 cm (range 5.1 cm to 6.4 cm)
	Mean weight:	2.23 g (range 1.88 g to 2.93 g)
	Acclimatization:	Acclimated to the test conditions for more than 14 days before use.
3.	**Test water:**	Laboratory mains supply. The water was pumped to the laboratory via a Purite AC9 activated carbon filter and a Purite ROPF 20 particulate filter, then heated to approximately 14°C, or chilled to the desired temperature within the laboratory
	Hardness:	46.3 - 51.7 mg/L as $CaCO_3$

B. STUDY DESIGN AND METHODS

1. In-life phase: 31 July – 04 October 1995

2. Exposure conditions

Test vessels:	122 L volume glass aquaria, containing 100 L of test medium
Experimental design:	Solvent control and single test concentration of 30 µg/L
Replicates:	One test vessel per group
Number of test organisms:	Solvent control: 40 fish Test item: 100 fish

Temperature:	12.0 – 14.2 °C
pH:	7.4 – 7.9
Dissolved oxygen:	62 – 100% ASV

3. Administration of the test item

Dose preparation and dosing

A radiodilution of aclonifen was prepared by mixing non-radiolabelled and (^{14}C)-aclonifen at a nominal ratio of 8.9/1 w/w (non-radiolabelled :radiolabelled) adding HPLC grade acetone. The total target concentration of aclonifen in the radiodilution was 4.2 mg/mL. The radiodilution was stored at approximately -20°C until use. The target specific radioactivity in the radiodilutions was 7.5 µCi/mg.

Every day during the uptake phase two volumes of a 0.7 mg/L (nominal) (^{14}C)-aclonifen stock solution were prepared by the addition of a volume of the radiodilution (as above) to 6 L of mains treated water. The solution was swirled then the mixing vessel topped up to the correct stock solution volume of 12 L. The mixing vessel contents were connected during their period of use in the test system. The nominal target exposure concentration of aclonifen in the test medium was 30 µg/L.

Test apparatus

The dilution water and prepared solvent stock solutions of the test item were pumped into the test vessels by means of Watson Marlow peristaltic pumps. The nominal flow rates of the dilution water and the stock solutions of the test item were 335 mL/min and 15 mL/min respectively.

Following the completion of the exposure phase, the fish were transferred to clean tanks, pre-filled with clean dilution water. Throughout the depuration phase the diluent pumps were adjusted to deliver an additional 15 mL/min (approximately) per test vessel.

4. Test organism assignment and treatment

Forty fish were allocated to the vessel containing the solvent control test medium and 100 fish were allocated to the test vessel containing (^{14}C)-aclonifen.

The fish were fed daily at a rate of approximately 2% wet body weight per day. Approximately two hours after feeding, the tanks were cleaned using a siphon tube to remove debris.

5. Measurements and observations

Samples of the stock solutions were taken daily. The test media from both test vessels was sampled daily during the uptake phase and daily until Day 16 and then on Days 19 and 20 during the depuration phase. Fish were removed from the control test vessel on Days 1 and 28 of uptake and Day 20 of depuration. From the test vessel containing (^{14}C)-aclonifen fish were removed on Days 0, 4, 10, 16, 22 and 28 of uptake and on Days 0, 2, 6, 10, 14 and 20 of depuration.

7. Statistics/Data evaluation

Uptake and depuration

The uptake rate constant k_1, was calculated using replicated data from sampled fish tissues (calculation performed using non-linear regression and software MINSQ, MicroMath Inc., USA); the depuration rate constant k_2 was calculated using the same software.

Bioconcentration

The static bioconcentration factors (BCFs) were calculated by dividing the fish ^{14}C-residue concentration by the mean measured concentration in the test medium. The kinetic bioconcentration factor (BCF_k) was calculated by dividing k_1 by k_2.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The mean measured concentration of (^{14}C)-residues during the 28-Day uptake phase of the test was 26.9 µg/L for a target concentration of 30 µg/L. Three of the 30 mean determinations (mean of triplicate samples) were less than 20% of the overall mean measured concentration and one mean determination was greater than 20% of the overall mean measured concentration.

Table: ^{14}C-residues (total radioactivity) as parent equivalents in the test media during the uptake phase

Time (days)	Measured concentration (µg/L)	Time (days)	Measured concentration (µg/L)
0 (-2 hours)	25	14	30
0 (+4 hours)	18	15	31
1	17	16	30
2	20	17	30
3	22	18	31
4	23	19	33
5	26	20	32
6	26	21	25
7	27	22	25
8	30	23	25
9	28	24	25
10	29	25	25
11	30	26	27
12	31	27	28
13	29	28	28
Mean measured concentration			**26.9**
Standard deviation			**3.99**

From the start to Day 3 of depuration the (^{14}C)-residue concentration fell from 7 to 4 µg/L. From Day 4 until the end of depuration the (^{14}C)-residue concentration was between zero and 2 µg/L.

B. BIOLOGICAL DATA

Throughout the exposure phase one fish in each of the control and aclonifen treated test medium died. There were no further observations of toxicity or mortalities throughout the exposure or depuration phase of the test.

Uptake of (^{14}C)-residues

The range of (^{14}C)-residues in fish tissues from Day 10 to Day 28 of uptake were 31.35 to 46.0, 72.7 to 102 and 49.1 to 77.9 µg/g for edible, non-edible and whole fish tissues respectively.

Depuration of (^{14}C)-residues

After two days of depuration, approximately 45% (14.2 µg/g), 50% (36.6 µg/g) and 46% (22.8 µg/g) of the (^{14}C)-residues, relative to those residues at the end of the uptake phase, were detected in edible, non-edible and total fish tissues.

By the end of the 20-Day depuration period, 0.9% (0.271 µg/g), 1.2% (0.840 µg/g) and 1.0% (0.479 µg/g) of the (^{14}C)-residues remained in edible, non-edible and total fish tissues, relative to those residues at the end of the uptake phase.

The time taken for 50% of the (^{14}C)-residues to be eliminated from fish tissues were 1.9, 2.3 and 2.0 days for edible, non-edible and whole fish tissues the corresponding 95% elimination values were 8.3, 9.7 and 8.8 days.

Table: ^{14}C-residues in edible and non edible parts and whole fish at mean measured concentration of 26.9 µg/L

Day	^{14}C-residue concentrations (µg/g)		
	Edible	Non edible	Total
Uptake phase			
0	4.44	7.36	5.65
4	22.1	52.7	34.5
10	33.2	89.4	57.3
16	46.0	102	77.9
22	40.1	99.2	63.5
28	31.35	72.7	49.1
Depuration phase			
0	28.4	70.3	45.9
2	14.2	36.6	22.8
6	4.80	14.2	8.50
10	1.31	3.54	2.23
14	0.602	1.73	1.05
20	0.271	0.84	0.479

Bioconcentration of (^{14}C)-residues

Static bioconcentration factors (BCF's) for the uptake period of 10 to 28 days ranged from 1165 to 1710, 2703 to 3792 and 1825 to 2896 for edible non-edible and whole fish tissues respectively.

Table: Bioconcentration factors for edible, non-edible tissues and total fish, exposed to (^{14}C)-aclonifen

Time (days)	Mean measured exposure concentration 26.9 µg/L		
	Edible	Non-edible	Total
0	165	274	210
4	822	1959	1283
10	1234	3323	2130
16	1710	3792	2896
22	1491	3688	2361
28	1165	2703	1825

The kinetic bioconcentration factors (BCF_k) were 1369, 3344 and 2248 for edible, non-edible and whole fish tissues respectively.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 305-I, 2012)	Achieved
Variation in water temperature	<±2°C	±1°C
Dissolved oxygen concentration (% saturation)	≥60%	≥60%
Limit in variation of measured test item concentration from the mean measured concentration	±20%	>20%
Test concentration	<limit of water solubility	Yes
Mortality or other adverse effects/disease	<10%	2.5% (max)

Three of the 30 mean determinations (mean of triplicate samples) for the measurement of test item concentrations were less than 20% of the overall mean measured concentration and one mean determination was greater than 20% of the overall mean measured concentration. Therefore, according to current validity criteria requirements the study is not valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Edible Tissue	Non-Edible Tissue	Whole Fish
Static bioconcentration factor (BCFs)	1710	3792	2896
Kinetic bioconcentration factor (BCFk)	1369	3344	2248
Depuration rate constant (day^{-1})	0.360	0.308	0.343

III. CONCLUSION

In fish continuously exposed to (^{14}C)-aclonifen at a mean measured concentration of 26.9 µg/L for a period of 28 days static bioconcentration factors (BCFs) for the uptake period of 10 to 28 days ranged from 1165 to 1710, 2703 to 3792 and 1825 to 2896 for edible non-edible and whole fish tissues respectively.

The kinetic bioconcentration factors (BCF_k) were 1369, 3344 and 2248 for edible, nonedible and whole fish tissues respectively.

The time taken for 50% of the (^{14}C)-residues to be eliminated from fish tissues were 1.9, 2.3 and 2.0 days for edible, non-edible and whole fish tissues. The corresponding 95% elimination values were 8.3, 9.7 and 8.8 days.

[redacted] *(1995)*

Assessment and conclusion by applicant:

In the previous submission (DAR, 2006), this study was evaluated and accepted as valid for risk assessment purposes. The study was conducted in accordance with OECD Guideline for Testing of Chemicals No. 305E (1981) and Draft OECD 305 (1992) and it was considered that all relevant validity criteria for the guidelines that were in force at the time of performing the study were satisfied.

The validity of the study has been re-evauluated against the current test guideline: OECD 305-I (2012) and the variation in measured concentrations exceeded the current validity criterion of ±20%. In addition, BCF_k was not corrected for fish growth and the lipid content of the fish was not determined so no lipid correction of BCF could be performed.

Therefore, as this study does not meet current OECD guideline validity criteria, it should be considered as supportive only and hence no summary for this study is provided.

A full assessment of the validity of this study is provided in KCA 8.2.2.3/05 (M-675783-01-1)

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.2.3/02
Report Author:	[redacted]
Report Year:	1995
Report Title:	Final report - Bioconcentration of (14C)-Aclonifen in Oncorhynchus mykiss under flow-through conditions
Report No:	C034500
Document No:	M-233029-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 305-I, 2012 BCF_k was not corrected for fish growth. Lipid content of fish not determined, no lipid correction of BCF. Variation in measured concentrations exceeded ±20%.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

The bioconcentration and depuration of (^{14}C)-aclonifen was determined in edible, non-edible and whole rainbow trout tissues using a flow-through test system.

The fish were continuously exposed to (^{14}C)-aclonifen at two mean measured concentrations, 4.24 and 37.7 µg/L for a period of eight days. Thereafter, the fish were transferred to clean tanks containing dilution water only for a depuration period of 14 days.

None of the fish exposed to (^{14}C)-aclonifen or in the solvent control vessel showed signs of toxicity throughout the test.

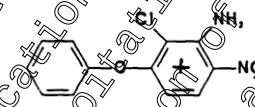
At both exposure concentrations a plateau of (^{14}C)-residues in fish tissues were reached within 1.8 to 3.5 days. Static bioconcentration factors (BCF_s) for the 4.24 and 37.7 µg/L exposure treatments over these periods averaged 841,1914 and 1284 for edible, non-edible and whole fish tissues respectively.

The calculated kinetic bioconcentration factors (BCF_k) for both exposure concentrations averaged 865, 1921 and 1301 for edible, non-edible and whole fish tissues respectively.

During depuration, 50% of the radioactivity present at the end of the uptake phase, was eliminated after an average of 0.868, 0.784 and 0.827 days for edible, non-edible and whole fish tissues respectively. By the end of the 14-Day depuration period an average of 0.6% of the radioactivity present at the end of the uptake phase, remained in the various fish tissues.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Non-radiolabelled test item:**	Aclonifen
	Batch no.:	MOY 1521
	Purity:	> 98%
	Appearance:	Yellow powder
	Date received:	Not provided
	Storage:	Approximately 4°C, in the dark
	Expiry date:	February 1998
2.	**Radiolabelled test item:**	(^{14}C)-Aclonifen
	Structure:	
		+ Position of ^{14}C-label
	Batch no.:	KWC-1846A
	Radiochemical purity:	99.1% (08-06-95)
	Specific activity:	19.8 mCi/mmol, 74.7 µCi/mg
	Date received:	Not provided
	Storage:	Approximately -20°C, in the dark
	Expiry date:	Not provided
2.	**Test Organism:**	Rainbow trout (*Oncorhynchus mykiss*)
	Age:	Juvenile
	Source:	[redacted]
	Feeding:	Proprietary food ([redacted]'s No. 4 Crumb)
	Mean fork length:	6.4 cm (range 5.3 cm to 7.0 cm)
	Mean weight:	3.3 g (range 1.7 g to 4.7 g)

	Acclimatization:	Acclimated to the test conditions for more than 14 days before use
3.	**Test water:**	Laboratory mains supply. The water was pumped to the laboratory via a Purite AC9 activated carbon filter and a Purite ROPF 20 particulate filter, then heated to approximately 16°C, or chilled to the desired temperature within the laboratory
	Hardness:	40.4 – 43.0 mg/L as $CaCO_3$

B. STUDY DESIGN AND METHODS

1. In-life phase: 31 July – 04 October 1995

2. Exposure conditions

Test vessels:	122 L volume glass aquaria, containing 100 L of test medium
Experimental design:	Solvent control, nominal test concentrations of 6 and 60 µg/L
Replicates:	One test vessel per group
Number of test organisms:	Solvent control: 40 fish Test item: 80 fish per concentration
Temperature:	14 - 16 °C
pH:	6.8 - 7.4
Dissolved oxygen:	78 - 99% ASV

3. Administration of the test item

Dose preparation and dosing

Two radiodilutions of aclonifen were prepared by mixing non-radiolabelled and (^{14}C)-aclonifen at a nominal ratio of 1:0.83 w/w (non-radiolabelled:radiolabelled) and 1:17.3 w/w (non-radiolabelled:radiolabelled). The total target concentration of aclonifen in the radiodilutions were 0.105 µg/µL and 1.05 µg/µL respectively. After the addition of the test item, approximately 5 mL of HPLC grade acetone was added to ensure thorough mixing. Excess organic solvent was removed using nitrogen convection, and the radiodilution stored at approximately -20°C until use. The target specific radioactivity in the radiodilutions was 68.588 µCi/mg.

On Day 7 of uptake the 0.105 mg/L stock solution was prepared by weighing the radiolabelled and non-radiolabelled test articles and dissolving in acetone. This solution was used to prepare the stock solution for the following 24-Hour exposure period.

On each day of the exposure phase (and during the pre-test) two 18 L volumes of each stock solution were prepared. Aliquots of the radiodilution were weighed and added to the dilution water with the addition of 1.8 mL acetone as an aid to dissolution of the test item.

Test apparatus

The dilution water and prepared solvent stock solutions of the test item and solvent control stock solution were pumped into the test vessels by means of Watson Marlow peristaltic pumps. The nominal flow rates of the dilution water and the stock solutions of the test item (and solvent control) were 330 mL/min and 20 mL/min respectively.

Following the completion of the exposure phase, the fish were transferred to clean tanks, pre-filled with clean dilution water. Throughout the depuration phase the diluent pumps were adjusted to deliver an additional 20 mL/min (approximately) per test vessel.

4. Test organism assignment and treatment

Forty fish were allocated to the vessel containing the solvent control test medium and 80 fish per vessel were allocated to the test vessels containing (^{14}C)-aclonifen.

The fish were fed daily at a rate of approximately 2% wet body weight per day. Approximately two hours after feeding, the tanks were cleaned using a siphon tube to remove debris.

5. Measurements and observations

The test media from all test vessels was sampled daily during the uptake and depuration phase. Fish were removed on Days 0, 1, 2, 4, 6, 7 and 8 of uptake and on Days 0, 1, 3 and 13 of depuration.

7. Statistics/Data evaluation

Uptake and depuration

The uptake rate constant k_1, was calculated using replicated data from sampled fish tissues (calculation performed using non-linear regression and software MINSQ, MicroMath Inc., USA); the depuration rate constant k_2 was calculated using the same software.

Statistical analysis of the uptake data was performed using Levenes test for homogeneity, one-way clarification of analysis of variance and Student-Newman-Keuls means comparisons.

Bioconcentration

The static bioconcentration factors (BCFs) were calculated by dividing the fish ^{14}C-residue concentration by the mean measured concentration in the test medium. The kinetic bioconcentration factor (BCF_k) was calculated by dividing k_1 by k_2.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The mean measured concentrations of (^{14}C)-residues during the eight day uptake phase of the test were 4.24 and 37.7 µg/L for the target concentrations of 6 and 60 µg/L respectively. Throughout depuration, the levels of radioactivity were below the limit of quantitation. The concentration of radioactivity in the control test medium was below the background determinations.

Table: ^{14}C-residues (total radioactivity) as parent equivalents in the test media during the uptake phase

Time (days)	Measured concentration (µg/L)			
	Nominal 6 µg/L	% of mean	Nominal 60 µg/L	% of mean
0	4.61	109	47.6	126
1	3.31	78	29.9	79
2	4.51	106	33.5	89
3	1.46	34	43.8	116

4	4.95	117	32.7	87
5	4.98	117	37.3	99
6	4.81	113	38.8	103
7	4.80	113	39.0	103
8	4.71	111	36	97
Mean measured concentration	**4.24**		**37.7**	
Standard deviation	**1.16**		**5.51**	

Chromatographic analysis of the water sampled from tanks at both exposure concentrations demonstrated that the major radiolabelled component shared chromatographic properties with the test substance. Low levels of two radiolabelled components were also detected, however due to their low concentration confirmation of their identity was not pursued.

Chromatographic analysis, using HPLC, of the extractable radioactive residues in the edible and non-edible fractions from rainbow trout (uptake phase Days 1 and 8, exposure concentration 4.24 µg/L), provided very similar qualitative and quantitative metabolite profiles. Identified/characterised extractable radioactive residues amounted to approximately 97% of the total radioactive residue (2.4 to 4.6 ppm) in the edible fraction and approximately 97% TRR (6 to 10 ppm) in the non-edible fraction at both time points. The major radioactive residue was identified by LC-MS as being the test substance, aclonifen.

Chromatographic analysis, using HPLC, of the extractable radioactive residues in the edible and non-edible fractions from rainbow trout (uptake phase days 1 and 8, exposure concentration 37.7 µg/L), provided very similar qualitative and quantitative metabolite profiles. Identified/characterised extractable radioactive residues amounted to approximately 100% TRR (31 to 35 ppm) in the edible fraction and approximately 93% TRR (70 to 69 ppm) in the non-edible fraction at both time points. The major radioactive residue was identified by LC-MS as being the test substance, aclonifen.

Chromatographic analysis, using HPLC, of the extractable radioactive residues in the edible and non-edible fractions from rainbow trout (depuration phase Day 18, both exposure concentrations), provided very similar qualitative and quantitative metabolite profiles. Identified/characterised extractable radioactive residues amounted to approximately 95% TRR (0.03 ppm) for fish sampled from the low exposure group and 19% TRR (0.03 ppm) for fish sampled from the high exposure group, the remainder of the radioactivity being present as polar metabolites whose identity was not pursued further due to limited sample availability. The major identified radioactive residue was test substance, aclonifen.

B. BIOLOGICAL DATA

Throughout the exposure and depuration phase there were no mortalities or any signs of toxicity to any fish.

Uptake of (^{14}C)-residues

Levels of (^{14}C)-residues reached an apparent plateau after approximately 1.8 and 3.5 days for the 4.24 and 37.7 µg/L exposure treatments respectively. Mean parent equivalent concentrations in test media containing 4.24 µg/L, throughout the plateau phase, ranged from 2.837 to 4.949 µg/g for edibles, 6.396 to 11.083 µg/g for nonedibles and 4.196 to 7.387 µg/g for total fish. The corresponding values for the 37.7µg/L exposure treatment were 28.037 to 42.813, 54.918 to 89.457 and 39.692 to 62.001 µg/g respectively.

Depuration of (^{14}C)-residues

After approximately 12 hours of depuration, approximately 59%, 51% and 55% of the (^{14}C)-residues, relative to the mean equilibrium concentrations during the uptake phase, were detected in edible, non-edible and total fish tissues respectively exposed to 4.24 µg/L. The corresponding values for fish exposed to 37.7 µg/L were 62%, 55% and 59% respectively.

By the end of the 14-Day depuration period, fish exposed to 4.24 µg/L, had 0.5%, 0.7% and 0.6% of the (^{14}C)-residues remaining in edible, non-edible and total fish tissues, relative to the mean equilibrium concentrations during the uptake phase. The corresponding values for fish tissues exposed to 37.7 µg/L were all 0.5%.

The time taken for 50% of the (^{14}C)-residues to be eliminated from fish tissues were 0.908, 0.673 and 0.766 days for edible, non-edible and whole fish tissues exposed to 4.24 µg/L and the corresponding values for fish exposed to 37.7 µg/L, 0.828, 0.895 and 0.888 days.

Table: ^{14}C-residues in edible and non-edible parts and whole fish during uptake and depuration of (^{14}C)-aclonifen at mean measured exposure concentrations of 4.24 and 37.7 µg/L

Time (Days)	^{14}C-residue concentrations (µg/g)					
	4.24 µg/L			37.7 µg/L		
	Edible	Non-edible	Total	Edible	Non-edible	Total
Uptake phase						
0.1	0.451	0.610	0.530	4.311	6.735	5.438
0.2	0.558	1.031	0.774	5.571	9.266	7.009
0.4	0.860	1.634	1.184	7.599	14.798	10.478
1.0	1.860	4.382	2.831	14.286	33.110	21.608
1.8	2.837	6.396	4.196	23.508	49.466	34.144
3.5	2.932	6.766	4.463	28.037	54.918	36.692
6.0	3.405	8.836	5.697	29.706	65.349	43.798
7.0	2.654	6.297	4.304	33.692	87.388	56.249
8.0	4.949	11.083	7.387	42.813	89.457	62.001
Depuration phase[1]						
0.5	1.978	4.032	2.860	20.903	40.724	29.523
1.0	1.692	3.188	2.302	14.340	42.011	26.435
2.0	0.800	1.137	0.960	7.150	13.671	10.062
4.0	0.246	0.678	0.429	2.275	6.212	3.817
14.0	0.015	0.052	0.030	0.157	0.386	0.256

[1]: Time refers to time after the start of depuration

Bioconcentration of (^{14}C)-residues

Static bioconcentration factors (BCF's) for uptake periods of 1.8 to 8 days for the 4.24 µg/L and 3.5 to 8 days for the 37.7 µg/L treatment, averaged 841, 1914 and 1284 for edible non-edible and whole fish tissues respectively.

Table: Static bioconcentration factors for edible, non-edible tissues and total fish, exposed to (^{14}C)-aclonifen

Time (Days)	Mean measured exposure concentration					
	4.24 µg/L			37.7 µg/L		
	Edible	Non-edible	Total	Edible	Non-edible	Total
0.1	106	144	125	114	179	144
0.2	132	243	183	1148	246	186
0.4	203	385	279	202	393	278

1.0	439	1033	668	379	878	573
1.8	669	1508	990	624	1312	906
3.5	691	1596	1053	744	1457	1053
6.0	803	2084	1344	788	1733	1162
7.0	625	1485	1015	894	2318	1492
8.0	1167	2614	1742	1136	2373	1645

The kinetic bioconcentration factor (BCF_k) for both exposure concentrations averaged 865, 1921 and 1301 for edible, non-edible and whole fish tissues respectively.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 305-I, 2012)	Achieved
Variation in water temperature	±2°C	<±1°C
Dissolved oxygen concentration (% saturation)	≥60%	≥60%
Limit in variation of measured test item concentration from the mean measured concentration	±20%	-65% to +26%
Test concentration	≤limit of water solubility	Yes
Mortality or other adverse effects/disease	<10%	0%

Two of the measured test item concentrations from each test vessel were outside of the ±20% of the overall mean measured concentration for each test concentration. Therefore according to current validity criteria requirements the study is not valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Edible Tissue	Non-Edible Tissue	Whole Fish
Static bioconcentration factor (BCFs)	841	1914	1284
Kinetic bioconcentration factor (BCFk)	865	1921	1301
Depuration rate constant (day^{-1})	0.800	0.902	1.343

III. CONCLUSION

From the observed data, a plateau of (^{14}C)-residues in fish tissues was reached within 1.8 to 3.5 days following the start of the exposure to (^{14}C)-aclonifen. Static bioconcentration factors (BCFs) for the 4.24 and 37.7 µg/L exposure treatments averaged 841, 1914 and 1284 for edible, non-edible and whole fish tissues respectively.

The calculated kinetic bioconcentration factors (BCFk) for both exposure concentrations averaged 865, 1921 and 1301 for edible, non-edible and whole fish tissues respectively.

During depuration, 50% of the radioactivity present at the end of the uptake phase, was eliminated after an average of 0.868, 0.783 and 0.827 days for edible, non-edible and whole fish tissues respectively. By the end of the 14-Day depuration period an average of 0.6% of the radioactivity present at the end of the uptake phase, remained in the various fish tissues.

[redacted] *(1995)*

Assessment and conclusion by applicant:

In the previous submission (DAR, 2006), this study was evaluated and accepted as valid for risk assessment purposes. The study was conducted in accordance with OECD Guideline for Testing of Chemicals No. 305E (1981) and Draft OECD 305 (1992) and it was considered that all relevant validity criteria for the guidelines that were in force at the time of performing the study were satisfied.

The validity of the study has been re-evauluated against the current test guideline, OECD 305-1 (2012) and the variation in measured concentrations exceeded the current validity criterion of ±20%. In addition, BCF_k was not corrected for fish growth and the lipid content of the fish was not determined so no lipid correction of BCF could be performed.

Therefore, as this study does not meet current OECD guideline validity criteria, it should be considered as supportive only and hence no summary for this study is provided.

A full assessment of the validity of this study is provided in KCA 8.2.2.3/05 (M-675783-01-1).

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.2.3/03
Report Author:	[redacted]
Report Year:	1992
Report Title:	Bio-accumulation of Aclonifen in Rainbow Trout Salmo gairdnerii Richardson
Report No:	C034783
Document No:	M-235556-01-2
Guideline(s) followed in study:	OECD: 305 E (1981)
Deviations from current test guideline:	OECD 305-1-2012. Yes. BCF_k was not corrected for fish growth. Lipid content of fish not determined, no lipid correction of BCF. Variation in measured concentrations exceeded ±20%.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The bioconcentration and depuration of aclonifen was determined in whole rainbow trout tissues using a flow-through test system.

The fish were continuously exposed to aclonifen at two nominal concentrations of 4.5 and 45.0 µg/L for a period of six days. Thereafter, the fish were transferred to clean tanks containing dilution water only for a depuration period of 12 days.

None of the fish exposed to aclonifen or in the control vessels showed signs of toxicity throughout the test.

Measurements for the analysis of residue in the whole fish body (based on kg fresh weight) at defined intervals showed at first a gradual rise of the Aclonifen quantity in both test concentrations. In the low concentration the maximum concentration was measured at 48 hours (with however, a subsequently lowered concentration in the water), and in the high concentration at 96 hours. The daily bio-concentration factors on the other hand, reached their maximum in both solutions at 96 hours with 1200 in the low and 993 in the high concentration. A noticeable steady-state plateau was not visible for the low concentration, but could perhaps lie between 48 and 96 hours. In the high concentration this takes place at 96 to 144 hours.

During the 12 day depuration phase without dosage of test substance, the concentration of aclonifen in the fish reduced within 30 hours to almost half of the value measured at the end of the uptake phase in the low concentration, in the high concentration to about one third.

The uptake rate constant (k_1), the depuration rate constant (k_2) and the steady-state bioconcentration factor (BCF) for the whole fish body were ascertained with the help of the approximate calculation given in Guideline OECD 305 E. For the low test concentration this resulted in a value of 1169, for the high concentration a value of 183. The BCF of the high concentration is as a result of this about a factor of 6 lower than the low concentration BCF. The theoretical BCF of the low concentration which was ascertained is comparable to the actual BCF at the period of the steady-state, whilst that of the high concentration is considerably less than the actual BCF value at the period of the steady-state.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Non-radiolabelled test item:**	Aclonifen
	Batch no.:	DA618
	Purity:	91.3%
	Appearance:	Green/yellow powder
	Date received:	29 May 1990
	Storage:	Approximately 7°C
	Expiry date:	Over 2 years
2.	**Test Organism:**	Rainbow trout (*Oncorhynchus mykiss*)
	Age:	Juvenile
	Source:	[redacted]
	Feeding:	Proprietary food ([redacted]'s No. 4 Crumb)
	Mean fork length:	6.4 cm (range 5.3 cm to 7.0 cm)
	Mean weight:	3.3 g (range 1.7 g to 4.7 g)

	Acclimatization:	Acclimated to the test conditions for more than 14 days before use
2.	**Test water:**	Laboratory mains supply filtered through activated charcoal activated carbon filter
	Hardness:	50 – 250 mg/L as $CaCO_3$

B. STUDY DESIGN AND METHODS

1. In-life phase: 22 April – 10 May 1992

2. Exposure conditions

Test vessels:	100 L glass aquaria, containing approximately 90 L of test medium
Experimental design:	Control, solvent control (50 µL/L acetone) and nominal test concentrations of 4.5 and 45.0 µg/L
Replicates:	One test vessel per group
Number of test organisms:	50 fish per test aquarium
Temperature:	14.5 – 16.7 °C
pH:	7.7 – 8.3
Dissolved oxygen:	70 - 97% ASV

3. Administration of the test item

Dose preparation and dosing

Tap water was pumped from a 700-L supply tank with a flow quantity of 500 mL/min/aquarium into the tank. This amounts to a water change approximately eight times per day. The test substance was continually dosed from a 20-L standard solution aquarium with a flow quantity of 5 mL/min/aquarium in such a way that it mixed evenly with the fresh water running into the aquarium.

The standard solutions in the 20-L aquaria were created every 48 hours with concentrations of 4.50 and 0.45 mg/L. This led to a dilution increment of 1:100. Acetone was used as the carrier solvent with a concentration of 0.05 mL/L in the test aquarium with 45.0 mg test substance per litre. Since the relation of test substance to carrier substance should remain the same, the Acetone concentration in the test aquarium with 4.5 g test substance amounted to 0.005 mL/L per litre.

In addition the test contained two control groups. The fish in the water control were only subject to the test water without the addition of any substance. The carrier substance concentration in the Acetone control was the same as that of the highest test concentration.

4. Test organism assignment and treatment

Fifty fish were allocated to each test vessel.

The fish were fed daily at a rate of approximately 2% wet body weight per day.

5. Measurements and observations

The test media from all test vessels was sampled .2, -1, -.075, 0, 0.25, 0.5, 1, 2, 4 and 6 days after addition of the fish to the test vessels. Fish were removed on Days 0.5, 1, 2, 4 and 6 of the uptake period.

7. Statistics/Data evaluation

Bioconcentration factors were determined following the recommendations of the Test Guideline.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The mean measured concentrations of aclonifen during the 6 day uptake phase of the test were 3.19 and 26.1 µg/L for the target concentrations of 4.5 and 45 µg/L respectively. With the exception of a single measured concentration of 2.35 µg/L in the high concentration at the start of the depuration period, throughout depuration the levels of aclonifen were below the limit of quantitation of the analytical method (0.5 µg/L).

The validated method is summarised in Document M-CA 4 (CA 4.1.2/98).

Table: Aclonifen concentrations in the test media during the uptake phase

Time (hours)	Measured concentration (µg/L)					
	4.5 µg/L	% Nom[1]	Deviation[2] (%)	45 µg/L	% Nom[1]	Deviation[2] (%)
0	3.81	84.6	+19.3	24.5	54.3	-6.5
6	3.20	71.2	+0.4	31.6	70.3	+21.1
12	<0.5	<0.5		32.3	71.1	+23.5
24	3.03	67.3	-5.1	29.9	66.6	+14.7
48	3.12	69.3	-2.3	23.2	51.5	-11.4
96	1.95	43.4	-38.8	19.0	42.3	-27.2
144	4.03	89.6	+26.4	22.4	49.8	-14.2
Mean measured concentration	**3.19**			**26.1**		

[1]: Percentage of nominal concentration
[2]: Percentage deviation from mean measured concentration

B. BIOLOGICAL DATA

Throughout the exposure and depuration phase there were no mortalities or any signs of toxicity to any fish.

Table: Aclonifen concentrations in whole fish at mean measured concentrations of 3.19 and 26.1 µg/L

Time (hours)	Measured concentration (µg/g)	
	3.19 µg/L	26.1 µg/L
Uptake phase		
12	2.18	12.01
24	2.36	11.48
48	2.74	15.17
96	2.34	18.19
144	1.83	18.24
Depuration phase		

174	<0.5	2.35
216	<0.5	<0.5
288	<0.5	<0.5
360	<0.5	<0.5
432	<0.5	<0.5

With the low test concentration there was no further accumulation of Aclonifen after 96 hours; a noticeable steady-state plateau was, however, not attained since the concentration in the fish body sank continuously in the period from 48 to 144 hours. Even so it cannot be ruled out that at the 96 h analysis a higher middle quantity of active agent in the fish would have been recorded, given a higher concentration of Aclonifen in the water. With the high test concentration it can be said that attainment of the state of equilibrium is reached at 96 hours.

Bioconcentration of aclonifen

Table: Static bioconcentration factors for whole fish, exposed to aclonifen

Time (hours)	Bioconcentration factors	
	3.19 µg/L	26.1 µg/L
12	699	372
24	779	383
48	878	655
96	1200	993
144	454	814

The kinetic bioconcentration factors (BCF_k) were determined to be 1169 and 183 for the nominal test concentrations of 4.5 and 45.0 µg/L respectively.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 305-I, 2012)	Achieved
Variation in water temperature	<±2°C	<±2.2°C
Dissolved oxygen concentration (% saturation)	≥60%	≥60%
Limit in variation of measured test item concentration from the mean measured concentration	±20%	-39% to +26%
Test concentration	<limit of water solubility	Yes
Mortality or other adverse effects/disease	<10%	0%

According to current validity criteria requirements the study is not valid due to the variation in water temperatures exceeding the allowable range, and measured concentrations being in excess of ±20% of the mean measured concentration.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	4.5 µg/L	45.0 µg/L

Static bioconcentration factor (BCFs) – maximum	1200	993
Kinetic bioconcentration factor (BCFk)	1169	183

III. CONCLUSION

Daily bioconcentration factors, reached their maximum in both solutions at 96 hours with 1200 in the low and 993 in the high concentration. A noticeable steady-state plateau was not visible for the low concentration, but could perhaps lie between 48 and 96 hours. In the high concentration this takes place at 96 to 144 hours.

During the 12 day depuration phase without dosage of test substance, the concentration of aclonifen in the fish reduced within 30 hours to almost half of the value measured at the end of the uptake phase in the low concentration, in the high concentration to about one third.

The uptake rate constant (k_1), the depuration rate constant (k_2) and the kinetic bioconcentration factor (BCF) for the whole fish body were ascertained with the help of the approximate calculation given in Guideline OECD 305 E. For the low test concentration this resulted in a value of 1169, for the high concentration a value of 183.

[redacted] *(1992)*

Assessment and conclusion by applicant

The validity of the study has been evaluated against the current test guideline, OECD 305-I (2012). Water temperatures exceeded the allowable range of ±2°C and the variation in measured concentrations exceeded the current validity criterion of ±20%. In addition, BCF_k was not corrected for fish growth and the lipid content of the fish was not determined so no lipid correction of BCF could be performed.

Therefore, as this study does not meet current OECD guideline validity criteria, it should be considered as supportive only and hence no summary for this study is provided.

A full assessment of the validity of this study is provided in KCA 8.2.2.3/05 (M-675783-01-1).

Assessment and conclusion by RMS

Data Point:	KCA 8.2.2.3/04
Report Author:	[redacted]; [redacted]; [redacted]
Report Year:	2019
Report Title:	Amendment no. 1 to final report - Aqueous exposure bioconcentration fish-test and biotransformation in fish (Oncorhynchus mykiss) - Aclonifen
Report No:	BAY-025/5-21/E
Document No:	M-667576-02-1
Guideline(s) followed in study:	OECD Test Guideline (TG) 305 SANCO/11 187/2013 rev. 3 (2013)
Deviations from current test guideline:	Current Guideline: OECD 305-I, 2012 None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to determine the bioaccumulative potential of the test item Aclonifen. The study was conducted under flow-through conditions according to the OECD Test Guideline 305 (2012) to determine a bioconcentration factor in rainbow trout (*Oncorhynchus mykiss*) with a nominal target concentration of 30 µg/L Aclonifen. The test item was applied as a blend of unlabelled and [^{14}C-] labelled in a ratio of approximately 1:1.

An untreated dilution water control was run in parallel to monitor natural mortalities and potential adverse effects of the test item. The test duration was 28 days uptake and 21 days depuration phase. Fish and water samples were collected during experimentation and analysed for Aclonifen (parent) to derive the uptake rate and depuration rate constants, as well as the bioconcentration factor in the state of equilibrium between uptake and elimination. The BCF was determined as steady-state BCF and as kinetic BCF.

Additionally, the biotransformation in fish was investigated by the qualitative and quantitative characterization of metabolites (≥ 10% of total radioactive residue and/or ≥ 0.05 mg/kg).

The mean water concentration was 28.3 µg/L parent test item and aside from Day 1, after introduction of the fish the water concentrations were within a range of ± 20% of the mean concentration during the whole exposure period.

The steady-state BCF (BCF$_{ss}$) was determined to be 1440 L/kg based on the average whole body concentrations at equilibrium phase between water and fish between Days 14 and 28. Lipid normalisation resulted in a lower lipid-normalised BCF$_{SSL}$ of 1364 L/kg, as the lipid content in treated fish was higher than 5% (5% is used as reference to which BCF is normalized to for inter-study and inter species comparability matters).

From the uptake rate constant of k_1 of 505, and the depuration rate constant k_2 of 0.376 the kinetic bioconcentration factor (BCF$_K$) was determined to be 1343 L/kg.

As fish grew during study, the BCF_K was corrected for growth-dilution effects into BCF_{Kg} 1425 L/kg incorporating the growth rate constant of 0.0214 d^{-1}. Lipid normalisation of BCF_{Kg} resulted in a BCF_{KgL} of 1349 L/kg.

The BCF_{KgL} for the whole fish is the most relevant BCF because it incorporates all measurements during uptake and depuration and the influence of the test fish growth and lipid content. The resulting BCF_{SSL} matches the BCF_{KgL} well, indicating a representative fitting of the fish matrix concentration data.

The time to reach a 50% tissue saturation in fish matrix was calculated to occur after 0.551 d (growth-corrected). The duration for further incorporation of test item to up to 95% of the steady state concentration was calculated to be reached after 9.73 d (growth-corrected). The half-life of the test item in fish was determined to be 1.33 d (growth-corrected). The elimination of 95% of test item in fish was determined to be 7.83 d (growth-corrected).

The metabolite analysis after 28 days exposure to 30 µg/L (nominal) Aclonifen, yielded one uncharacterized metabolite, which accounted for < 3% of TRR, but reaching an absolute concentration of 1.04 mg/kg in fish tissue.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Non-radiolabelled test item:** Aclonifen
   Batch no.: PLA1000563
   Purity: > 99.5% Aclonifen in dried material
   1.81% water in undried material
   (~ 97.7% a.s. in technical test item)
   Appearance: Yellow crystalline solid, powder
   Date received: 24 May 2018
   Storage: 25 ± 5 °C
   Expiry date: 02 May 2020

2. **Radiolabelled test item:** [aniline-U-L-^{14}C] Aclonifen
   Structure:

   * Position of ^{14}C-label
   Batch no.: KML 10557
   Radiochemical purity: > 99% Aclonifen in dried material
   Specific activity: 6.59 MBq/mg
   Date received: 29 January 2019
   Storage: ≤ -68°C
   Expiry date: 29 January 2020 (defined by Fraunhofer IME)

2. **Test Organism:** Rainbow trout (*Oncorhynchus mykiss*)
   Age: Juvenile

Source:	[redacted]
Feeding:	Commercial fish diet Inicio Plus, 2 mm biomar, Denmark
Total length:	7.47 ± 0.43 cm

3. Test water: De-chlorinated local tap water. The tap water was sourced from the Schmallenberg district water production plants, mostly fed by small springs and percolation. The purification process occured on-site at Fraunhofer IME and includes filtration with activated charcoal, passage through a lime-stone column, and aeration to the point of oxygen saturation. To avoid copper contamination, plastic water pipes are used in the test facilities.

B. STUDY DESIGN AND METHODS

1. In-life phase: 14 March – 02 May 2019

2. Exposure conditions

Test vessels:	100 L glass aquaria filled with 75 L of test solution
Experimental design:	Control and single test concentration of 30 µg/L
Replicates:	One test vessel per group
Number of test organisms:	109 fish per test group
Temperature:	13.0 – 15.0 °C
pH:	6.76 – 7.87
Dissolved oxygen:	69 – 108% ASV

3. Administration of the test item

Dose preparation and dosing

At the beginning of experimentation an adequately concentrated stock solution of the test item in solvent (acetonitrile) was prepared for the whole period of performance. For the radioactive stock solution 232.2 mg of the radiolabelled test item were dissolved in 50 mL acetonitrile. A second stock solution of 708.7 mg non-radio labelled test item was prepared by diluting the Aclonifen in 50 mL acetonitrile. The complete radio labelled solution was mixed with 28 mL of the non-radio labelled solution and filled up to 230 mL with acetonitrile to obtain a concentration of 33.2 mg Aclonifen/L as stock solution.

An intermediate dilution was prepared for every day to enrich the flow through water with test item to the desired concentration. Therefore, 5 mL of the stock was transferred into a separate 500 mL glass bottle, which was stored at ≤ -18 °C until use.

For the daily preparation of the 1st intermediate stock solution (ISS), the solvent was evaporated from the 500 mL bottle by flushing with nitrogen. 200 mL dilution water and 2.7 mL HCL (37%) was added and the solution was stirred with a magnetic agitator. Thereafter, the bottle was filled to approximately 500 mL with dilution water and put into an ultrasonic bath overnight with a pulse protocol of 1 h sonication and 2 h pause.

The 2nd ISS was prepared the next day by transferring the complete mix of the 1st ISS into a 10 Lbrown glass bottle with screw caps, which already contained 2 L of dilution water. The mixture was stirred all the time with a magnetic agitator. The empty 500 mL bottle was rinsed 2 times with dilution water, the wash was combined into the total volume of the 2nd ISS. The latter mix was filled to a total volume of 10 L with dilution water and was stirred overnight.

The next day the 2nd ISS (10 L) was mixed with 80 L dilution water to prepare the daily reservoir (total volume of 90 L) for the aquarium (1:10 dilution) in a stainless steel basin covered from light.

Test apparatus

The test vessels were 100 L glass aquaria filled with 75 L of test solution (treatment) or dilution water (control). To achieve a 5-fold exchange per day, as recommended by OECD 305, a continuous flow of 15.6 L/h test solution (uptake phase) or water (depuration phase) was maintained throughout the test using a metering pump system. The same flow through rates of dilution water were applied in the control vessel. The metering pump system was set to combine 3.5 L/h of the daily dilution with 12.1 L/h dilution water resulting in a constantly applied test concentration of 30 µg/L in the treatment vessel during the uptake phase.

4. Test organism assignment and treatment

During the uptake phase, the fish were continuously exposed to the test item. Thereafter, the remaining fish population was transferred into a new aquarium containing the test item-free dilution water for 21 days (depuration phase).

The fish were fed each day Inicio Plus, 2 mm biomar, Denmark at a level of 1.5% of the body weight. Uneaten food and faeces were siphoned from the vessels within one hour after feeding.

5. Measurements and observations

The oxygen concentration (WTW Oximeter inoLab Oxi 7310), temperature, and pH (WTW pH-Meter inoLab pH 7310) of the test solutions and control water was measured daily.

Total Organic Carbon (TOC) including organic carbon from particles and dissolved organic carbon, were measured as Non-Purgeable Organic Carbon (NPOC) at the beginning of the test (24 and 48 h prior to test initiation of uptake phase), before introduction of the fish and at least once a week during both uptake and depuration phases.

During uptake, water samples were taken at least three times a week and directly before fish sampling to monitor the concentration of the test item in the control and test vessels. During the depuration phase, water samples were taken at Day 0 and three times per week thereafter, until measured concentrations of the test item during the depuration phase were below the LOQ.

Fish samples were taken on Days 1, 3, 7, 14, 21 and 28 of the exposure phase and at Days 1 (29), 2 (30), 4 (32), 7 (35), 14 (42) and 21 (49) of the depuration phase.

Each fish was analysed individually for the test item (in total five replicates per sampling date). From the control group, one replicate sampled at the beginning and end of the exposure phase as well as at the end of the depuration phase was analyzed and no background contamination was observed. Three additional fish were sampled at the end of the uptake period as well as the end of the depuration period for lipid analysis, respectively. The additional fish needed for lipid analysis at the start of the uptake period were sampled from the stock population.

7. Statistics/Data evaluation

The kinetic bioconcentration parameters were calculated accordingly to the suggested mathematical operations given in the OECD TG 305 for BCF determination using Microsoft Excel 2016®, SigmaPlot® and SigmaSat®.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The mean achieved concentration of test item in treatment water was 28.3 ± 3.54 µg/L parent test item corresponding to 30.1 ± 3.51 µg/L TRR. The control water did not contain any trace of test item (values all < LOQ). During uptake phase of the experiment, the first measured value was below the 20% tolerance which can be explained by the introduction of a high amount of biomass.

Table: Measured aclonifen concentrations in the aqueous test media during the uptake phase

Time (days)	Aclonifen concentration by LC-MS/MS (µg/L)	Aberrance from mean (%)	TRR concentration measure by LSC (µg/L)
-3	26.1	-7.53	28.1
-2	33.6	18.9	36.0
-1	30.6	8.44	20.1
0	30.6	8.23	31.8
1	21.0	-25.7	21.7
3	28.7	1.57	29.7
5	30.1	7.50	31.3
7	24.4	-13.6	25.5
11	31.9	13.0	34.9
12	31.7	12.2	33.7
14	33.9	19.90	33.8
18	28.1	-0.61	27.7
20	26.4	-6.59	29.4
24	27.4	-2.97	30.3
22	26.2	-7.11	30.3
25	23.2	-17.85	26.9
27	25.8	-8.68	29.3
28	28.5	0.94	32.5
Mean concentration (µg/L)	**28.3**		**30.1**
Standard deviation (µg/L)	**3.54**		**3.51**
Standard deviation (%)	**12.5**		**11.7**
Minimum concentration (µg/L)	**21.0**		**21.7**
Maximum concentration (µg/L)	**33.9**		**36.0**

Measured concentrations in the aqueous test media declined from 5.11 µg/L on Day 1 of the depuration phase to less than the LOQ of the analytical method by Day 4 of depuration.

B. BIOLOGICAL DATA

Test conditions

During the test, the water temperature in the aquaria ranged between 13.0 °C – 15.0 °C in both test vessels and was within the range of 15 °C ± 2 °C recommended by OECD 305.

The remaining ambient parameters were also in the range of the guideline cited. The pH in the test vessels ranged between 6.76 and 7.87 and the oxygen saturations in both vessels were between 69.0 and 108%.

Non-purgeable organic carbon (NPOC) as a measure of total organic carbon (TOC) was only determined in the control vessel, no analysis for the treatment group was possible due to the 14C-labelled test item. NPOC ranged between 0.135 and 1.572 mg/L (OECD 305 guideline requirement < 2.0 mg/L) with the exception of a single value of 1.61 mg/L determined on Day 42. The maximum value was a single observation, which occurred in the holding and dilution water during the depuration phase and was thus evaluated to have no effect to the test system.

Fish health and behaviour

All fish were in a vivid and healthy condition and showed no abnormal behaviour during the study. Fish were immature at the start of the study, and at termination still no signs of sex differentiation were visible. No mortality occurred during the study.

Uptake of test item in fish and BCF_{SS}

By considering the mean concentration of data points of Day 14, 21 and 28, a mean steady-state-concentration of 40694 ± 6950 µg/L was determined. Day 7 was not included in the calculation, as data at this time point displayed a stronger scatter and the calculated (growth-corrected) time to reach a 95% tissue saturation was t_{95Ug} = 9.37 d.

Based on the considered means of the concentration in fish divided by the concentration in water (C_f/C_w), a BCF_{ss} of 1440 ± 246 L/kg was calculated.

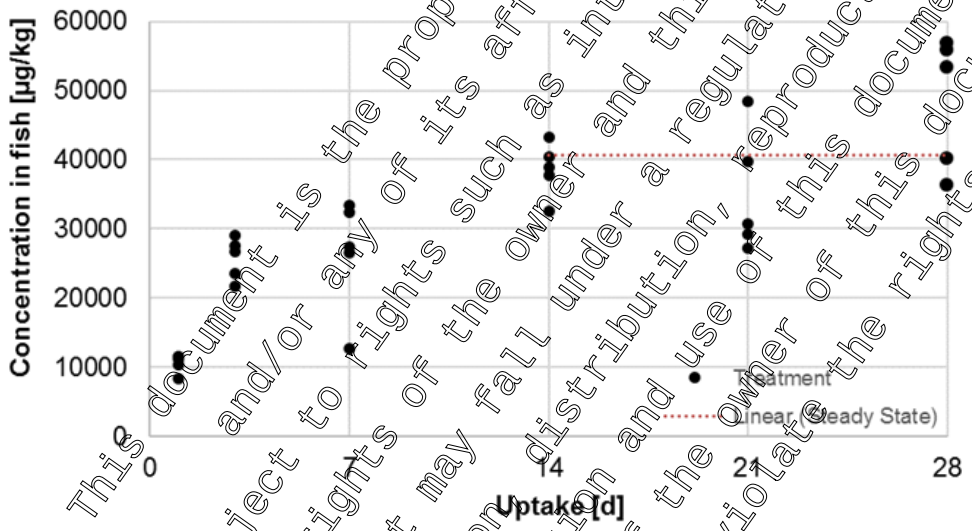
Table: Concentration of test item in single fish during uptake phase

Time (day)	C_f (µg/kg)	Mean C_f (µg/kg)	Steady state (µg/kg)	Steady state SD (µg/kg)	Steady state RSD (µg/kg)	Deviation from SS (%)	C_f / TWA
1	10348	10584	N/A	N/A	N/A	-74	366
	8410						298
	11500						407
	11213						397
	11448						405
3	23525	25683	N/A	N/A	N/A	-36.9	833
	26666						944
	27511						974
	21739						770
	28974						1026
7	32385	26485	N/A	N/A	N/A	-34.9	1146
	27437						971
	33382						1182
	12660						448
	26559						940
14	38805	38566	30245	7218	23.9	-5.23	1377
	37057						1337
	32525						1151
	40483						1433
	43171						1528
21	27237	35057	33369	6215	18.6	-13.9	964
	30727						1088

	48451						1715
	39668						1404
	29201						1034
28	53312	48459	40694	6950	17.1	19.1	1887
	56857						2013
	55776						1974
	36246						1282
	40103						1420
BCF_{ss}							**1440**
SD BCF_{ss}							**246**
% RSD BCF_{ss}							**17.08**

C_f: Concentration of test item in fish
SD: Standard Deviation
RSD: Relative Standard Deviation
TWA: Time Weighted Average
N/A: Not applicable
BCF_{ss}: Bioconcentration factor at steady state

Figure: Concentration of test item in single fish during uptake phase



Depuration of test item in fish and BCF_k

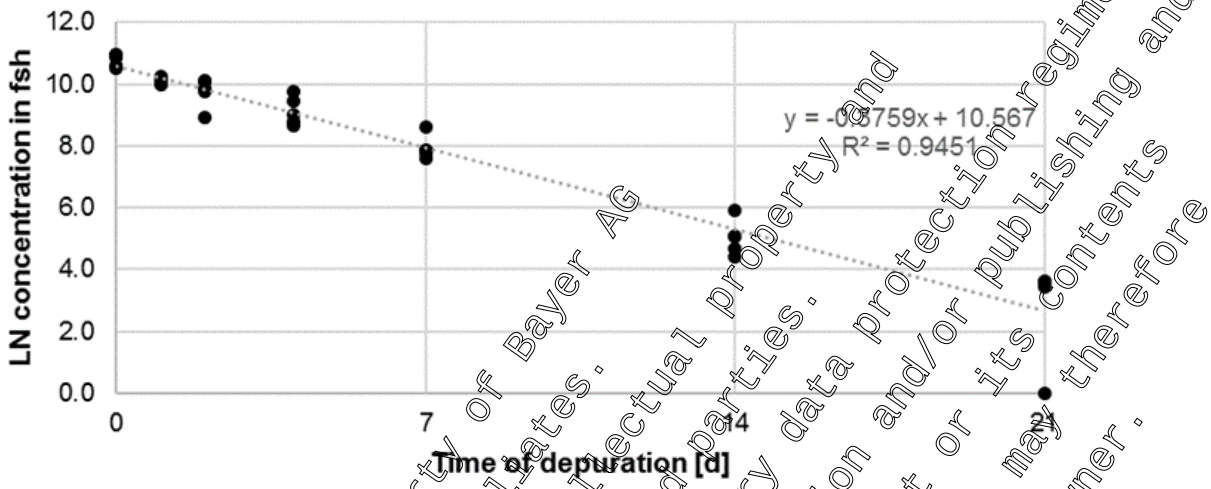
The results from the depuration phase allowed to determine the depuration rate constant k_2 with a linear fit (R^2 = 0.945) of the ln-transformed concentration data.

The respective depuration constant k_2 of 0.376 d^{-1} was determined from the slope of single values.

Based on k_2, the uptake rate constant k_1 was determined to be 505.

A BCF_K of 1343 L/kg was determined by division of uptake and depuration rate constant.

Figure: Ln-linear fit of the fish matrix concentrations of aclonifen during depuration phase



Growth correction

The slope from growth data of the treatment group was used to correct the BCF_K with the treatment growth rate constant of $K_g = 0.0214$. The latter constant was subtracted from k_2 to obtain the growth corrected k_{2g} of 0.354.

Based on the corrected depuration rate constant, the growth-corrected BCF_{Kg} was determined to be 1425 L/kg.

Lipid correction

The lipid content of fish from the stock at the start of the experiment was about 5.70%. Over the experimental phase of the study, a mean lipid content of 5.28 ± 9.80% was determined in Aclonifen-exposed specimens and was comparable to the control group with a lipid content of 5.81 ± 26.3%. For lipid normalization, the lipid content of treated animals was used.

The BCF_{SS} of 1440 L/kg results in a lipid-corrected BCF_{SSL} of 1364 L/kg. For the BCF_{Kg} of 1425 L/kg, lipid correction resulted in a BCF_{KgL} of 1349 L/kg.

Calculation of time to steady-state for Aclonifen

The time span to reach half of the steady state concentration was calculated based on the previously determined $C_{f,ss}$ of 40694 µg/kg of Aclonifen. Half of the maximum concentration of the test item in fish matrix (t_{50U}) was reached after 0.603 days (i.e. 14.5 h), or when also considering the correction for growth (k_1 and k_2) 0.551 days (i.e. 13.2 h), respectively.

A 95% tissue saturation (t_{95U}) was calculated to be reached after 20.2 days (i.e. 486 h), or when correcting for growth (t_{95Ug}) 9.37 days (i.e. 225 h).

Half-life of Aclonifen

Based on the slope and intercept of the linear function of the ln-transformed C_f values, the substance specific half-life (t_{50D}) was determined to be 1.25 days (i.e. 30.1 h). Considering also the growth rate constant, the respective elimination time (t_{50Dg}) increases to 1.33 days (i.e. 31.9 h).

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 305-I, 2012)	Achieved
Variation in water temperature	<±2°C	<±2°C
Dissolved oxygen concentration (% saturation)	≥60%	≥60%
Limit in variation of measured test item concentration from the mean measured concentration	±20%	±20%*
Test concentration	<limit of water solubility	Yes
Mortality or other adverse effects/disease	<10%	0%

*Aside from a drop of the concentration after fish introduction and the measurement of the concentration at 24 hours which was considered to be due to the introduction of a high amount of biomass

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of calculated parameters

Bioconcentration Parameter	Treatment value
k_g (growth rate constant, day^{-1})	0.0214 (SE 0.0027; 95% CI 0.005)
k_1 (uptake rate constant, L/kg day^{-1})	505 (SE 297; 95% CI 609)
k_2 (depuration rate constant, day^{-1})	0.326 (SE 0.0158; 95% CI 0.032)
k_{2g} (growth-corrected depuration rate constant, day^{-1})	0.354
$C_{f,SS}$ (Concentration in fish at steady-state, µg/kg)	40694 (SD 6950; RSD 17.1%)
C_w (Concentration in water (TWA), uptake phase, µg/L)	28.3 (SD 3.54; RSD 12.5%)
L_N (overall lipid normalisation factor, unitless)	0.0528
BCF_{SS} (steady-state bioconcentration factor, L/kg)	1440 (SD 246; RSD 17.1%)
BCF_{SSL} (lipid-normalised, steady-state bioconcentration factor, L/kg)	1364
BCF_K (kinetic bioconcentration factor, L/kg)	1343
BCF_{Kg} (growth–corrected kinetic bioconcentration factor, L/kg)	1425
BCF_{KgL} (growth- and lipid–corrected kinetic bioconcentration factor, L/kg)	1349
t_{50U} (time to reach 50% of $C_{f,SS}$, day)	0.603 (14.5 h)
t_{50Ug} (growth-corrected time to reach 50% of $C_{f,SS}$, day)	0.551 (13.2 h)
t_{95U} (time to reach 95% of $C_{f,SS}$, day)	20.2 (486 h)
t_{95Ug} (growth-corrected time to reach 95% of $C_{f,SS}$, day)	9.37 (225 h)
t_{50D} (half-life, day)	1.25 (30.1 h)
t_{50Dg} (growth-corrected half-life, day)	1.33 (31.9 h)
t_{95D} (time required to reach 95% depuration, day)	7.38 (177 h)
t_{95Dg} (growth-corrected time required to reach 95% depuration, day)	7.83 (188 h)

SE: Standard error
SD: Standard deviation
RSD: Relative standard deviation

III. CONCLUSION

In fish continuously exposed to [^{14}C]-aclonifen at a mean measured concentration of 28.3 µg/L for a period of 28 days the steady-state BCF (BCF_{SS}) was determined to be 1440 L/kg based on the average

whole body concentrations at equilibrium phase between water and fish between Days 14 and 28. Lipid normalisation resulted in a lower lipid-normalised BCF_{SSL} of 1364 L/kg.

From the uptake rate constant of k_1 of 505, and the depuration rate constant k_2 of 0.376 the kinetic bioconcentration factor (BCF_K) was determined with 1343 L/kg.

As fish grew during study, the BCF_K was corrected for growth-dilution effects into BCF_{Kg} 1425 L/kg incorporating the growth rate constant of 0.0214 d^{-1}. Lipid normalisation of BCF_{Kg} resulted in a BCF_{KgL} of 1349 L/kg.

The result that the BCF_{Kg} and the BCF_{SS}, as well as BCF_{KgL} and BCF_{SSL} are very similar indicates that the steady state was truly reached during the experiment and that uptake and depuration processes follow first order kinetics.

The BCF_{KgL} for the whole fish is the most relevant BCF because it incorporates all measurements during uptake and depuration and the influence of the test fish growth and lipid content.

The time to reach a 50% tissue saturation in fish matrix was calculated to occur after 0.551 d (growth-corrected). The duration for further incorporation of test item to up to 95% of the steady state concentration was calculated to be reached after 9.73 d (growth-corrected). The half-life of the test item in fish was determined to be 1.33 d (growth-corrected). The elimination of 95% of test item in fish was determined to be 7.83 d (growth-corrected).

The metabolite analysis after 28 days exposure to 30 µg/L (nominal) Aclonifen, yielded one uncharacterized metabolite, which accounted for <3% of TRR, but reaching an absolute concentration of 1.04 mg/kg in fish tissue.

[redacted] *(2019)*

Assessment and conclusion by applicant:
All validity criteria were satisfied and therefore this study can be considered to be valid.

The steady-state BCF (BCF_{SS}) was determined to be 1440 L/kg. Lipid normalisation resulted in a lower lipid-normalised BCF_{SSL} of 1364 L/kg.

From the uptake rate constant of k_1 of 505, and the depuration rate constant k_2 of 0.376 the kinetic bioconcentration factor (BCF_K) was determined with 1343 L/kg.

As fish grew during study, the BCF_K was corrected for growth-dilution effects into BCF_{Kg} 1425 L/kg incorporating the growth rate constant of 0.0214 d^{-1}. Lipid normalisation of BCF_{Kg} resulted in a BCF_{KgL} of 1349 L/kg.

The BCF_{KgL} of 1349 L/kg for the whole fish is the most relevant BCF because it incorporates all measurements during uptake and depuration and the influence of the test fish growth and lipid content. This value should be used for risk assessment purposes.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.2.3/05
Report Author:	[redacted]
Report Year:	2019
Report Title:	Aclonifen: Endpoint selection for the bioconcentration of aclonifen in rainbow trout, Oncorhynchus mykiss
Report No:	VC/19/016/02
Document No:	M-675783-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	--
Previous evaluation:	
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	

Executive Summary

This paper represents the selection of the relevant endpoint for the bioconcentration of aclonifen in aquatic organisms.

A total of four fish bioaccumulation tests with aclonifen have been conducted to determine the bioaccumulation potential of aclonifen to rainbow trout, *Oncorhynchus mykiss*. In each study, the bioaccumulation test consisted of two phases: the exposure (or uptake) phase and post exposure (or depuration) phase.

The first study ([redacted], 1992, M-235556-01-2) was performed according to OECD test guideline 305E (1981) and included two test concentrations of 4.5 and 45.0 µg/L with an uptake phase of 6 days followed by a 12-Day depuration phase.

Studies two and three were performed at the same test facility according to OECD test guideline 305E (1981) and OECD draft guideline 305 (1992). The second study ([redacted] 1992, M-235029-01-1) used two test concentrations of 6.0 and 60 µg/L with an uptake phase of 8 days followed by a 14-Day depuration phase. Based on the results of the second study, the third study ([redacted] 1992, M-174910-01-1) included a single test concentration of 30 µg/L only. A 28-Day uptake phase followed by a 20-Day depuration period was employed in the third study.

The fourth and final study ([redacted] 2019, M-667576-02-1) was performed according to the current OECD Test Guideline (OECD 305 I (2012)). A single test concentration of 30 µg/L was included and the test was performed using a 28-Day uptake period followed by a 21-Day depuration period.

The data generated in each of these studies have been re-evaluated to define the relevant bioconcentration endpoint.

Deficiencies have been identified in three of the four bioconcentration studies that have been performed on aclonifen ([redacted], 1992, M-235556-01-2; [redacted] 1992, M-235029-01-1; and [redacted] 1992, M-174910-01-1). These studies were performed in accordance with the relevant test

guidelines at the time of performing the studies, however due to scientific advances in both the performance and evaluation of bioconcentration studies, the deficiencies identified when comparing these studies to current guideline requirements are of sufficient magnitude to raise serious concerns with respect to the validity of the reported BCF values.

The recently completed study ([redacted] 2019, M-667576-02-1) was performed according to the latest OECD test guideline, OECD 305-I, 2012, and satisfied all the requirements of that guideline. It is therefore considered that the determined BCF results are an accurate and reliable estimate of the bioconcentration potential for aclonifen.

A growth-corrected, lipid normalised bioconcentration factor (BCF_{KgL}) of 1349 L/kg is therefore considered the most relevant BCF for aclonifen in aquatic organisms.

[redacted] *(2019)*

Assessment and conclusion by applicant:
The re-evaluation of the available study data is considered to be acceptable and hence the conclusions drawn are considered to be valid.
A growth-corrected, lipid normalised bioconcentration factor (BCF_{KgL}) of 1349 L/kg is therefore considered the most relevant BCF for aclonifen in aquatic organisms.

Assessment and conclusion by RMS:

CA 8.2.3 Endocrine disrupting properties

Data Point:	KCA 8.2.3/01
Report Author:	[redacted]; [redacted]
Report Year:	2020
Report Title:	Appendix I - Assessment of the endocrine disrupting properties of the active substance aclonifen in accordance with Commission Regulation (EU) 2018/605
Report No:	M-676736-01-1
Document No:	M-676736-01-1
Guideline(s) followed in study:	in Accordance with Commision Regulation (EU) 2018/605
Deviations from current test guideline:	
Previous evaluation:	
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	

Executive Summary

The potential of aclonifen to interact with endocrine systems in aquatic organisms has been reviewed, to facilitate an assessment of whether aclonifen may be judged to be an endocrine disrupter (ED) within the framework of European legislation.

Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 has been published (EFSA/ECHA, JRC, 2018). This guidance document describes how to gather, evaluate and consider all relevant information for the assessment, conduct a MoA analysis, and apply a WoE approach, in order to establish whether the ED criteria are fulfilled. The guidance states that a substance shall be considered as having endocrine disruption properties if it meets all of the following criteria:

i. It shows an adverse effect in an intact organism or its progeny, which is a change in the morphology, physiology, growth, development, reproduction, or life span of an organism, system, or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other influences.
ii. It has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system.
iii. The adverse effect is a consequence of the endocrine mode of action.

Standard toxicology and ecotoxicology studies conducted to meet to the data requirements under Regulation (EU) 283/2013 have been submitted in this renewal dossier. A literature search was conducted to find relevant studies in the open literature conducted in the last 10 years. Further in vitro studies have been conducted to investigate EATS-mediated endocrine activity.

A summary of all relevant studies is provided in the excel spreadsheet Appendix E.

T-modalities

Based on the available endpoints for non-target organisms, there is no specific evidence suggestive of T-mediated endocrine activity/adversity of aclonifen. However, the available evidence is not sufficient to conclude either on T-mediated activity or on T-mediated adversity in non-target organisms. Further data need to be generated.

Based on scenario 2a (iii) of the ED Guidance, the endocrine activity was not sufficiently investigated for the T-modality. Therefore, according to the ED Guidance, a level-3 study should be performed. The available level-3 test guidelines are the Amphibian Metamorphosis Assay (AMA; OECD TG 231) and the Xenopus Eleutheroembryonic Thyroid Assay (XETA; OECD TG 248).

EFSA recently (ECHA ED Expert Meeting on December 3rd, 2019) considered that the XETA can be used for evaluating the T-modality if "information is available that one of the mechanisms of action which the assay is able to detect can be involved:

- Metabolism by defodinases,
- Clearance/ hepatic metabolism,
- Thyroid receptor agonist,
- Thyroid receptor antagonist"

As indicated in the mammalian ED assessment, enhanced hepatic clearance of thyroid hormones is considered to be the most likely MoA for the changes in thyroid hormones and thyroid hypertrophy

induced by aclonifen. The XETA is therefore considered as the appropriate assay to evaluate the T-modality for aclonifen.

EAS-modalities

The EAS-modalities were not sufficiently investigated in non-target organisms, and further data must be generated.

According to the ED Guidance, in case further data with non-target organisms are needed to elucidate the endocrine activity due to the EAS-modalities, level-3 studies with fish according to the OECD TG 229 and TG 230 are recommended, the preferred assay being the Fish Short Term Reproduction Assay (FSTRA, OECD TG 229).

However, according to EFSA ([redacted], Abstract WE201, SETAC Europe Meeting, 26-30 May 2019, Helsinki, Finland), there might be cases where substances having anti-androgenic properties are not detected in tests where adult fish are exposed, which is the case of the OECD TG 229 and TG 230. This is because fish are exposed after the sexual differentiation occurred.

Similarly, alteration of steroidogenesis can conceivably result in changes in steroid hormone balance, and this may affect sexual differentiation in fish. Such effects might not be captured in studies with adult fish conducted according to the OECD TG 229 and TG 230.

The Fish Sexual Development Test (FSDT, OECD TG 234) is considered as an alternative solution for substance acting as androgen antagonists ([redacted] 2019). It is also the preferred test to assess substances acting on steroidogenesis, *e.g.* aromatase-inhibiting chemicals ([redacted] 2012, Comparative Biochemistry and Physiology, Part C 155: 407-415).

For aclonifen, there is indication from the *in vivo* assays that this substance has anti-androgenic activity and affects steroidogenesis. Although the *in vitro* endocrine activity *vid* the A and S modalities are not replicated *in vivo* in mammals, the most appropriate assay to elucidate the endocrine activity due to these two modalities in fish would be Fish Sexual Development Test (FSDT; OECD TG 234).

The FSDT also allows detecting substances acting as estrogens and anti-estrogen, and it is also responsive to certain thyroid-disrupting chemicals (see OECD GD 150, section C.2.9).

Overall conclusion on the ED assessment for non-target organisms

For aclonifen, the available data are not sufficient to conclude on EATS-mediated activity in non-target organisms. Further data need to be generated according to scenario 2a (iii) of the ED Guidance.

For the T-modality, a study according to the OECD TG 248 (*Xenopus* Eleutheroembryonic Thyroid Assay - XETA) is proposed. According to EFSA (December 2019), there are two possible options:

- If the XETA is negative, the ED criteria are not met for the T-modality for non-target organisms.
- If the XETA is positive, according to Figure 1 of the ED Guidance, a MoA Analysis should be performed, and further testing might be needed. In this case, an AMA should be performed if information is available that the substance may interfere with the THs synthesis (*i.e.* inhibitor of the sodium iodide symporter, NIS), which might be considered applicable in the case of aclonifen (see Table 2 and section 3.1.5).

For the EAS-modalities, a study according to the OECD TG 234 (Fish Sexual Development Test - FSDT) is proposed in order to address the A and S modalities more specifically than with an FSTRA.

Assessment and conclusion by applicant:

The review of the available data is considered to be acceptable and hence the conclusions drawn are considered to be valid.

Assessment and conclusion by RMS:

CA 8.2.4 Acute toxicity to aquatic invertebrates

CA 8.2.4.1 Acute toxicity to *Daphnia magna*

Data Point:	KCA 8.2.4.1/01
Report Author:	[redacted]
Report Year:	1991
Report Title:	The acute toxicity of Aclonifen to daphnia magna
Report No:	R007749
Document No:	M-174313-01-1
Guideline(s) followed in study:	OECD: 202, 1
Deviations from current test guideline:	Current Guideline: OECD 202, 2004 Water hardness was higher than recommended but was not considered to have affected study results
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The acute toxicity of aclonifen to *Daphnia magna*, was determined in a 48-hour, static exposure. Test solutions were prepared using stock solutions prepared in 10% Tween 80-acetone. Twenty *Daphnia* per test group were exposed to an untreated control, solvent control and nominal Aclonifen concentrations of 0.10, 0.18, 0.32, 0.56, 1.0, 1.8, 3.2, 5.6 and 10 mg/L. The total test period was 48 hours. Samples for analytical confirmation of actual exposure concentrations were taken at the start and after 48 hours of exposure.

Dissolved oxygen, pH, and temperature were measured in the controls and each test concentration at the beginning of the test and end of the test. Daily observations were made of immobilisation.

Results are based on nominal concentration since all test concentrations analysed showed measured concentrations to range from 81 to 97% of nominal with the exception of the 3.2 mg/L test sample at 48 hours which had a measured concentration of 74% of nominal. At 3.2 mg/L, settlement of undissolved material may have been the reason for the low measured value.

The 48-Hour EC_{50} of Aclonifen to *Daphnia magna* was determined to be 1.2 mg/L (confidence limits 1.0 – 1.5 mg/L). The NOEC was 0.32 mg/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Item: Aclonifen Technical
Batch no.: DA 618
Active Ingredient / Purity: 91.3%
Appearance: Green-yellow powder
Date received: 20 June 1990
Storage: In original container in darkness at +4°C
Expiry date: December 1990

2. Test Organism: *Daphnia magna* (Straus)
Age: <24-hours
Source: [redacted]
Feeding: Daily with fry fish food (Liquifry®) and a suspension of mixed algae, predominantly *Chlorella* spp.

3. Test water: Dechlorinated (with sodium thiosulphate) and aged laboratory tap water
Total hardness: Approximately 350 mg/L as $CaCO_3$

B. STUDY DESIGN AND METHODS

1. In-life phase: 03 – 05 July 1990

2. Exposure conditions
Test vessels: Glass jars, containing 200 mL test solution
Experimental design: Nine test concentrations (0.032, 0.056, 0.10, 0.18, 0.32, 0.56, 1.0, 1.8 and 3.2 mg/L) plus one control and one solvent control (100 µL/L) each in duplicate
Loading: 20 mL of media per *Daphnia*
Temperature: 21 ± 1°C
pH: 7.9 – 8.5
Dissolved oxygen: 7.9 – 8.5 mg O_2/L
Aeration: None
Photoperiod: 16 h light: 8 h dark

3. Administration of the test item

Preliminary solution in 10% Tween 80- acetone.

4. Test organism assignment and treatment

Daphnia were placed in the test solutions after addition of the test item.

5. Measurements and observations

The number of immobilised daphnids was assessed after 24 and 48 hours from the beginning of the test. The criterion for the effect (immobility) was the inability to swim within 15 seconds after gentle agitation.

Dissolved oxygen concentrations and pH values were measured in all the test groups and the control and solvent control vessels at the beginning and at the end of the test. The temperature was recorded at 0, 24 and 48 hours.

Samples were taken from the solvent control and 0.032, 0.10, 0.32, 1.0 and 3.2 mg/L test concentrations for analysis. The samples were collected at 0 and 48 hours (end of the test).

5. Statistics/Data evaluation

The 24 and 48-hour EC_{50} and associated 95% confidence limits were calculated following the method described by [redacted] (1952). The No Observed Effect Concentration (NOEC) was determined by visual inspection of the data.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Measured concentrations for all exposure levels analysed (with the exception of the 3.2 mg/L level), remained within the range 81 - 97% of nominal throughout the study. At 3.2 mg/L, the measured concentration fell from 93% at 0 hours to 74% at 48 hours, indicating that some settlement of undissolved test substance had occurred. This is not unexpected given that the water solubility value for Aclonifen is quoted as 2.5 mg/L (as advised by the Sponsor). Nominal concentrations have been retained for the calculation of EC_{50} values however, since the overwhelming evidence is that near nominal concentrations were maintained across the exposure range during the study. The measured concentration for the 3.2 mg/L level at 48 hours has not been used for the calculation of EC_{50} values since this practice would not take into consideration the effect of near nominal concentrations at the start of the study.

Table: Measured concentrations from the exposure of *Daphnia magna* to Aclonifen

Nominal Concentration (mg/L)	0 Hours		48 Hours	
	Measured concentration (mg/L)	% of nominal	Measured concentration (mg/L)	% of nominal
Control	ND	-	ND	-
0.032	0.028	88	0.026	81
0.10	0.096	96	0.086	86
0.32	0.309	97	0.290	91
1.0	0.896	90	0.885	88
3.2	2.964	93	2.363	74

ND: None Detected (Limit of detection = 0.01 mg/L)

The validated method is summarised in Document M-CA4 (CA 4.1.2/62).

B. BIOLOGICAL DATA

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

Table: Percentage immobilisation from the exposure of *Daphnia magna* to Aclonifen

Nominal Concentration (mg/L)	Cumulative immobilisation							
	24 Hours				48 Hours			
	R_1	R_2	Total	%	R_1	R_2	Total	%
Control	0	0	0	0	0	0	0	0
Solvent control	0	0	0	0	0	0	0	0
0.032	0	0	0	0	0	0	0	0
0.056	0	0	0	0	0	0	0	0
0.10	0	0	0	0	0	0	0	0
0.18	0	0	0	0	0	0	0	0
0.32	0	0	0	0	1	0	1	5
0.56	1	0	1	5	2	1	3	15
1.0	1	0	1	5	4	1	5	25
1.8	3	1	4	20	6	8	14	70
3.2	10	10	20	100	10	10	20	100

$R_1 – R_2$ = Replicates 1 to 2

The single immobilised *Daphnia* at nominal 1.0 mg/L was within guideline limits for background mortality and was not considered to be biologically significant.

All chemical and physical parameters in the definitive test were within expected ranges.

Based on the observed immobilisation, the EC_{50} values at each observation point were determined to be:

Table: EC_{50} values from the exposure of *Daphnia magna* to Aclonifen

Time (Hours)	EC_{50} (mg/L)	95% confidence limits (mg/L)
24	2.0	1.8 – 2.3
48	1.2	1.0 – 1.5
No Observed Effect Concentration (48 hours) = 0.92 mg/L		

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 202, 2004)	Achieved
Mortality in controls	<10%	0%
Dissolved oxygen concentration at the end of the test	>3 mg/L	8.4 mg/L

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal Concentration (mg/L)
EC_{50} (48 hours)	1.2
95% confidence limits	1.0 – 1.5

NOEC	0.32

III. CONCLUSION

The 48-Hour EC_{50} of Aclonifen technical to *Daphnia magna* was determined to be 1.2 mg/L (95% confidence limits 1.0 – 1.5 mg/L). The NOEC was 0.32 mg/L.

[redacted] (1991)

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The 48-Hour EC_{50} of Aclonifen technical to Daphnia magna based on nominal test concentrations was determined to be 1.2 mg/L (95% confidence limits 1.0 – 1.5 mg/L). The NOEC was 0.32 mg/L.

In addendum 1 to the draft assessment report of aclonifen dated 17 March 2008, the Rapporteur Member State recalculated the EC_{50} based on the arithmetic mean measured concentrations to be 0.952 mg/L but stated: "Thus, the mean measured concentration was 2.66 mg/L, corresponding to 83% of the nominal concentration during the course of the test. The corresponding values for the four other analysed concentrations are: 84% (at nominal 0.032 mg as/L), 91% (0.1 mg as/L), 94% (0.3 mg as/L), and 89% (1.0 mg as/L). Therefore, the EC_{50} calculation based on nominal concentrations is considered to be appropriate."

EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2015)[1] recommends that mean measured concentrations are calculated using the geometric mean rather than the arithmetic mean. A summary of the measured, arithmetic mean and geometric mean measured concentrations is provided in the following table:

Table: Measured concentrations from the exposure of *Daphnia magna* to Aclonifen

Nominal Concentration (mg/L)	Measured Concentration							
	0 Hours		48 Hours		Arithmetic Mean		Geometric Mean	
	mg/L	% nom	mg/L	% nom	mg/L	% nom	mg/L	% nom
0.032	0.028	88	0.026	81	0.027	84	0.027	84
0.1	0.096	96	0.086	86	0.091	91	0.091	91
0.32	0.309	97	0.29	91	0.300	94	0.299	94
1	0.896	90	0.885	88	0.891	89	0.890	89
3.2	2.964	93	2.363	74	2.664	83	2.646	83

% nom: Percentage of nominal concentration

Given that both the arithmetic and geometric mean measured test concentrations were within the range of 83% to 94% of nominal values it was considered that recalculation of the study endpoints based on the arithmetic or geometric mean measured concentrations was not necessary. Consequently, the EC_{50} of 1.2 mg/L is used for risk assessment.

Assessment and conclusion by RMS:

[1] EFSA (European Food Safety Authority), 2015. Technical report on the outcome of the pesticides peer review meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2015:EN-924. 62 pp.

CA 8.2.4.2 Acute toxicity to an additional aquatic invertebrate species

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

CA 8.2.5 Long-term and chronic toxicity to aquatic invertebrates

CA 8.2.5.1 Reproductive and development toxicity to *Daphnia magna*

Data Point:	KCA 8.2.5.1/01
Report Author:	[redacted]
Report Year:	1991
Report Title:	An assessment of the effects of aclonifen on the reproduction of Daphnia magna
Report No:	R007153
Document No:	M-174321-01-1
Guideline(s) followed in study:	OECD: 202, part 2
Deviations from current test guideline:	Current Guideline: OECD 211, 2012 Validity criterion relating to the mean number of living offspring per parent animal was not satisfied
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

The objectives of this study were to determine the effects of Aclonifen on the survival and reproduction of the water flea *Daphnia magna* under semi-static exposure conditions to determine the No Observed Effect Concentration (NOEC) and the lethal concentrations/effect concentrations causing 50% of inhibition (EC_{50}).

Daphnia magna were exposed to aclonifen at nominal concentrations of 0.018, 0.056, 0.18, 0.56 and 1.8 mg/L for a period of 21 days. Renewal of test media was performed on Days 2, 5, 7, 9, 12, 14, 16 and 19.

Dissolved oxygen, pH, and temperature were measured before and after each test media renewal.

The live and dead Daphnia of the parental (P1) generation, observations on the general condition and size of Daphnia as compared with controls were recorded daily, as well as the number of Daphnia with eggs or young in the brood pouch. The numbers of live and dead filial (F1) Daphnia and the number of discarded unhatched eggs were determined at each test media renewal time.

Water samples from the control and each concentration were taken on Days 0 (fresh media), 2, 5, 7, 9, 12, 14, 16, 19 and 21 (old media) for analysis of aclonifen content by HPLC method.

The test item was chemically stable in water with measured concentrations consistent at approximately 86% of nominal throughout the study. The results of the study were based on the mean measured test concentrations which were calculated to be 0.016, 0.045, 0.16, 0.47 and 1.6 mg/L.

Based on measured concentrations, the EC_{50} (immobilisation) with 95% confidence limits in *Daphnia magna* exposed to aclonifen was calculated to be 0.10 (0.084-0.13) mg/L.

Based on the total number of live young produced, the NOEC was found to be 0.016 mg/L.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen Technical 2-chloro-6-nitro-3-phenoxyaniline
	Batch no.:	DA 618
	Purity:	91.3%
	Appearance:	Green-yellow powder
	Date received:	20 June 1990
	Storage:	In original container in darkness at +4°C.
	Expiry date:	December 1990
2.	**Test Organism:**	*Daphnia magna* (Straus)
	Source:	[redacted]
	Age:	~24 hours
	Feeding:	Cultures were fed daily with a mixture of fry fish food (Liquifry®) and a suspension of mixed algae (predominantly *Scenedesmus* sp and *Selenastrum* sp).
3.	**Test water:**	Dechlorinated (with sodium thiosulphate) and aged laboratory tap water. Total hardness approximately 350 mg/L as $CaCO_3$. This value is slightly higher than the recommended range but is not considered to have had any significant effects on the results of this test.

B. STUDY DESIGN AND METHODS

1. In-life phase: 01 – 22 August 1990

2. Exposure conditions

Test vessels:	Glass jars each containing 400 mL of test solution
Experimental design:	5 test concentrations (0.018, 0.056, 0.18, 0.56 and 1.8 mg/L) plus 1 control and 1 solvent control (100 µL/L)
Replicates:	4 replicates per control and treatment group, each containing 10 daphnids

Loading:	40 mL test solution per organism
Temperature:	21 ± 1°C
pH:	8.0 – 8.2
Aeration:	None. The diluent only was aerated prior to test media preparation
Photoperiod:	16 h light : 8 h dark

3. Administration of the test item

The test item was prepared using a preliminary solution in 10% Tween 80-acetone.

Test media renewal was performed 3 times per week (Days 2, 5, 7, 9, 12, 14, 16 and 19).

4. Test organism assignment and treatment

Daphnia were placed in the test solutions after addition of the test substance. The adult Daphnia were transferred to fresh media by wide-bore pipette before the contents of each vessel were passed through a fine mesh. Young daphnids (live and dead) and unhatched eggs collected on the mesh were counted using a stereo microscope and then discarded.

Young daphnids were considered to be dead if no sign of movement was apparent during microscopic examination. Adult *Daphnia* which were unable to swim for approximately 15 seconds after gentle agitation (i.e. immobile), were considered to be dead. An immobilisation criterion for the young daphnids was considered to be inappropriate due to the large numbers of offspring produced in the flasks.

Each vessel received approximately 5 mL of a mixed unicellular algal culture (*Scenedesmus* sp and *Selenastrum* sp) supplemented with fry fish food (Liquifry®), daily. Feeding was at a level to maintain a green tinge in the test solutions thereby ensuring that food was available continuously. Equal amounts of food were given to each vessel.

5. Measurements and observations

Temperature was recorded daily for each flask. Dissolved oxygen, pH and temperature were measured before and after each test media renewal

Verification of test concentrations was carried out on Days 0 (fresh media), 2, 5, 7, 9, 12, 14, 16, 19 and 21 (old media).

The live and dead *Daphnia* of the "parental" (P_1) generation were counted daily and recorded together with observations on the general condition and size of the *Daphnia* as compared with the controls. The number of *Daphnia* with eggs or young in the brood pouch was also determined daily. At each test media renewal the numbers of live and dead "filial" (F_1) *Daphnia* were recorded. The number of discarded unhatched eggs was also determined at this time.

6. Statistics/Data evaluation

EC_{50} values for immobilisation (mortality) of the parental *Daphnia* were calculated according to the method of [redacted] (1952).

EC_{50} values for the effects on reproduction were determined by fitting logistic response curves to the data.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

All calculations and estimations throughout this study are based on mean measured concentrations. Data from samples collected on Days 0, 14 and 21 have been omitted from the calculation of mean measured values, however, for the following reasons:

(a) Samples collected on Day 0 were from freshly prepared media. In order to give a "worst case" analysis only values for "expired" media have been considered for this exercise.

(b) Samples collected on Day 14 could not be analysed on the day of collection due to instrument failure. Although the samples were stored deep frozen (-20°C) and analysed subsequently, the results were considered to be unreliable and were discarded.

(c) All samples collected on Day 21 appeared to be approximately 160% of the nominal concentration. However, there was no corresponding increase in toxic effects to support this chemical evidence and consequently, it was considered preferable to omit these data from the calculations in order to avoid raising the "mean measured" values to misleadingly high levels.

Overall the test substance was chemically stable in water with measured concentrations consistent at approximately 86% of nominal throughout the study.

Table: Mean measured test concentrations from the exposure of *Daphnia magna* to Aclonifen in a 21-Day reproduction test

Nominal concentration (mg/L)	Mean measured concentration[1] (mg/L)	% Nominal
0.018	0.016	87
0.056	0.045	80
0.18	0.16	90
0.56	0.47	84
1.8	1.6	89

[1]: Mean measured concentration based on analytical results for expired media on Days 2-19. Data from samples on Days 14 and 21 have not been included due to difficulties with the analysis

The validated method is summarised in Document M-CA4 (CA 4.1.2/63).

B. BIOLOGICAL DATA

Progressive mortalities on the parental generation occurred throughout the study, particularly at 0.16 mg/L.

Effects on parental generation were statistically significant at 0.045 mg/L and above after 21 days exposure. Parental *Daphnia* in the 0.16 mg/L group (only) appeared to be smaller in size compared with the control *Daphnia* from Day 7 onwards. The adults became gravid only after 16 days with fecundity thereafter being very low.

Numbers of unhatched and dead young were insignificant in all controls and treatment groups.

Table: Summary of effects from the exposure of *Daphnia magna* to Aclonifen in a 21-Day reproduction test

Concentration (mg/L)				

Nominal	Measured	% survival of P1	Number of live young per female	Number of dead young per female	Number of unhatched eggs per female
Control		98	44	0	< 1
Solvent control		98	45	0	< 1
0.018	0.016	98	43	< 1	< 1
0.056	0.045	88	29	0	< 1
0.18	0.16	38	1	0	< 1
0.56	0.47	0	-	-	< 1
1.8	1.6	0	-	-	< 1

The 21-day EC_{50} (immobilisation) value for the parental generation was calculated to be 0.1 mg/L

Impairment of reproduction occurred at exposure concentrations of 0.045 mg/L and above with a 21-day EC_{50} (reproduction) value calculated to be 0.055 mg/L.

The NOEC is estimated to be 0.016 mg/L based on the total number of live young produced. Thus, the lowest concentration exhibiting significant adverse effects is 0.045 mg/L.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 211, 2012)	Achieved*
Control mortality	≤20%	3%
Mean number of living offspring per parent animal surviving at the end of the test	≥60	45

*Based on solvent control

The study was conducted in accordance with OECD 202 (1984), Part 2 and all relevant validity criteria for the guidelines that were in force at the time of performing the study were satisfied.

In terms of the current guideline, OECD 211 (2012), the control mortality satisfied the current validity criterion. However the validity criterion relating to the mean number of living offspring per parent animal was not satisfied. Overall, as the test only fulfilled only one of the two validity criteria; with regards to the OECD Guideline 211 (2012) the study is not valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Measured concentration (mg/L)	95% Confidence limits
21-Day EC_{50} (immobilization)	0.10	0.084 – 0.13
21-Day EC_{50} (reproduction)	0.055	0.048 – 0.063
LOEC (reproduction)	0.045	-
NOEC (reproduction)	0.016	-

III. CONCLUSION

Based on measured concentrations, the EC_{50} (immobilisation) with 95% confidence limits in *Daphnia magna* exposed to aclonifen was calculated to be 0.10 (0.084-0.13) mg/L.

Based on the total number of live young produced, the NOEC was found to be 0.016 mg/L.

[redacted] *(1991)*

Assessment and conclusion by applicant:

In the previous submission (DAR, 2006), this study was evaluated and accepted as valid for risk assessment purposes. The study was conducted in accordance with OECD 202 (1984), Part 2, and all relevant validity criteria for the guidelines that were in force at the time of performing the study were satisfied.

In terms of the current guideline, OECD 211 (2012), the control mortality satisfied the current validity criterion. However, the validity criterion relating to the mean number of living offspring per parent animal was not satisfied. The study is therefore not valid.

As this study does not meet current OECD guideline validity criteria, it should be considered as supportive only

Assessment and conclusion by RMS:

Data Point:	RCA 8.2.5.1/02
Report Author:	[redacted]
Report Year:	2017
Report Title:	Amendment no. 4 - Effects of aclonifen tech. (BCS-AG74518) on development and reproductive output of the waterflea Daphnia magna under continuous static-renewal exposure and under peak exposure conditions
Report No:	EBCL0002
Document No:	M-573305-02-1
Guideline(s) followed in study:	EU Directive 91/414/EEC Regulation 1107/2009 (Europe) US EPA OCSPP 850.1300
Deviations from current test guideline:	Current Guideline: OECD 211, 2012 None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A chronic toxicity test was performed to identify possible effects of the test item on development, reproductive capacity and behaviour of *Daphnia magna* over 21 days under static-renewal exposure or peak exposure, expressed as chronic NOEC for parental health and reproductive output.

Daphnia magna were exposed to aclonifen at nominal concentrations of 0, 5.12, 12.8, 32, 80 and 200 µg a.s./L for a period of 21 days. In addition, a single peak-exposure concentration of 200 µg a.s./L

was provided two times for a duration of 24 hours, on study Days 0 and 7, alternating with recovery periods in untreated water.

As endpoints, the total living offspring per introduced parent animal, the parental age at first offspring emergence as well as the rate of parental survivors and their body-length and dry body mass at the end of the study were recorded as data for NOEC/LOEC calculation. Additional body length measurements were performed on Days 0, 7 and 14.

For verification of the actual test item concentrations during exposure, water-samples from start and end of three representative exposure-intervals were analysed. For the peak exposure scenario, water samples from start and end of both exposure periods were analysed.

The accompanying chemical analysis of aclonifen in the freshly prepared test solutions at start of the chosen exposure intervals revealed recoveries between 92% and 152% (mean: 116%) of the corresponding nominal concentrations. The corresponding concentrations of the aged test solutions at the end of the exposure intervals ranged between 81% and 139% (mean 106%) of nominal. The measured test concentrations partly exceeded 120% of nominal. Therefore, all reported results were based on measured time-weighted mean concentrations.

The lowest chronic NOEC for 21 days of static renewal exposure of *Daphnia magna* to aclonifen (tech.) was 6.27 µg a.s./L based on parent body length at study Day 14. However, the observed effects at 14.2 µg/L on the body length were not statistically signicant on Day 7 or Day 21. Therefore, these effects are not considered to be biologically relevant and the overall NOEC for the study is 14.2 µg/L based on length and dry weight. The non relevance of the effects on length at Day 14 is confirmed by the EC_{10} of 38.5 µg/L which is similar the the EC_{10} at Day 21 (37.4 µg/L).

The lowest EC_{10} was 14.5 µg a.s./L, based on final dry body mass.

The overall chronic NOEC concentrations for peak-exposure of *Daphnia magna* to aclonifen (tech.), provided two times for a duration of 24 hours, on study Days 0 and 7, alternating with recovery periods in untreated water was less than the tested peak exposure concentration of 221 µg a.s./L. This NOEC is based on parent body length at study Days 14 and 21.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen tech. (BCS-AG74518)
	Batch no.:	AE F068300-01-14
	Purity:	99.5% w/w
	Appearance:	Yellow powder
	Date received:	Not provided
	Storage:	+10 to +30° C
	Expiry date:	26 November 2016
2.	**Test Organism:**	*Daphnia magna*
	Source:	[redacted]
	Age:	First instar, less than 24 hours old neonates

	Feeding:	Three times per week with living cells of the green alga *Desmodesmus subspicatus* in aqueous suspension
3.	**Test water:**	Elendt M7

B. STUDY DESIGN AND METHODS

1. In-life phase: 28 June – 28 July 2016

2. Exposure conditions

Test vessels:	250 mL glass beakers (DIN 12332), filled with 100 mL of the test solution, corresponding to a fluid level of approximately 4 cm height; during exposure covered with transparent glass plates
Experimental design:	5 test concentrations (5.12, 12.8, 32.0, 80.0 and 200 µg a.s./L) plus 1 control and 1 solvent control (100 µL DMF/L) for the continuous static-renewal exposure plus a supplemental single peak exposure concentration of 200 µg a.s./L provided two times for a duration of 24 hours on study Days 0 and 7, alternating with recovery periods in untreated water.
Replicates:	Ten replicates per control and treatment group with one daphnid per replicate, all assigned in randomised order
Loading:	100 mL test solution per organism
Temperature:	20.3 – 21.0°C
pH:	7.9 – 8.0
Aeration:	None
Photoperiod:	16 h light : 8 h dark
Light intensity:	1000 – 1500 lux

3. Administration of the test item

Aqueous solutions of the test item were prepared in artificial test water (Elendt M7) immediately before start of each exposure interval, by addition of 200 µL of a corresponding DMF stock solution to 2 litres of the artificial dilution water followed by 65 – 180 minutes of stirring by a magnetic stirrer.

Test media renewal was performed 3 times per week (Days 2, 5, 7, 9, 12, 14, 16 and 19).

4. Test organism assignment and treatment

Daphnia were placed in the test solutions within 30 minutes of addition of the test item. On Mondays, Wednesdays and Fridays, immediately after new test solutions had been prepared, each parent animal was gently transferred to its corresponding fresh test solution inside a minimised volume of the old test solution.

Each vessel received living cells of unicellular green alga *Desmodesmus subspicatus* (strain SAG 86/81) at a daily amount of 0.1 - 0.2 mg TOC per test vessel with 100 mL, corresponding to 1 x 10^8 cells/L.

5. Measurements and observations

Sublethal effects on parental animals and offspring were assessed by visual comparison of untreated control animals and treated animals, including existence of aborted eggs and neonates mortality.

The parental survival was determined by counting of mobile daphnids, defined as animals with swimming movements (slight movements of antennae were not interpreted as swimming movement) within approximately 15 seconds after gentle agitation of the test vessel.

The onset of maturity as indicated by first brood release was recorded individually for each parent female. The number of neonates per adult reproductive day was counted daily (visual enumeration) from first brood release. Once counted the offspring were discarded.

Parental body length was manually measured for surviving parental individuals via a stage micrometer and stereo dissecting microscope (to the nearest 0.05 mm), taken from the apex of the helmet to the base of the posterior spine. This was performed immediately after termination of exposure, as well as for all surviving parent animals after 7 and 14 days of exposure and for 10 representative 1st neonates, chosen from the used breeding stock at start of exposure.

Parental dry-body masses at study termination was manually measured as dry-body-mass on a digital balance (to the nearest 0.01 mg) for surviving parental individuals, immediately after completion of lyophilisation (3 days at 25°C / 0.030 mbar).

Dissolved oxygen, pH and temperature were measured before and after each test media renewal.

Verification of test concentrations was carried out on freshly prepared test media on study days 0, 7 and day 16 for static renewal exposure and on study days 0 and 7 for pulse exposure, immediately before distribution to the test vessels from batch preparation for each treatment and control group. For the aged test media, sampling was performed on study days 2, 9 and 19 for static renewal exposure, and on days 2 and 9 for pulse exposure, immediately after termination of exposure as composite from all replicates of a treatment group and control group.

6. Statistics/Data evaluation

As the study covered pure water control and an additional solvent control, adequate analysis for detection of statistically significant differences between controls was performed (Shapiro-Wilk´s Test on Normal Distribution, STUDENT-t test for Homogeneous Variances on a 5% level of significance [two-sided probability]). Wherever such pre-testing on homogeneity of controls revealed no significant differences all treatments were related to pooled controls. Otherwise, all treatments were compared with both controls separately.

If applicable, at least the EC_{10} including the associated 95 percent confidence limits for parental immobilisation and total living offspring was calculated by Probit analysis (for linear regression), or 3-parameters normal CFD (for non-linear regression). A dose response relationship curve (displayed as sigmoid, shaped over the logarithm of the concentration) was modelled. Wherever possible, computation of 95% confidence limits was included.

For the determination of NOEC and LOEC values, all grouped data were analysed on variance homogeneity (e.g. Levene´s Test) and normal distribution (e.g. Shapiro-Wilks Test) followed by parametric or non-parametric procedures.

All statistical analysis was performed using ToxRat-Professional©, Vers.3.2.1 (ToxRat Solutions GmbH, Germany).

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Chemical analysis of aclonifen in the freshly prepared test solutions at start of the chosen exposure intervals revealed recoveries between 92% and 152% (mean: 116%) of the corresponding nominal concentrations.

The corresponding concentrations of the aged test solutions at the end of the exposure intervals ranged between 81% and 139% (mean 106%) of nominal.

All measured values for the untreated control groups were found to be below the lowest analytical standard concentration during analysis of the test samples (0.501 µg/L).

The measured test concentrations partly exceeded 120% of nominal. Therefore all reported results were based on measured time-weighted mean concentrations.

Table: Measured test concentrations from the exposure of *Daphnia magna* to Aclonifen in a 21-Day reproduction test

Nominal concentration (µg a.s./L)	Measured concentration (µg a.s./L)						TWA mean measured concentration (µg a.s./L)
	Day 0	Day 2	Day 7	Day 9	Day 16	Day 19	
	Fresh media	Aged media	Fresh media	Aged media	Fresh media	Aged media	
Control[1]	<0.501	<0.501	<0.501	<0.501	<0.501	<0.501	-
Solvent control[1]	<0.501	<0.501	<0.501	<0.501	<0.501	<0.501	-
Scenario A (continuous 21-days static-renewal exposure)							
5.12	6.56	5.57	7.78	7.13	6.56	4.91	6.27
12.8	13.2	14.8	17.7	16.0	14.7	10.7	14.2
32.0	32.1	37.3	42.9	37.9	37.1	26.1	34.8
80.0	99.1	75.7	82.2	76.8	73.3	71.7	78.1
200	200.9	192.8	210.8	243.6	n.s.	n.s.	212
Scenario B (double 24 hours peak-exposure)							
Nominal concentration (µg a.s./L)	**Day 0**	**Day 1**	**Day 7**	**Day 8**			**TWA mean measured concentration (µg a.s./L)**
	Fresh media	**Aged media**	**Fresh media**	**Aged media**			
200	201.6	196.5	252.9	234.0			221

[1]: Aclonifen was not detected in the control samples in a concentration higher than 0.501 µg/L, which was used as the lowest standard concentration during this study (multiplied with the dilution factor).
n.s.: No sample

The validated method is summarised in Document M-CA4 (CA 4.1.2/89).

B. BIOLOGICAL DATA

While one animal (10%) of the introduced parent animals of the pure water control group died prematurely, all parent animals from solvent control group survived unaffected. Statistical pre-testing on homogeneity of controls (Shapiro-Wilk´s Test on Normal Distribution and STUDENT-t test for Homogeneous Variances) revealed no significant differences between control groups. Therefore, biological results were related to pooled-control groups.

For water quality monitoring, temperatures, pH values and dissolved oxygen concentrations of the exposure solutions, as well as conductivity, hardness and alkalinity of the used test media, were regularly

controlled throughout the study as recommended by the underlying guidelines. As measurements show, the physical / chemical properties corresponded to the required values. Thus, the study conditions and breeding quality met the required quality criteria.

Table: Summary of effects from the exposure of *Daphnia magna* to Aclonifen in a 21-Day reproduction test

Treatment		Parental endpoints							Reproductive endpoints		
Nominal conc.	TWA-mean measured conc.	Body length (mm)				Dry body mass (mg)	Survival (%)	Total offspring per parent animal (n)	Parent age at first offspring emergence (days)	Offspring behaviour	
µg a.s./L	µg a.s./L	d0	d7	d14	d21	d21	d21			Affected	Dead
Control		0.91	2.95	3.64	4.00	0.64	90	69.6	9.5	none	none
Solvent control		-	3.14	3.69	4.03	0.64	100	85.9	9.4	none	none
Pooled controls		-	3.04	3.66	4.01	0.64	95	77.8	9.4	none	none
Scenario A (continuous 21 days static-renewal exposure)											
5.12	6.27	-	3.05	3.51	3.92	0.61	100	73.2	10.2	2 eggs	none
12.8	14.2	-	2.83	3.35	3.77	0.55	100	73.3	10.2	none	none
32.0	34.8	-	2.68	3.31	3.65	0.48	100	64.7	9.9	none	none
80.0	78.1	-	1.77	1.96	2.95	0.26	40	1.7	20.0	none	none
200	212	-	1.32	-	-	-	0	0.0	-	none	none
Scenario B (double 24 hours peak-exposure)											
200	221	-	2.95	3.47	3.87	0.61	80	66.6	9.9	none	none

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 211, 2012)	Achieved*
Control mortality	≤20%	5%
Mean number of living offspring per parent animal surviving at the end of the test	≥60	77.8

*Based on pooled controls

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Time weighted mean measured concentration (µg a.s./L)			
	NOEC	LOEC	EC_{10}	95% confidence limits
Scenario A (continuous 21 days static-renewal exposure)				
Total number of living offspring produced per introduced parent animal	34.8	78.1	32.9	24.5 – 44.2
Immobilization of the parent animals	34.8	78.1	60.6	n.d.
Parental age at first offspring emergence	34.8	78.1	n/a	-
Parent body length at study Day 7	14.2	34.8	19.3	12.2 – 30.5
Parent body length at study Day 14	6.27	14.2	38.5	29.3 – 50.7
Parent body length at study Day 21	14.2	34.8	37.4	25.6 – 54.6

Final dry body mass of surviving parental animals	14.2	34.8	14.5	5.3 – 39.4
Scenario B (double 24 hours peak-exposure)				
Total number of living offspring produced per introduced parent animal	≥ 221	-	-	
Immobilization of the parent animals	n/a	-	-	-
Parental age at first offspring emergence	≥ 221	-	-	-
Parent body length at study Day 7	≥ 221	-	-	-
Parent body length at study Day 14	< 221	-	-	-
Parent body length at study Day 21	< 221	-	-	-
Final dry body mass of surviving parental animals	≥ 221	-	-	-

n/a: calculation not applicable
n.d.: not determined either due to mathematical reasons or value is beyond the tested concentrations by more than factor 1000

III. CONCLUSION

The lowest chronic NOEC for 21 days of static renewal exposure of *Daphnia magna* to aclonifen (tech.) was 6.27 µg a.s./L based on parent body length at study Day 14. However, the observed effects at 14.2 µg/L on the body length were not statistically significant on Day 7 or Day 21. Therefore, these effects are not considered to be biologically relevant and the overall NOEC for the study is 14.2 µg/L based on length and dry weight. The non relevance of the effects on length at Day 14 is confirmed by the EC_{10} of 38.5 µg/L which is similar the the EC_{10} at Day 21 (37.4 µg/L).

The lowest EC_{10} was 14.5 µg a.s./L based on final dry body mass.

The overall chronic NOEC concentrations for peak-exposure of *Daphnia magna* to aclonifen (tech.), provided two times for a duration of 24 hours, on study Days 0 and 7, alternating with recovery periods in untreated water was less than the tested peak exposure concentration of 221 µg a.s./L. This NOEC is based on parent body length at study Days 14 and 21.

[redacted] *(2017)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The overall chronic NOEC for 21 days of static renewal exposure of *Daphnia magna* to aclonifen (tech.) is 14.2 µg a.s./L. This NOEC is based on parent body length and dry weight at study Day 21. The corresponding LOEC is 34.8 µg a.s./L.

The lowest EC_{10} was 14.5 µg a.s./L based on final dry body mass.

The overall chronic NOEC concentrations for peak-exposure of *Daphnia magna* to aclonifen (tech.), provided two times for a duration of 24 hours, on study Days 0 and 7, alternating with recovery periods in untreated water was less than the tested peak exposure concentration of 221 µg a.s./L. This NOEC is based on parent body length at study Days 14 and 21.

EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2019)[2] recommends that the lowest of the EC_{10} and NOEC values be used for risk assessment purposes. In this study, as the NOEC was lower than the EC_{10}, the NOEC of 14.2 µg a.s./L should be used for risk assessment.

EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2015)[3] recommends that measured concentrations are calculated using the geometric mean. A summary of the TWA and geometric mean measured concentrations is provided in the following table:

Table: Measured concentrations from the exposure of *Daphnia magna* to Aclonifen

Nominal concentration (µg a.s./L)	Measured concentration (µg a.s./L)			
	Time-weighted average	% Nominal	Geometric Mean	% Nominal
Scenario A (continuous 21 days static-renewal exposure)				
5.12	6.27	122	6.31	123
12.8	14.2	111	14.3	112
32	34.8	109	35.2	110
80	78.1	98	78.8	98
200	212	106	211.2	106
Scenario B (double 24 hours peak-exposure)				
200	221	111	220	110

Given that the geometric mean measured test concentrations were within 1% of the TWA measured test concentrations it was considered that recalculation of the study endpoints based on the geometric mean measured concentrations was not necessary. Consequently, the NOEC of 14.2 µg a.s./L is used for risk assessment.

Assessment and conclusion by RMS:

[2] EFSA (European Food Safety Authority), 2019. Technical report on the outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2019:EN-1673. 117 pp. doi:10.2903/sp.efsa.2019.EN-1673

[3] EFSA (European Food Safety Authority), 2015. Technical report on the outcome of the pesticides peer review meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2015:EN-924. 62 pp.

Data Point:	KCA 8.2.5.1/03
Report Author:	[redacted]
Report Year:	2019
Report Title:	Aclonifen: Toxicity to the water flea Daphnia magna straus under laboratory conditions (21 d reproduction test - Pulsed exposure-scenario)
Report No:	EBCL0211
Document No:	M-670399-01-1
Guideline(s) followed in study:	OECD -GUIDELINES FOR THE TESTING OF CHEMICALS NO. 211: Daphnia magna Reproduction Test, Adopted by the Council at 2nd October 2012 and EPA OCSPP-NUMBER 850.1300, U.S. ENVIRONMENTAL PROTECTION AGENCY, 2016. SERIES 850 - ECOLOGICAL EFFECTS TEST GUIDELINES: DAPHNID CHRONIC TOXICITY TEST
Deviations from current test guideline:	Current Guideline: OECD 211, 2012 Some environmental parameters varied by more than the allowed range. The impact of these deviations is considered to be minor with no impact on the study outcome.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A chronic toxicity test was performed to identify possible effects of the aclonifen on development, reproductive capacity and behaviour of *Daphnia magna* over 21 days under a pulsed exposure scenario was conducted.

Daphnia magna were exposed to the solutions containing nominal concentrations of aclonifen at 237, 356, 533, 800 and 1200 µg a.s./L together with a control and a solvent control twice for 48 hours within the 21 day test duration. The first pulse was set between day 0 and day 2 and the second pulse was set between day 7 and day 9. Between pulses daphnia were kept in untreated test medium with semi-statically renewal of test medium. The mortality, the time of the first production of offspring, the number of offspring and body length were compared with the corresponding parameters in the controls. Assessments on other effects (mobility of parental daphnia, appearance of aclonifen solution) were performed each day

As endpoints, the total living offspring per introduced parent animal, the total living offspring per adult surviving to day 21 (test end) as well as body length of parental survivors at the end of the study were recorded as data for NOEC/LOEC calculation. Additional body length measurements were performed on day 0, 2 and 9 (end of pulsed exposure).

The measured content of aclonifen was between 78 and 112% of nominal in the fresh samples with a mean recovery of 100% of nominal in these initial samples. In the aged samples the measured content was between 74 and 112% of nominal with a mean measured recovery of 96% of nominal. The toxicological endpoints were evaluated using nominal active substance concentrations and the actual

concentrations (based on the geometric mean of active substance of each measured concentration), since some values are found to be below 80% of nominal.

Results are expressed based on geometric mean measured concentrations. The NOEC for mortality was calculated as 807 µg a.s./L. The day 21 LC_{10} was determined to be 573 µg a.s./L and the LC_{50} was 1076 µg a.s./L.

The number of alive offspring produced by adults alive from test start showed a statistically significant decrease of reproduction in the test item (active substance) concentration of 213 µg a.s./L and above. Therefore, the NOEC was determined as <213 µg a.s./L. The EC_{10} and EC_{20} could not be determined statistically as inhibition was >20% for all concentration levels.

The number of alive offspring produced by adults alive at day 21 (test end) showed statistically significant decrease of reproduction in the test item (active substance) concentration of 213 µg a.s./L and above. The NOEC was determined as <213 µg a.s./L. The EC_{10} and EC_{20} could not be determined statistically as inhibition was >20% at this time point for all concentration levels.

The NOEC for body length was <213 µg a.s./L at day 21 (test end) and the LOEC was 213 µg a.s./L. No value for EC_{10} could be determined statistically, as inhibition was between 11.7 and 29.4% at this time point for all concentration levels. The EC_{20} was determined to be 529 µg a.s./L.

The overall NOEC was <213 µg a.s./L, including all parameters (mortality of adults, reproduction and body length).

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test item**
   - **Batch no.:** Aclonifen
   - **Purity:** PTDI001324
   - **Appearance:** 99.9% w/w
   - **Storage:** Yellow solid
   - **Expiry date:** Ambient +15 to +25° C
   - 13 November 2020

2. **Test Organism:** *Daphnia magna*, Straus, Clone V
   - **Source:** [redacted]
   - **Age:** First instar, less than 24 hours old neonates
   - **Feeding:** Three times per week with living cells of the green alga *Desmodesmus subspicatus* in aqueous suspension. Also fed with suspension of Tetra Min Baby® at every media renewal

3. **Test water:** Elendt M4

B. STUDY DESIGN AND METHODS

1. **In-life phase:** 27 March to 26 April 2019

2. Exposure conditions

Test vessels:	100 mL glass beakers, filled with 50 mL of the test solution, during exposure covered with transparent glass plates
Experimental design:	5 test concentrations (237, 356, 533, 800 and 1200 µg a.s./L) plus 1 control and 1 solvent control (100 µL DMF/L) 10 replicates per test treatment, each containing 1 daphnid
Replicates:	Ten replicates per control and treatment group with one daphnid per replicate, all assigned in randomised order
Loading:	50 mL test solution per organism
Temperature:	18.7 - 21.1°C
pH:	7.46 – 9.41
Dissolved oxygen	≥8.5 mg/L
Aeration:	None
Photoperiod:	16 h light : 8 h dark
Light intensity:	1432 lux

3. Administration of the test item

The necessary amount of aclonifen for preparing the stock solution S1 was prepared by dissolving 120 mg of the aclonifen in 5.00 mL DMF (dimethylformamide). The solution was homogenised by shaking. Lower test solutions were prepared by dilution of the appropriate solution with DMF. 50 µL of each dilution solution was applied into one litre Elendt M4. All solutions were prepared freshly on each day of application. The concentration of the solvent DMF was 50 µL/L in the aclonifen treatment and in the solvent control. The preparation procedure was done on test start (first pulse) and repeated at day 7 (second pulse). Decreasing yellow discolouration of solutions was observed with decreasing concentration on each application day. No precipitation was observed. Between pulses daphnia were kept in untreated test medium with renewal of test solutions every Monday, Wednesday and Friday.

4. Test organism assignment and treatment

Daphnia were placed in the test solutions within 30 minutes of addition of the aclonifen. On Mondays, Wednesdays and Fridays, immediately after new test solutions had been prepared, each parent animal was gently transferred to its corresponding fresh test solution inside a minimised volume of the old test solution.

Each vessel received living cells of unicellular green alga *Desmodesmus subspicatus* (strain SAG 86/81) at a daily amount of 0.1 - 0.2 mg TOC per test vessel with 100 mL, corresponding to 1 x 10^8 cells/L.

5. Measurements and observations

All dead animals were counted and removed daily. The presence of eggs in the brood pouch, males or winter eggs were recorded. Similarly, if there were obvious differences in condition and size of the parental generation between the test concentrations and the controls, these differences were reported.

Additionally, mobility of parental daphnia was observed daily. Immobile parental daphnia were not discarded but checked for recovery the next day.

A determination of the body length of 5 representative alive parental daphnia was performed for control, solvent control and each treatment group at test start, and at the end of each peak (days 2 and 9) for

modelling purposes only. At test end determination of the body length of all daphnids alive was performed.

Dissolved oxygen, pH, total hardness and temperature were measured on day 0, at each test medium renewal and at the end of the test.

Verification of test concentrations and stability of aclonifen was carried out on analytical samples taken from all aclonifen concentrations and controls at test start and 7 days from fresh solutions from bulk solutions and after 2 days and 9 days from aged solutions from pooled replicates to cover beginning and end of both peaks. A retained sample was also taken. Stability control samples were taken from additional vessels with test solutions without food and daphnids, which will last from day 0 to day 2 (peak 1), the other from day 7 to day 9 (peak 2), corresponding to the pulsed exposure scenario. All samples were stored deep frozen until they were transferred to the analytical laboratory. Sample analysis was performed by direct injection of test medium samples after dilution and quantification by HPLC-MS/MS detection.

6. Statistics/Data evaluation

LOEC is the lowest aclonifen concentration tested showing a statistically significant difference from the control(s) for all endpoints. NOEC is the highest aclonifen concentration tested below the LOEC for the respective endpoint. $LC_{10, 20, 50}$/$LC_{10, 20, 50}$ is the aclonifen concentration causing 10, 20, 50% variation in the respective endpoint of the test organism population (estimated by probit and Weibull analysis).

Controls were pooled for statistical evaluation of all parameters, since no statistically significant difference was found between control and solvent control. For the calculation of NOEC, LOEC and $EC_{10, 20, 50}$/$LC_{10, 20, 50}$ ToxRat Professional 3.3.0 was used.

Mortality of adult Daphnia

A test for normality of the data was performed by calculating the Shapiro-Wilk's statistic, a test for homogeneity of the data was performed according to Levene. The NOEC and LOEC were determined by using a multiple comparison method (Step-down Cochran-Armitage Test Procedure; $p \leq 0.05$). As LOEC the lowest statistically significant concentration was defined. The $LC_{10, 20, 50}$-values were determined by Weibull analysis using linear max. likelihood regression.

Reproductive output per parent animal from test start

A test for normality of the data was performed by calculating the Shapiro-Wilk's statistic, a test for homogeneity of the data was performed according to Levene. The NOEC and LOEC were determined by using a multiple comparison method (Williams Multiple Sequential t-test Procedure; $p \leq 0.05$). As LOEC the lowest statistically significant concentration was defined. No value for $EC_{10, 20}$ could be determined statistically, as inhibition was above 20% at this time point for all concentration levels. The EC_{50}-value was determined by Weibull analysis using linear max. likelihood regression.

Reproductive output per parent animal from test end

A test for normality of the data was performed by calculating the Shapiro-Wilk's statistic, a test for homogeneity of the data was performed according to Levene. The NOEC and LOEC were determined by using a multiple comparison method (Multiple Welch´s t-test with Bonferroni-Holm adjustment; left-sided, $p<0.05$). As LOEC the lowest statistically significant concentration was defined. No value for

$EC_{10,\ 20}$ could be determined statistically, as inhibition was above 20% at this time point for all concentration levels. The EC_{50}-value was determined by Weibull analysis using linear max. likelihood regression.

Body length at test end

A test for normality of the data was performed by calculating the Shapiro-Wilk's statistic, a test for homogeneity of the data was performed according to Levene. The NOEC and LOEC were determined by using a multiple comparison method (Williams Multiple Sequential t-test Procedure; $p \leq 0.05$). As LOEC the lowest statistically significant concentration was defined. No value for $EC_{10,\ 50}$ could be determined statistically, as inhibition was between 11.7 and 29.4% at this time point for all concentration levels. The EC_{20}-value was determined by probit analysis using linear max. likelihood regression.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The measured content of aclonifen was between 78 and 112% of nominal in the fresh samples with a mean recovery of 100% of nominal in these initial samples. In the aged samples the measured content was between 74 and 112% of nominal with a mean measured recovery of 96% of nominal. The toxicological endpoints were evaluated using nominal active substance concentrations and the actual concentrations (based on the geometric mean of active substance of each measured concentration), since some values are found to be below 80% of nominal.

The validated method is summarised in Document M-CA4 (CA 4.1.2/96).

Table: Measured test concentrations from the exposure of *Daphnia magna* to Aclonifen in a 21-Day reproduction test

Nominal concentration (µg a.s./L)		Measured concentration (µg a.s./L)					
		Day 0	Day 2	Day 7	Day 9	Geometric mean measured (µg a.s./L)	Geometric mean (%)
As test item	As aclonifen	Fresh media	Aged media	Fresh media	Aged media		
Control	-	n.d.	n.d.	n.d.	n.d.	-	-
Solvent control	-	n.d.	n.d.	n.d.	n.d.	-	-
237	237	258	249	185	176	213	90
356	356	381	394	308	272	335	94
533	532	575	575	485	450	516	97
800	799	881	829	791	728	807	101
1200	1200	1340	1340	1210	1100	1248	104

n.d. = not detectable; LOQ = 23.7 µg a.i./L Aclonifen; LOD = 7.11 µg a.i./L

B. BIOLOGICAL DATA

Mortality of adult Daphnia

In the controls and up to and including the aclonifen concentration level of 800 µg/L no mortality above the allowed control mortality of 20% was observed. Significant mortality (70%) was observed in the highest aclonifen concentration of 1200 µg/L.

Reproductive output

In the solvent control 1444 alive and 0 dead offspring were counted during the test duration in all replicates. In the control 1231 alive and 0 dead offspring were counted during the test duration in all replicates. In the highest aclonifen concentration 72 alive and 6 dead offspring were counted. The mean sum of total offspring (dead and alive) per alive adult at the end of the test ranged from 26.0 (1200 µg/L) to 151.6 (solvent control). The CV of the mean of living offspring was found to be 22.5% in the solvent control and 25.6% in the control.

The first offspring in the solvent control and control was observed on day 8. The first offspring at the concentration level 237 µg/L was observed on day 9, at 356 µg/L on day 10, at 533 µg/L on day 11, at 800 µg/L on day 15 and at 1200 µg/L on day 17.

Reproductive output per parent animal from test start

The mean number of alive offspring at test end per adult from test start was 144.4 in the solvent control, 123.1 in the control and 7.2 in the highest aclonifen concentration. Statistically significant inhibitory effects were determined for this parameter at all aclonifen concentrations. The inhibition in the highest aclonifen concentration of 1200 µg/L was 94.6% compared to the pooled controls.

Reproductive output per parent animal alive at test end

The mean number of alive offspring at test end per adult alive at test end was 151.6 in the solvent control, 123.1 in the control and 24.0 in the highest aclonifen concentration. Statistically significant inhibitory effects were determined for this parameter at all test item concentrations. The inhibition in the highest item concentration of 1200 µg/L was 82.4% compared to the pooled controls.

Body length at test start

At test start the body length of five representative daphnids from the apex (without anal spine) to the helmet was measured. The measurement of body length was done for modelling purposes only and no statistical evaluation was performed.

Body length at day 2 (end of first pulse) and day 9 (end of second pulse)

At the end of the first pulse the body length of the same five representative daphnids chosen at test start from the apex (without anal spine) to the helmet was measured. At the end of the second pulse the body length of the same five representative daphnids chosen at test start from the apex (without anal spine) to the helmet was measured. The measurement of body length was done for modelling purposes only and no statistical evaluation was performed.

Body length at test end

At the end of the test the body length of each surviving adult daphnid from the apex (without anal spine) to the helmet was measured. Statistically significant inhibitory effects were determined for body length at all aclonifen concentrations compared to the pooled controls.

Behaviour and appearance of adult Daphnids

Adult daphnids in the test concentration levels 237, 356 and 533 µg/L appeared to be smaller compared to the controls throughout the test. They were additionally found to be paler compared to the controls between day 8 and 11 at 237 µg/L, between day 8 and 13 at 356 µg/L and from day 8 until test end at 533 µg/L.

Adult daphnids in the test concentration level 800 µg/L appeared to be smaller and weak compared to the controls from day 2 until day 7 and were smaller and paler compared to the controls from day 8 until test end. Adult daphnids in the test concentration level 1200 µg/L appeared to be smaller and weak compared to the controls between day 2 and 7, as well as between day 12 and 17. Between day 8 and 11 they were additionally estimated to be paler compared to the controls and covered with algae, from day 18 until test end they were smaller and paler compared to the controls.

Table: Summary of effects from the exposure of *Daphnia magna* to Aclonifen in a 21-Day reproduction test

Geometric mean measured conc (µg a.s./L)	Body length (mm)				Adult survival at day 21	Total offspring	Mean offspring per parent at test start	% inhibition to pooled control	Mean offspring per parent alive at day 21	% inhibition to pooled control
	d0	d7	d9	d21	(n)	(n)	(n)		(n)	
Control	1.010	1.839	3.190	4.258	10	1231	123.1		123.1	
Solvent control	1.038	1.859	3.529	4.443	9	1444	144.4	-	151.6	-
Pooled control	1.024	1.849	-	4.346		1338	133.8	-	136.6	-
213	1.011	1.260	2.615	3.837*	10	914	91.4*	31.7	91.4*	33.1
335	1.045	1.276	2.422	3.748*	9	734	73.4*	45.1	81.1*	40.6
516	1.045	1.314	2.251	3.482*	10	721	72.1*	46.1	72.1*	47.2
807	1.097	1.242	2.016	3.153*	8	430	43.0*	67.9	52.3*	61.7
1248	1.040	1.279	1.544	3.067*	1	72	7.2*	94.6	24.0*	82.4

* Statistically significant from pooled controls

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 211, 2012)	Achieved*
Control mortality	≤20%	10% (solvent control) 0% (control)
Mean number of living offspring per parent animal surviving at the end of the test	≥60	151.6 (solvent control) 123.1 (control)

*Based on pooled controls

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Geometric mean measured concentration of aclonifen (µg a.s./L)				
	NOEC	LOEC	EC/LC$_{10}$ (95% CI)	EC/LC$_{20}$ (95% CI)	EC/LC$_{50}$ (95% CI)
Mortality of adult Daphnia	807 [a]	1248 [a]	573 [b] (217 – 754)	736 [b] (404 - 917	1076 [b] (851 – 1495)
Alive offspring per adult (day 0)	<213 [c]	213 [c]	n.d. [d]	n.d. [d]	447 [e] (205 – 663)
Alive offspring per alive adult (day 21)	<213 [f]	213 [f]	n.d. [d]	n.d. [d]	489 [b]
Body length (day 21)	<213 [c]	213 [c]	n.d. [d]	529 [g] (383 – 670)	n.d. [d]

Controls were pooled, since no statistically significant difference was found between control and solvent control

a Following Step-down Cochran-Armitage test (descending order, $p<0.05$)
b Calculated by Weibull analysis using linear max. likelihood regression
c Following Williams Multiple Sequential t-test Procedure ($p<0.05$)
d No value for EC$_{10}$, EC$_{20}$ could be determined statistically as inhibition was >20% at this time point for all concentration levels
e Calculated by Weibull analysis using linear max. likelihood regression
f Following Multiple Welch´s t-test with Bonferroni-Holm adjustment (left-sided, $p<0.05$)
g Calculated by probit analysis using linear max. likelihood regression
n.d. Not determined

III. CONCLUSION

Results are expressed based on geometric mean measured concentrations.

The NOEC for mortality was calculated as 807 µg a.s./L and the LOEC was 1248 µg a.s./L. The day 21 LC$_{10}$ was determined to be 573 µg a.s./L, the LC$_{20}$ was 736 µg a.s./L and the LC$_{50}$ was 1076 µg a.s./L.

The number of alive offspring produced by adults alive from test start showed a statistically significant decrease of reproduction in the test item (active substance) concentration of 213 µg a.s./L and above. Therefore, the NOEC was determined as < 213 µg a.s./L and the LOEC was 213 µg a.s./L. The EC$_{10}$ and EC$_{20}$ could not be determined statistically, as inhibition was >20% for all concentration levels.

The number of alive offspring produced by adults alive at day 21 (test end) showed statistically significant decrease of reproduction in the test item (active substance) concentration of 213 µg a.s./L and above. The NOEC was determined as 213 µg a.s./L and the LOEC was 213 µg a.s./L. The EC$_{10}$ and EC$_{20}$ could not be determined statistically, as inhibition was >20% at this time point for all concentration levels.

The NOEC for body length was <213 µg a.s./L at day 21 (test end) and the LOEC was 213 µg a.s./L. No value for EC$_{10}$ could be determined statistically, as inhibition was between 11.7 and 29.4% at this time point for all concentration levels. The EC$_{20}$ was determined to be 529 µg a.s./L.

The overall NOEC was <213 µg a.s./L, including all parameters (mortality of adults, reproduction and body length).

[redacted] *(2019)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

Results are expressed based on geometric mean measured concentrations.

The NOEC for mortality was calculated as 807 µg a.s./L. The day 21 LC_{10} was determined to be 573 µg a.s./L and the LC50 was 1076 µg a.s./L.

The number of alive offspring produced by adults alive from test start showed a statistically significant decrease of reproduction in the test item (active substance) concentration of 213 µg a.s./L and above. Therefore, the NOEC was determined as < 213 µg a.s./L. The EC_{10} and EC_{20} could not be determined statistically as inhibition was >20% for all concentration levels.

The number of alive offspring produced by adults alive at day 21 (test end) showed statistically significant decrease of reproduction in the test item (active substance) concentration of 213 µg a.s./L and above. The NOEC was determined as < 213 µg a.s./L. The EC_{10} and EC_{20} could not be determined statistically as inhibition was >20% at this time point for all concentration levels.

The NOEC for body length was 213 µg a.s./L at day 21 (test end) and the LOEC was 213 µg a.s./L. No value for EC10 could be determined statistically, as inhibition was between 11.7 and 29.4% at this time point for all concentration levels. The EC_{20} was determined to be 529 µg a.s./L.

EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2019)[4] recommends that the lowest of the EC_{10} and NOEC values be used for risk assessment purposes. In this study, as the NOEC was lower than the EC_{10}, the NOEC of <213 µg a.s./L should be used for risk assessment.

Assessment and conclusion by RMS:

[4] EFSA (European Food Safety Authority), 2019. Technical report on the outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2019:EN-1673. 117 pp. doi:10.2903/sp.efsa.2019.EN-1673

Data Point:	KCA 8.2.5.1/04
Report Author:	[redacted]
Report Year:	2019
Report Title:	Aclonifen: Toxicity to the water flea Daphnia magna straus under laboratory conditions (21 d reproduction test - Pulsed exposure-scenario)
Report No:	S19-00213
Document No:	M-670403-01-1
Guideline(s) followed in study:	OECD -GUIDELINES FOR THE TESTING OF CHEMICALS NO. 211: Daphnia magna Reproduction Test, Adopted by the Council at 2nd October 2012 and US SPP-NUMBER 850.1300, U.S. ENVIRONMENTAL PROTECTION AGENCY, 2016. SERIES 850 - ECOLOGICAL EFFECTS TEST GUIDELINES: DAPHNID CHRONIC TOXICITY TEST
Deviations from current test guideline:	Current Guideline: OECD 211, 2012 Some environmental parameters varied by more than the allowed range. The impact of these deviations is considered to be minor with no impact on the study outcome.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A chronic toxicity test was performed to identify possible effects of the aclonifen on development, reproductive capacity and behaviour of *Daphnia magna* over 21 days under a pulsed exposure scenario was conducted.

Daphnia magna were exposed to the solutions containing nominal concentrations of aclonifen at 237, 356, 533, 800 and 1200 µg a.s./L together with a control and a solvent control twice for 48 hours within the 21 day test duration. The first pulse was set between day 0 and day 2 and the second pulse was set between day 14 and day 16. Between pulses daphnia were kept in untreated test medium with semi-statically renewal of test medium. The mortality, the time of the first production of offspring, the number of offspring and body length were compared with the corresponding parameters in the controls. Assessments on other effects (mobility of parental daphnia, appearance of aclonifen solution) were performed each day.

As endpoints, the total living offspring per introduced parent animal, the total living offspring per adult surviving to day 21 (test end) as well as body length of parental survivors at the end of the study were recorded as data for NOEC/LOEC calculation. Additional body length measurements were performed on day 0, 2 and 16 (end of pulsed exposure).

The initial measured content of aclonifen was between 101 and 120% and in the aged samples the measured content was between 97 and 112% of nominal. Therefore, the ecotoxicological endpoints were evaluated using nominal active substance.

Results are expressed based on nominal exposure concentrations. The NOEC for mortality was calculated as 799 µg a.s./L. The day 21 LC10 was determined to be 726 µg a.s./L and the LC_{50} was 1090 µg a.s./L.

The number of alive offspring produced by adults alive from test start showed a statistically significant decrease of reproduction in the test item (active substance) concentration of 237 µg a.s./L and above. Therefore, the NOEC was determined as <237 µg a.s./L. The EC_{10} and EC_{20} could not be determined statistically as inhibition was >20% for all concentration levels.

The number of alive offspring produced by adults alive at day 21 (test end) showed statistically significant decrease of reproduction in the test item (active substance) concentration of 237 µg a.s./L and above. The NOEC was determined as <237 µg a.s./L. The EC_{10} and EC_{20} could not be determined statistically as inhibition was >20% at this time point for all concentration levels.

The NOEC for body length was <237 µg a.s./L at day 21 (test end) and the LOEC was 237 µg a.s./L. No value for EC_{50} could be determined statistically, as inhibition was <50% at the highest nominal test concentration (1200 µg a.s./L). The EC_{10} was determined to be 304 µg a.s./L and the EC_{20} was 816 µg a.s./L.

The overall NOEC was <237 µg a.s./L (including all parameters (mortality of adults, reproduction and body length).

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test item**
   - **Batch no.:** Aclonifen
   - **Purity:** PTDF001324
   - **Appearance:** 99.9% w/w
   - **Storage:** Yellow solid
   - **Expiry date:** Ambient +15 to +25°C
   - 13 November 2020

2. **Test Organism:** *Daphnia magna*. Straus, Clone V
   - **Source:** [redacted]
   - **Age:** First instar, less than 24 hours old neonates
   - **Feeding:** Three times per week with living cells of the green alga *Desmodesmus subspicatus* in aqueous suspension. Also fed with suspension of Tetra Min Baby® at every media renewal

3. **Test water:** Elendt M4

B. STUDY DESIGN AND METHODS

1. **In-life phase:** 27 March to 25 April 2019

2. Exposure conditions

Test vessels:	100 mL glass beakers, filled with 50 mL of the test solution, during exposure covered with transparent glass plates
Experimental design:	5 test concentrations (237, 356, 533, 800 and 1200 µg a.s./L) plus 1 control and 1 solvent control (100 µL DMF/L) 10 replicates per test treatment, each containing 1 daphnid
Replicates:	Ten replicates per control and treatment group with one daphnid per replicate, all assigned in randomised order
Loading:	50 mL test solution per organism
Temperature:	18.7 - 21.1°C
pH:	7.46 – 9.41
Dissolved oxygen	≥8.5 mg/L
Aeration:	None
Photoperiod:	16 h light : 8 h dark
Light intensity:	1432 lux

3. Administration of the test item

The necessary amount of aclonifen for preparing the stock solution S1 was prepared by dissolving 120 mg of the aclonifen in 5.00 mL DMF (dimethylformamide). The solution was homogenised by shaking. Lower test solutions were prepared by dilution of the appropriate solution with DMF. 50 µL of each dilution solution was applied into one litre Elendt M4. All solutions were prepared freshly on each day of application. The concentration of the solvent DMF was 50 µL/L in the aclonifen treatment and in the solvent control. The preparation procedure was done on test start (first pulse) and repeated at day 14 (second pulse). Decreasing yellow discolouration of solutions was observed with decreasing concentration on each application day. No precipitation was observed. Between pulses daphnia were kept in untreated test medium with renewal of test solutions every Monday, Wednesday and Friday.

4. Test organism assignment and treatment

Daphnia were placed in the test solutions within 30 minutes of addition of the aclonifen. On Mondays, Wednesdays and Fridays, immediately after new test solutions had been prepared, each parent animal was gently transferred to its corresponding fresh test solution inside a minimised volume of the old test solution.

Each vessel received living cells of unicellular green alga *Desmodesmus subspicatus* (strain SAG 86/81) at a daily amount of 0.1 - 0.2 mg TOC per test vessel with 100 mL, corresponding to 1 x 10^8 cells/L.

5. Measurements and observations

All dead animals were counted and removed daily. The presence of eggs in the brood pouch, males or winter eggs were recorded. Similarly, if there were obvious differences in condition and size of the parental generation between the test concentrations and the controls, these differences were reported.

Additionally, mobility of parental daphnia was observed daily. Immobile parental daphnia were not discarded but checked for recovery the next day.

A determination of the body length of 5 representative alive parental daphnia was performed for control, solvent control and each treatment group at test start, and at the end of each peak (days 2 and 16) for

modelling purposes only. At test end determination of the body length of all daphnids alive was performed.

Dissolved oxygen, pH, total hardness and temperature were measured on day 0, at each test medium renewal and at the end of the test.

Verification of test concentrations and stability of aclonifen was carried out on analytical samples taken from all aclonifen concentrations and controls at test start and 7 days from fresh solutions from bulk solutions and after 2 days and 9 days from aged solutions from pooled replicates to cover beginning and end of both peaks. A retained sample was also taken. Stability control samples were taken from additional vessels with test solutions without food and daphnids, which will last from day 0 to day 2 (peak 1), the other from day 7 to day 9 (peak 2), corresponding to the pulsed exposure scenario. All samples were stored deep frozen until they were transferred to the analytical laboratory. Sample analysis was performed by direct injection of test medium samples after dilution and quantification by HPLC-MS/MS detection.

6. Statistics/Data evaluation

LOEC is the lowest aclonifen concentration tested showing a statistically significant difference from the control(s) for all endpoints. NOEC is the highest aclonifen concentration tested below the LOEC for the respective endpoint. $LC_{10, 20, 50}$/$EC_{10, 20, 50}$ is the aclonifen concentration causing 10, 20, 50% variation in the respective endpoint of the test organism population (estimated by probit analysis).

Controls were pooled for statistical evaluation of all parameters, since no statistically significant difference was found between control and solvent control. For the calculation of NOEC, LOEC and $EC_{10, 20, 50}$/$LC_{10, 20, 50}$ ToxRat Professional 3.3.0 was used.

Mortality of adult Daphnia

A test for normality of the data was performed by calculating the Shapiro-Wilk's statistic, a test for homogeneity of the data was performed according to Levene. The NOEC and LOEC were determined by using a multiple comparison method ($p \leq 0.05$). As LOEC the lowest statistically significant concentration was defined. The $LC_{10, 20, 50}$-values were determined by probit analysis using linear max. likelihood regression.

Reproductive output per parent animal from test start to test end

A test for normality of the data was performed by calculating the Shapiro-Wilk's statistic, a test for homogeneity of the data was performed according to Levene. The NOEC and LOEC were determined by using a multiple comparison method (Williams Multiple Sequential t-test Procedure; $p \leq 0.05$). As LOEC the lowest statistically significant concentration was defined. No value for $EC_{10, 20}$ could be determined statistically, as inhibition was above 20% at this time point for all concentration levels. The EC_{50}-value was determined by Weibull analysis using linear max. likelihood regression.

Body length at test end

A test for normality of the data was performed by calculating the Shapiro-Wilk's statistic, a test for homogeneity of the data was performed according to Levene. The NOEC and LOEC were determined by using a multiple comparison method (Williams Multiple Sequential t-test Procedure; $p \leq 0.05$). As LOEC the lowest statistically significant concentration was defined. The $EC_{10, 20}$ values were determined by Weibull analysis using linear max. likelihood regression. No value for EC_{50} could be

determined statistically, as inhibition was <50% at this time point for the highest concentration level. Therefore, EC_{50}-value was determined to be greater than the highest concentration level.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The measured content of aclonifen was between 101 and 120% of nominal in the fresh samples with a mean recovery of 112% of nominal in these initial samples. In the aged samples the measured content was between 97 and 112% of nominal with a mean measured recovery of 103% of nominal. The toxicological endpoints were evaluated using nominal active substance concentrations since concentrations in aged solutions decreased by <20% from initial measured concentrations.

The validated method is summarised in Document M-CA4 (CA 4.1.2/07).

Table: Measured test concentrations from the exposure of *Daphnia magna* to Aclonifen in a 21-Day reproduction test

Nominal concentration (µg a.s./L)		Measured concentration (µg a.s./L)							
		Day 0		Day 2		Day 14		Day 16	
As test item	As aclonifen	Fresh media	% nominal	Aged media	% nominal	Fresh media	% nominal	Aged media	% nominal
Control	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-
Solvent control	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-
237	237	239	101	229	97	261	110	238	100
356	356	407	114	399	112	396	111	371	104
533	532	590	111	558	105	598	112	523	98
800	799	881	110	825	103	941	118	784	98
1200	1200	1330	111	1340	112	1440	120	1200	100

n.d. = not detectable; LOQ = 23.7 µg a.i./L Aclonifen; LOD = 7.11 µg a.i./L

B. BIOLOGICAL DATA

Mortality of adult Daphnia

In the controls and up to and including the aclonifen concentration level of 800 µg/L no mortality above the allowed control mortality of 20% was observed. Significant mortality (60%) was observed in the highest aclonifen concentration of 1200 µg/L.

Reproductive output

In the solvent control 1258 alive and 0 dead offspring were counted during the test duration in all replicates. In the control 1173 alive and 0 dead offspring were counted during the test duration in all replicates. In the highest aclonifen concentration 186 alive and 0 dead offspring were counted. The mean sum of total offspring (dead and alive) per alive adult at the end of the test ranged from 28.8 (1200 µg/L) to 129.9 (solvent control). The CV of the mean of living offspring was found to be 20.7% in the solvent control and 17.8% in the control.

The first offspring in the solvent control and control were observed on day 10. The first offspring at the concentration level 237, 356, 533 and 800µg/L were observed on day 11 and at 1200 µg/L on day 12.

Reproductive output per parent animal from test start

The mean number of alive offspring at test end per adult from test start was 125.8 in the solvent control, 117.3 in the control and 18.6 in the highest aclonifen concentration. Statistically significant inhibitory effects were determined for this parameter at all aclonifen concentrations. The inhibition in the highest aclonifen concentration of 1200 µg/L was 84.7% compared to the pooled controls.

Reproductive output per parent animal alive at test end

The mean number of alive offspring at test end per adult alive at test end was 129.9 in the solvent control, 117.3 in the control and 28.8 in the highest aclonifen concentration. Statistically significant inhibitory effects were determined for this parameter at all test item concentrations. The inhibition in the highest item concentration of 1200 µg/L was 76.6% compared to the pooled controls.

Body length at test start

At test start the body length of five representative daphnids from the apex (without anal spine) to the helmet was measured. The measurement of body length was done for modelling purposes only and no statistical evaluation was performed.

Body length at day 2 (end of first pulse) and day 14 (end of second pulse)

At the end of the first pulse the body length of the same five representative daphnids chosen at test start from the apex (without anal spine) to the helmet was measured. At the end of the second pulse the body length of the same five representative daphnids chosen at test start from the apex (without anal spine) to the helmet was measured. The measurement of body length was done for modelling purposes only and no statistical evaluation was performed.

Body length at test end

At the end of the test the body length of each surviving adult daphnid from the apex (without anal spine) to the helmet was measured. Statistically significant inhibitory effects were determined for body length at all aclonifen concentrations compared to the pooled controls.

Behaviour and appearance of adult Daphnids

Adult daphnids in the test concentration levels 237, 356 and 533 µg/L appeared to be smaller compared to the controls throughout the test.

Adult daphnids in the test concentration level 800 µg/L appeared to be smaller and inactive compared to the controls from test start until day 18 and from day 19 to the end of the study were paler compared to the controls and covered with algae.

Adult daphnids in the test concentration level 1200 µg/L appeared to be smaller and inactive compared to the controls throughout the study. On day 19, they were observed to be paler compared to the controls and covered with algae.

Table: Summary of effects from the exposure of *Daphnia magna* to Aclonifen in a 21-Day reproduction test

Geometric mean measured conc. (µg a.s./L)	Body length (mm)				Adult survival at day 21	Total offspring	Mean offspring per parent at test start	% inhibition to pooled control	Mean offspring per parent alive at day 21	% inhibition to pooled control
	d0	d2	d16	d21	(n)	(n)	(n)		(n)	
Control	1.038	1.267	3.794	4.093	10	1258	117.3	-	117.3	-

Solvent control	1.046	1.159	3.817	4.183	9	1173	125.8	-	129.9	-
Pooled control	1.042	1.213	3.806	4.136			121.6	-	123.3	-
237	1.025	1.117	3.219	3.778	10	875	87.5*	28.0	87.5*	29.0
356	0.970	1.109	3.253	3.662	10	770	77.0*	36.7	77.0*	37.6
533	0.971	1.009	3.050	3.471	10	729	72.9*	40.0	72.9*	40.9
800	0.963	1.076	3.209	3.438	8	432	43.2*	64.5	49.8*	59.6
1200	0.949	1.050	2.882	3.014	4	186	18.6*	84.7	28.8*	76.6

* Statistically significant from pooled controls

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 211, 2012)	Achieved*
Control mortality	≤20%	10% (solvent control) 0% (control)
Mean number of living offspring per parent animal surviving at the end of the test	≥60	129.9 (solvent control) 117.3 (control)

*Based on pooled controls

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal concentration of aclonifen (µg a.s./L)				
	NOEC	LOEC	EC/LC$_{10}$ (95% CI)	EC/LC$_{20}$ (95% CI)	EC/LC$_{50}$ (95% CI)
Mortality of adult Daphnia	799 [a]	1200 [a]	726 [g] (339 - 881)	834 [g] (518 – 1000)	1090 [g] (901 - 1680)
Alive offspring per adult (day 0)	<237 [c]	237 [c]	n.d. [d]	n.d. [d]	556 [e] (n.c.)
Alive offspring per alive adult (day 21)	<237 [c]	237 [c]	n.d. [d]	n.d. [d]	586 [b] (n.c.)
Body length (day 21)	<237 [c]	237 [c]	304 [b] (n.c.)	816 [b] (n.c.)	>1200 [g]

Controls were pooled, since no statistically significant difference was found between control and solvent control

a Following Step-down Cochran-Armitage test (descending order, p<0.05)
b Calculated by Weibull analysis using linear max. likelihood regression
c Following Williams Multiple Sequential t-test Procedure (p<0.05)
d No value for EC$_{10}$, EC$_{20}$ could be determined statistically as inhibition was >20% at this time point for all concentration levels
e Calculated by Weibull analysis using linear max. likelihood regression
f Calculated by probit analysis using linear max. likelihood regression
g No value for EC$_{10}$, EC$_{20}$ could be determined statistically as inhibition was >20% at this time point for all concentration levels
n.c. Not calculable
n.d. Not determined

III. CONCLUSION

Results are expressed based on geometric mean measured concentrations.

The NOEC for mortality was calculated as 799 µg a.s./L and the LOEC was 1200 µg a.s./L. The day 21 LC_{10} was determined to be 726 µg a.s./L, the LC_{20} was 834 µg a.s./L and the LC_{50} was 1090 µg a.s./L.

The number of alive offspring produced by adults alive from test start showed a statistically significant decrease of reproduction in the test item (active substance) concentration of 237 µg a.s./L and above. Therefore, the NOEC was determined as <237 µg a.s./L and the LOEC was 237 µg a.s./L. The EC_{10} and EC_{20} could not be determined statistically, as inhibition was >20% for all concentration levels.

The number of alive offspring produced by adults alive at day 21 (test end) showed statistically significant decrease of reproduction in the test item (active substance) concentration of 237 µg a.s./L and above. The NOEC was determined as <237 µg a.s./L and the LOEC was 237 µg a.s./L. The EC_{10} and EC_{20} could not be determined statistically as inhibition was >20% at this time point for all concentration levels.

The NOEC for body length was <237 µg a.s./L at day 21 (test end) and the LOEC was 237 µg a.s./L. The EC_{10} was determined to be 304 µg a.s./L and the EC_{20} was determined to be 816 µg a.s./L. The EC_{50} was assessed as >1200 µg a.s./L (the highest tested concentration) as inhibition was less than 50% at this concentration.

The overall NOEC was <237 µg a.s./L (including all parameters (mortality of adults, reproduction and body length).

[redacted] *(2019)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

Results are expressed based on nominal concentrations as concentrations in aged solutions decreased by <20% from initial measured concentrations.

The NOEC for mortality was calculated as 799 µg a.s./L. The day 21 LC_{10} was determined to be 726 µg a.s./L, the LC_{20} was 834 µg a.s./L and the LC_{50} was 1090 µg a.s./L.

The number of alive offspring produced by adults alive from test start showed a statistically significant decrease of reproduction in the test item (active substance) concentration of 237 µg a.s./L and above. Therefore, the NOEC was determined as <237 µg a.s./L. The EC_{10} and EC_{20} could not be determined statistically as inhibition was >20% for all concentration levels.

The number of alive offspring produced by adults alive at day 21 (test end) showed statistically significant decrease of reproduction in the test item (active substance) concentration of 237 µg a.s./L and above. The NOEC was determined as <213 µg a.s./L. The EC_{10} and EC_{20} could not be determined statistically as inhibition was >20% at this time point for all concentration levels.

The NOEC for body length was <237 µg a.s./L at day 21 (test end) and the LOEC was 237 µg a.s./L. The EC_{10} was determined to be 304 µg a.s./L and the EC_{20} was determined to be 816 µg a.s./L. The EC_{50} was assessed as >1200 µg a.s./L (the highest tested concentration) as inhibition was less than 50% at this concentration.

EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2019)[5] recommends that the lowest of the EC_{10} and NOEC values be used for risk assessment purposes. In this study, as the NOEC was lower than the EC_{10}, the NOEC of <237 µg a.s./L should be used for risk assessment.

Assessment and conclusion by RMS:

CA 8.2.5.2 Reproductive and development toxicity to an additional aquatic invertebrate species

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

CA 8.2.5.3 Development and emergence in Chironomus riparius

Data Point:	KCA 8.2.5.3/01
Report Author:	[redacted]
Report Year:	1996
Report Title:	Aclonifen - Toxicity to the sediment dwelling chironomid larvae (chironomus riparius) - 28 days
Report No:	R007434
Document No:	M-174918-01-1
Guideline(s) followed in study:	BBA: Draft guideline 1995
Deviations from current test guideline:	Current Guideline: OECD 219, 2004 At test initiation the stock solutions were added to each vessel just above the water and not below as specified in the test guideline. This deviation was not considered to have affected study integrity and validity.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to estimate the toxicity of aclonifen on the sediment dwelling life stage of *Chironomus riparius* in a sediment-water system. A total of 600 organisms (25 per replicate 4 replicates

[5] EFSA (European Food Safety Authority), 2019. Technical report on the outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2019:EN-1673. 117 pp. doi:10.2903/sp.efsa.2019.EN-1673

per concentration) were exposed to 4 concentrations of aclonifen (12, 41. 143, and 500 µg/L), a solvent (acetone) control and a dilution water-sediment control for an exposure period of 21 days.

One hour after test initiation analytical verification of the nominal test concentrations in the overlying dilution water showed the measured values were close to the nominal concentrations (84-105% recovery). Seven days after application of the test item the concentrations of aclonifen in the dilution water were significantly reduced with 8-15% of the initial measured values recovered at the three highest concentration levels. The recovery at the lowest nominal concentration of 12 µg/L was below the limit of quantification of 2.5 µg/L for the test substance. A final analytical verification at test termination showed 3% recovery of the initial measured value at the highest nominal concentration of 500 µg/L, the recoveries at the three lower test concentrations were all below the limit of quantification for the test item at this time.

The results of the test were reported in terms of the initial measured test concentrations which were as follows; 10.8, 34.4, 150 and 472 µg/L.

As the final emergence of adult midges was observed 14 days after test initiation it was decided to terminate the test after 21 days rather than 28 days exposure to the test item.

Emergence of adult midges from first instar larvae was not significantly reduced at any of the concentrations tested. There was also no significant effect on the developmental rate of adult midges at any of the concentrations tested.

The No Observed Effect Concentration (NOEC) was therefore determined to be 472 µg/L. The Lowest Observed Effect Concentration (LOEC) was in excess of the highest test concentration of 472 µg/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen
   Batch no.: 95 335 02
   Purity: 999 g/kg
   Appearance: Yellow powder
   Date received: Not provided
   Storage: In the dark in an air-tight container, at room temperature (approximately 20°C)
   Expiry date: February 1998

2. **Test Organism:** *Chironomus riparius*
   Age: 1st instar larvae (2 to 3 days old)
   Source: [redacted]
   Feeding: 10 - 20 mL of a 10 mg/mL solution of a fish flake food (Tetramin®) three to four times weekly

3. **Test water:** Reconstituted water (80% DSW, 20% LC-oligo). Dilution water was prepared from municipal water (reverse-osmozed, deionized and filtered through activated charcoal and 0.22 µm filters)

Hardness:	160 ± 20 mg/L as $CaCO_3$

4. Sediment:

Artificial sediment (according to OECD 207) was prepared as follows (on the basis of dry weights):

- 10% sphagnum peat (as close to pH 5.5 to 6.0 as possible, no visible plant remains, air dried and finely ground)
- 20% kaolin clay (kaolinite content preferably above 30%)
- 70% industrial sand (fine sand predominates with more than 50 per cent of the particles between 50 and 200 microns).
- The pH of the final mixture of the sediment was adjusted to 6.0 ± 0.5 by addition of calcium carbonate (chemically pure quality)

The dry constituents were blended in the correct proportions and mixed thoroughly in a Turbula mixer (model T50A) for one hour. A small quantity of dilution water was added to moisten the artificial sediment before it was used for the study (100 mL per 200 g of sediment).

B. STUDY DESIGN AND METHODS

1. In-life phase: 20 June – 11 July 1996

2. Exposure conditions

Test vessels:	3 L glass beakers measuring 10 -13 cm in diameter and with a test solution height of approximately 27.5 cm
Experimental design:	Control, solvent control and nominal test concentrations of 12, 41, 143, and 500 µg/L
Replicates:	Four replicates per control and treatment group each containing 25 test organisms
Temperature:	21.1 – 21.9 °C
pH:	7.1 – 7.9
Aeration:	Gentle aeration provided through a glass Pasteur pipette situated approximately 2.5 cm above the sediment layer (approximately 1 bubble/sec). When adding the larvae, the aeration of the water was stopped. One day after adding the larvae, the aeration was provided again.
Dissolved oxygen:	≥5.4 mg/L
Photoperiod:	16 hours light: 8 hours darkness
Light intensity:	1037 – 1046 lux

3. Administration of the test item

A stock solution for the highest test item concentration (500 µg/L) was prepared by dissolving 50 mg of test item in 5 mL of acetone. The test solution for each replicate of this concentration was prepared by adding volumes of this stock solution to the water column of each test vessel. Stock solutions for the three lower test concentrations of 12, 41 and 143 µg/L were prepared by serial dilution of the first stock solution with acetone and subsequent addition to the overlying dilution water. The final concentration of solvent at each concentration level was 0.05 mL/L.

4. Preparation of test vessels

An appropriate quantity of wet artificial sediment to obtain a depth of approximately 2 cm was filled into each test beaker and left to stand for 24 hour in a fume cupboard.

To avoid a separation of the ingredients in the sediment and to minimize turbidity of the overlying water, the dilution water was then poured into each beaker very slowly, taking care not to disturb the sediment.

The test vessels were prepared 1 week before test initiation and were acclimatized under the test conditions. The test vessels contained 200 g of sediment and 2.5 l of dilution water (depth approximately 20.0 cm). The exact volume of water added was recorded, and the level marked outside on the test vessel.

The test vessels were positioned in a temperature controlled water bath in order to minimize any temperature variations.

The test vessels were covered with perspex sheets to minimize evaporation and with a nylon mesh to prevent escape of emerged midges. Water levels were not topped up during the study.

5. Test organism assignment and treatment

Egg masses were removed from the culture aquaria six days before test initiation (Day 0-6) and deposited in glass tubes each containing culture medium. Hatch was observed three days later. First instar larvae then (2-3 days old) were introduced into the test vessels two days later (one day before test initiation). Twenty five larvae were allocated to each test vessel using a blunt Pasteur pipette.

One day after adding the larvae, the appropriate volume (0.125 mL) of each stock solution was added to the water column of the test system. The additions were made just above the water surface using a pipette, the water column was then gently stirred to ensure homogeneous distribution without disturbing the sediment. Test initiation corresponded to the time of addition of the test item.

6. Measurements and observations

The endpoints of the study were the day of first emergence, the time distribution (peak) of emergence of male and female midges, and the total number of fully emerged male and female midges.

The test vessels were observed at least three times per week to make a visual assessment of any behavioural differences compared with the controls. During the period of emergence, a daily check of emerged midges was performed. The sex and number of adults emerging was recorded at each observation time. After identification, the midges were removed from the vessels. Any egg masses deposited prior to the termination of the test were recorded and removed to prevent re-introduction of larvae into the sediment. Only the number of fully emerged male and female midges were counted. Any visible pupae which failed to emerge were counted and recorded separately.

The larvae were fed at least 3 times per week at a rate of approximately 1 mg fish food per day per larvae beginning on the day of larvae were introduced into the test vessel. A 3 mL suspension of Tetramin® in dilution water (20 mg/mL) was added to each test vessel up until Day 15 of the test. The volume was then reduced to 1.5 mL per test vessel until Day 20.

Temperature, pH, conductivity and total water hardness of the dilution water were recorded at preparation of the test vessel.

The oxygen concentration, water temperature and pH were recorded in all test vessels at the start and end of the study. These parameters were subsequently recorded once a week for the duration of the test. Light intensity incident on the test vessels was also recorded at these times.

Samples of the water column in the dilution water-sediment control, solvent control and nominal concentrations of 12, 41, 143 and 500 µg/L were collected for analysis 1 hour, 7 days and 21 days after test initiation (application of test substance). Each sample volume was composed of a pool of the four replicate test vessels at each concentration level.

7. Statistics/Data evaluation

For each of the parameters emergence rate and development rate, the dilution water control group was compared to the solvent control group using a t-test. No significant differences were observed for either parameter therefore the control groups were pooled (pooled control group) for subsequent comparisons.

Statistical analysis was performed using the group mean for each parameter. Bartlett's test (using untransformed values) and the Kruskal-Wallis non parametric one-way analysis of variance by ranks were used to perform the analysis.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

One hour after test initiation analytical verification of the nominal test concentrations in the overlying dilution water showed the measured values were close to the nominal concentrations (84-105% recovery). Seven days after application of the test item the concentrations of aclonifen in the dilution water were significantly reduced with 8-15% of the initial measured values recovered at the three highest concentration levels. The recovery at the lowest nominal concentration of 12 µg/L was below the limit of quantification of 2.5 µg/L for the test substance. A final analytical verification at test termination showed 3% recovery of the initial measured value at the highest nominal concentration of 500 µg/L, the recoveries at the three lower test concentrations were all below the limit of quantification for the test item at this time.

The results of the test were reported in terms of the initial measured test concentrations which were as follows; 10.8, 34.4, 150 and 472 µg/L.

Table: Measured test concentrations from the exposure of *Chironomus riparius* to Aclonifen in a sediment-water system

Nominal concentration (µg/L)	Measured Concentration (µg/L)		
	T0 + 1 hour	T0 + 7 days	T0 + 21 days
Control	<LOQ	<LOQ	<LOQ
Solvent control	<LOQ	<LOQ	<LOQ
12	10.8	<LOQ	<LOQ

41	34.4	4.2	<LOQ
143	150	11.3	<LOQ
500	472	70.5	16.3

LOQ: Limit of Quantitation = 2.5 µg/L

The validated method is summarised in Document M-CA4 (CA 4.1.2/86).

B. BIOLOGICAL DATA

The first emergence of adult midges was observed in the control groups and at the each of the test concentrations 10 days after application (test initiation).

As the final emergence of adult midges was observed 14 days after test initiation it was decided to terminate the test after 21 days rather than 28 days exposure to the test substance. This was 7 days after the final recorded emergence in any test vessel, therefore this reduction in exposition time was not thought to have influenced the test results in any way.

In the exposed groups 36 - 96% emergence was recorded from individual replicate test vessels and the per vessel development rate for larvae in these groups ranged from 0.092 to 0.097.

The variation in ratio of males to females between replicates was greater than between test groups and no specific tendencies were observed between the controls and the test groups except that a high percentage of males emerged from two out of 4 replicates of the dilution water-sediment control group. No specific tendencies were observed with increasing test concentrations. Therefore male and female emergence data was pooled for subsequent statistical analysis.

Statistical analysis showed no significant difference between the rate of development (DR) of first instar larvae to adult midge in the exposed groups compared to the pooled control group. Bartlett's test indicated homogenous variance for this parameter and analysis of variance (ANOVA) was used to perform the analysis.

Table: Rates of emergence and development of *Chironomus riparius* following exposure Aclonifen

Initial measured concentration (µg/L)	Mean rate of emergence (standard deviation)	Mean rate of development (standard deviation)
Control	0.870 (0.038)	0.097 (0.001)
Solvent control	0.780 (0.083)	0.094 (0.004)
10.8	0.800 (0.033)	0.094 (0.002)
34.4	0.870 (0.105)	0.096 (0.001)
150	0.870 (0.020)	0.097 (0.001)
472	0.750 (0.268)	0.096 (0.001)

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 219, 204)	Achieved*
Emergence in controls	≥70%	87%
Day of emergence	12 - 23	10 - 12
Oxygen concentration at end of test	≥60% ASV	≥61% ASV
pH of overlying water at end of test	6 - 9	7.1 - 7.7
Variation in water temperature	±1.0 °C	0.5 °C

*Based on dilution water control

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Initial concentration (µg/L)		
	EC_{50}	NOEC	LOEC
28-day emergence	>472	472	>472
Development rate	>472	472	>472

III. CONCLUSION

Emergence of adult midges from first instar larvae was not significantly reduced at any of the concentrations tested. There was also no significant effect on the developmental rate of adult midges at any of the concentrations tested.

The No Observed Effect Concentration (NOEC) based on initial measured concentrations was therefore determined to be 472 µg/L. The Lowest Observed Effect Concentration (LOEC) was in excess of the highest test concentration of 472 µg/L.

[redacted] *(1996)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The No Observed Effect Concentration (NOEC) determined from the exposure of *Chironomus riparius* to aclonifen in a spiked water test based on initial measured concentrations was determined to be 472 µg/L. The Lowest Observed Effect Concentration (LOEC) was in excess of the highest test concentration of 472 µg/L.

Due to the lack of toxic effects, EC_{10}, EC_{20} and EC_{50} values can all be estimated as being greater than 472 µg/L.

Assessment and conclusion by RMS:

CA 8.2.5.4 Sediment dwelling organisms

Data Point:	KCA 8.2.5.4/01
Report Author:	[redacted]; [redacted]
Report Year:	2004
Report Title:	Sediment-water chironomid toxicity test using spiked sediment Aclonifen (AE F068300)
Report No:	C039873
Document No:	M-227300-01-1
Guideline(s) followed in study:	OECD: 218 (Draft, 12/2002)
Deviations from current test guideline:	Current Guideline: OECD 218, 2004 None
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to estimate the toxicity of aclonifen on the sediment dwelling life stage of *Chironomus riparius* in a sediment-water system. A total of 560 organisms (20 per replicate 4 replicates per concentration) were exposed to 5 concentrations of aclonifen (10, 32, 100, 320 and 1000 mg/kg), a solvent (acetone) control and a dilution water-sediment control for an exposure period of 28 days. Dosing of the test system was via spiked sediment.

Analysis for the test material was performed on Days -2 (preparation of the sediment), 0 and 28. Samples on Day -2 were analysed for the concentration of the test material in the sediment only in order to confirm correct dosing of the test system. Samples were taken for analysis of the sediment, overlying water and interstitial water on Days 0 and 28.

Analysis of the sediment on Day -2, the day the sediment was prepared showed the measured concentrations to range from 110% to 119% of nominal. Analysis of sediment on Day 0 and 28 showed the measured concentrations to range from 86% to 99% and from 83% to 99% respectively. Given that the measured concentrations were in excess of 80% of nominal throughout the test it was considered justifiable to calculate the results based on nominal test concentrations only.

The 28-Day EC_{50} (reduction in emergence) based on nominal test concentrations was 110 mg/kg with 95% confidence limits of 66 -190 mg/kg. The No Observed Effect Concentration was 32 mg/kg. The EC_{50} (development rate) based on nominal test concentrations was greater than 100 mg/kg.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen (AE F 068300)
   Batch no.: OP2150250
   Purity: 98.6% w/w
   Appearance: Yellow powder
   Date received: 29 April 2003

	Storage:	Room temperature in the dark
	Expiry date:	07 April 2005
2.	**Test Organism:**	*Chironomus riparius*
	Age:	1st instar larvae (2 to 3 days old)
	Source:	[redacted]
	Feeding:	Tetramin® flake food at approximately 250 mg per vessel per day. The Tetramin® flake food was prepared as a suspension in water and an appropriate volume added to the overlying water.
3.	**Test water:**	Reconstituted water (Elendt M4)
4.	**Sediment:**	A defined formulated sediment was used with the following composition: • Industrial quartz sand 76% w/w • Kaolinite clay 20% w/w • Sphagnum moss peat 4% w/w The peat was air dried and homogenised to give a particle size of less than 1 mm. The organic carbon content of the final mixture was 2.1%. Calcium carbonate was added to bring the pH range within 7.0 ± 0.5.

B. STUDY DESIGN AND METHODS

1. In-life phase: 13 May – 31 December 2003

2. Exposure conditions

Test vessels:	600 mL glass beakers approximately 8 cm in diameter
Experimental design:	Control, solvent control and nominal test concentrations of 10, 32, 100, 320 and 1000 mg/kg
Replicates:	Four replicates per control and treatment group each containing 20 test organisms
Temperature:	20.3 – 22.0 °C
pH:	7.5 – 8.7
Aeration:	Gentle aeration provided via narrow bore glass tubes situated approximately 2 - 3 cm above the sediment layer (approximately 1 bubble/sec). When adding the larvae, the aeration of the water was stopped. One day after adding the larvae, the aeration was provided again.
Dissolved oxygen:	≥3.7 mg O_2/L
Photoperiod:	16 hours light: 8 hours darkness with 20 minute dawn and dusk transition periods
Light intensity:	479 - 534 lux

3. Administration of the test item

Approximately 2 days prior to the start of the test, the test item was prepared by a preliminary solution in acetone.

Amounts of test item (1.60 and 5.00 g) were each separately dissolved in acetone with the aid of ultrasonication and the volume adjusted to 50 mL to give solvent stock solutions of 1.60 and 5.00 g/50 mL. Serial dilutions were made from these to give further solvent stock solutions of 0.50, 0.16 and 0.050 g/50 mL. An aliquot (25 mL) of each of the 0.050, 0.16, 0.50, 1.6 and 5.00 g/50 mL solvent stock solutions was separately added to the surface of approximately 100 g of artificial sediment. The acetone was then allowed to evaporate off from each of the preparations prior to being incorporated into a final dry weight of 2.50 kg of artificial sediment to give test concentrations of 10, 32, 100, 320 and 1000 mg/kg (dry weight). Each concentration had 950 mL of deionised reverse osmosis water added to give a nominal moisture content of 40% and the pH of the prepared sediment adjusted to pH 6.97 to 7.09 by the addition of calcium carbonate.

4. Preparation of test vessels

The prepared sediment was dispensed to glass beakers to give a 2 cm layer and was then covered with a 8 cm depth of reconstituted water (sediment:water ratio, 1:4). Four replicates were prepared for each of the control, solvent control, 10, 32, 100, 320 and 1000 mg/kg test concentrations, plus an additional two replicates of each for sacrificing on Day 10 of the exposure period and two for analysis on Days 0 and 28. A plastic disc was placed over the sediment and the reconstituted water poured gently onto the surface of the disc in order to avoid disturbance of the sediment. The disc was removed after addition of the water. The test vessels were then aerated (approximately 1 bubble/second) and the vessels left for 2 days prior to addition of the test organisms in order to allow settlement and equilibration of test concentrations between the sediment and water phases.

5. Test organism assignment and treatment

After the 2-day equilibration period the aeration was stopped and 20 larvae were placed in each test and control vessel and maintained in a temperature controlled room at approximately 21°C with a photoperiod of 16 hours light and 8 hours darkness with 20 minute dawn and dusk transition periods. The aeration was switched back on after approximately 24 hours having allowed the larvae to settle in the sediment.

6. Measurements and observations

The measured end-point for the study was the number of live, emerged adult midges. The number of emerged adult midges was recorded daily until termination of the study after 28 days. The sex of the individual midges was also determined after emergence. Any egg masses produced prior to termination were also recorded and removed from the test vessels to prevent re-introduction of larvae into the sediment. The number of visible pupae that failed to emerge were counted separately. Any abnormal behaviour was also recorded.

On Day 10 of the exposure period, two of the extra replicates prepared for the control and each test concentration were sacrificed for the determination of larval survival and weight. The sediment was sieved and live and dead larvae counted. The dry weight of the surviving larvae per test vessel was determined and the mean individual dry weight per vessel calculated.

Room temperature and light intensity were recorded daily throughout the test. Dissolved oxygen concentrations, water temperature and pH were recorded daily hi each test vessel throughout the test. The water hardness was determined in one vessel from the solvent control and 1000 mg/kg on Days 0 and 28 and the ammonia concentration on Day 28.

The concentration and stability of the test material in the whole sediment, pore water and overlying water were verified by chemical analysis on Days 0 and 28. Analysis for the concentration of the test material in the sediment was also performed on Day -2 (the day of sediment preparation) to confirm correct dosing of the test system.

7. Statistics/Data evaluation

The 28-Day EC_{50} (reduction in emergence) value and associated confidence limits were calculated by the maximum-likelihood probit method ([redacted] 1971) using the ToxCalc computer software package ([redacted] 1999).

Probit analysis is used where two or more partial responses to exposure are shown.

Statistical analysis of the emergence data was performed using a Dunnett's multiple comparison procedure for comparing several treatments with a control ([redacted] 1995). All statistical analyses were performed using the SAS computer software package (SAS 1999 - 2001).

An EC_{50} (development rate) was estimated by inspection of the data.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Analysis of the sediment on Day -2, the day the sediment was prepared showed the measured concentrations to range from 110% to 119% of nominal. Analysis of sediment on Days 0 and 28 showed the measured concentrations to range from 86% to 99% and from 83% to 99% respectively. Given that the measured concentrations were in excess of 80% of nominal throughout the test it was considered justifiable to calculate the results based on nominal test concentrations only.

Analysis of the overlying and interstitial water on Days 0 and 28 indicated that some test item leached from the sediment into the water phase over the duration of the test. Comparison of the Day 0 and Day 28 values showed a general trend for lower measured concentrations after 28 Days which was in line with published data that the test item was unstable in water in non-sterile systems (The Pesticide Manual, ed. CDS [redacted] (1997)).

Table: Measured test concentrations from the exposure of *Chironomus riparius* to Aclonifen in a sediment-water system

Nominal Concentration (mg/kg)	Measured concentration									
	Sediment						Overlying water		Interstitial water	
	Day -2		Day 0		Day 28		Day 0	Day 28	Day 0	Day 28
	mg/kg	%	mg/kg	%	mg/kg	%	mg/L	mg/L	mg/L	mg/L
Solvent control	<LOQ		<LOQ	-	<LOQ	-	<LOQ	<LOQ	<LOQ	<LOQ
10	11.0	110	9.32	93	8.58	86	0.0470	0.00212	0.0731	0.0203
32	35.8	112	31.8	99	26.7	83	0.110	0.0125	0.146	0.0969
100	119	119	89.2	89	97.1	97	0.638	0.0632	1.18	0.588
320	364	114	275	86	298	93	0.779	0.143	3.27	1.177
1000	1180	118	927	93	989	99	1.02	0.333	2.39	2.222

LOQ = Limit of Quantitation
0.238 mg/kg for sediment
0.00076 mg/L for overlying water

0.0951 mg/L for interstitial water

The validated method is summarised in Document M-CA4 (CA 4.1.2/65).

B. BIOLOGICAL DATA

Inspection of the Day 10 larval survival and growth data showed no significant differences in larval survival and growth, in terms of mean larval dry weight, between the solvent control and the 10 and 32 mg/kg test groups. However, significant differences in larval survival but not growth were observed between the solvent control and 100 mg/kg test group. Also, significant differences in larval survival and weight was observed between the solvent control and the 320 and 1000 mg/kg test groups.

The 28-day EC_{50} (reduction in emergence) based on nominal test concentration was 110 mg/kg sediment. The EC_{50} (development rate) based on nominal test concentrations was greater than 100 mg/kg. Although the development rates for the 320 and 1000 mg/kg groups were similar to the solvent control and other test group values, it was considered inappropriate to use the data for comparison due to the low numbers of adult midges that emerged at these concentrations. Statistical analysis of the emergence ratio data showed no significant differences between the 10 and 32 mg/kg test groups compared to solvent control. There were significant differences between solvent control and the 100 mg/kg test group.

Statistical analysis of the numbers of male and female adult midges emerged showed no biological significance between the numbers of males and females.

Table: Emergence and development of *Chironomus riparius* following exposure Aclonifen

Nominal concentration (mg/kg)	Mean emergence (%)	Mean rate of development (standard deviation)
Control	89	-
Solvent control	86	0.0695 (0.00117)
10	86	0.0692 (0.00207)
32	81	0.0711 (0.00176)
100	53	0.0669 (0.00183)
320	5	0.069 (0)
1000	4	0.0679 (0.00156)

The No Observed Effect Concentration was 32 mg/kg on the basis that no biologically significant reduction in emergence was observed after 28 days and additionally no sub-lethal effects were observed at 32 mg/kg.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 218, 204)	Achieved
Emergence in controls*	≥70%	86%
Day of emergence*	12 - 23	13 - 19

Oxygen concentration at end of test	≥60% ASV	≥71% ASV
pH of overlying water at end of test	6 - 9	8.3 – 8.6
Variation in water temperature	±1.0 ºC	±0.85 ºC

*Based on solvent control

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Nominal concentration (mg/kg)		
	EC_{50}	NOEC	L_xOEC
28-day emergence [95% confidence limits]	110 [66 – 190]	32	100
Development rate	>100	100	-

III. CONCLUSION

The toxicity of aclonifen to the sediment-dwelling larvae of *Chironomus riparius* has been investigated and gave a 28-Day EC_{50} (emergence) of 110 mg/kg with 95% confidence limits of 66 -190 mg/kg. The No Observed Effect Concentration was 32 mg/kg. The EC_{50} (development rate) based on nominal test concentrations was greater than 100 mg/kg.

[redacted] *(2004)*

Data Point:	KCA 8.2.5.4/02
Report Author:	[redacted]
Report Year:	2019
Report Title:	Aclonifen (AE F068300): Sediment-water Chironomid toxicity test using spiked sediment - Statistical re-analysis of [redacted] 2004 (M-227300-01-1) study
Report No:	VC/19027/006
Document No:	M-674905-01-1
Guideline(s) followed in study:	Not applicable. Report is a re-evaluation of previously generated study data
Deviations from current test guideline:	Not applicable
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The report for the sediment-water toxicity test using spiked sediment of Aclonifen to *Chironomus riparius* (M-227300-01-1, [redacted] 2004) only provided EC_{50} values for the test item. Data from the study has been re-analysed in order to provide EC_{10}, EC_{20} and EC_{50} values along with the LOEC and NOEC.

Statistical analyses of the available data resulted in the calculation of the following EC_x, LOEC and NOEC values:

Parameter	Cumulative emergence (0 – 28 d)				
	EC_{10}	EC_{20}	EC_{50}	LOEC	NOEC
Value (mg/kg)	35.832	53.859	117.451	100	32
Lower 95%-cl	5.915	14.094	58.150	-	-
Upper 95%-cl	68.851	97.036	238.686	-	-

All computations were carried out in ToxRat Professional version 3.3.0 (ToxRat Solutions GmbH, 2018).

[redacted] *(2019)*

Assessment and conclusion by applicant:
All validity criteria were satisfied and therefore this study can be considered to be valid.

The original study report only provided EC_{50} values for the test item. Data from the study has been re-analysed in order to provide EC_{10}, EC_{20} and EC_{50} values along with the LOEC and NOEC for emergence.

The 28-Day EC_{10}, EC_{20} and EC_{50} (reduction in emergence) based on nominal test concentrations was 36, 54 and 117 mg/kg respectively. The No Observed Effect Concentration was 32 mg/kg.

EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2019)[6] recommends that the lowest of the EC_{10} and NOEC values be used for risk assessment purposes. In this study, as the NOEC was lower than the EC_{10}, the NOEC of 32 mg/kg should be used for risk assessment.

Assessment and conclusion by RMS:

CA 8.2.6 Effects on algal growth

CA 8.2.6.1 Effects on growth of green algae

[6] EFSA (European Food Safety Authority), 2019. Technical report on the outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2019:EN-1673. 117 pp. doi:10.2903/sp.efsa.2019.EN-1673

Data Point:	KCA 8.2.6.1/01
Report Author:	[redacted]
Report Year:	1990
Report Title:	The algistatic activity of Aclonifen CME127
Report No:	R007145
Document No:	M-174303-01-1
Guideline(s) followed in study:	EU (=EEC): Official Journal L133; OECD: 201
Deviations from current test guideline:	Current Guideline: OECD 201, 2011 Initial cell density higher than recommended concentration of 2 - 5 x 10³ cells/mL. Validity criteria not satisfied.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

A study was performed to assess the inhibitory effect of Aclonifen CME 127 on the growth of the unicellular freshwater green alga *Scenedesmus subspicatus*. The test was run with a control, solvent control and nominal test concentrations of 0.00125, 0.0025, 0.0050, 0.010 and 0.020 mg/L, each in triplicate. Observations of cell growth were recorded daily (0, 24, 48, 72 and 96 hours) to determine the potential effect on growth rate and biomass (area under the curve) relative to the control.

Verification of test concentrations showed that the measured test concentrations when viewed as an overall mean (results from 0 and 96 hours) were within the desired limits (ie >80% of nominal values) and therefore, the nominal test concentrations were used for the calculation of EC values.

The 96-Hour E_bC_{50} was calculated to be 0.0067 mg/L and the E_rC_{50} (0 – 24 h) was 0.0069 mg/L. The NOEC was 0.0025 mg/L, based on nominal test concentrations.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen, CME 127
   Batch no.: DA618
   Purity: 99.3 %
   Appearance: Yellow crystalline solid
   Date received: 26 June 1990
   Storage: In dark glass jar at +4° C
   Expiry date: Not provided

2. **Test Organism:** *Scenedesmus subspicatus*
   Strain: CCAP 276/20
   Source: [redacted]

Pre-culture:	Sterile nutrient medium was inoculated from a master culture and incubated under continuous illumination (approximately 7000 lux) and aeration at 24 °C to give an algal suspension in log phase growth characterised by an absorbance of 0.067 (@ 665 nm). The suspension was diluted to an absorbance of 0.021 prior to use. This suspension had a mean cell density of 6.47 x 10^4 cells/mL.
3. Test water:	Nutrient medium as per guideline

B. STUDY DESIGN AND METHODS

1. In-life phase: 16 – 20 July 1990

2. Exposure conditions

Test vessels:	250 mL conical flasks containing 100 mL test solution and loosely stoppered to reduce evaporation.
Experimental design:	5 test concentrations (0.00125, 0.0025, 0.0050, 0.010 and 0.020 mg/L) plus 1 control and 1 solvent control (100 µL acetone/litre)
Replicates:	Three replicate vessels were prepared for each control and treatment group
Initial cell density:	6.47 x 10^4 cells/mL
Temperature:	24 °C
pH:	7.9 – 8.9
Aeration:	None. Gaseous exchange and suspension of algal cells maintained by orbital shaker
Photoperiod:	Continuous
Light intensity:	Approximately 8000 lux

3. Administration of the test item

1.0 g test item was dissolved in auxiliary solvent (acetone) and the volume made up to 10 mL. This stock solution was then further diluted to give a 0.02 g/10 mL stock solution from which serial dilutions were made. 10 µL aliquots of the appropriate concentrations were dispensed to each 100 mL of algal suspension to give the required test series.

4. Measurements and observations

Samples were taken at 0, 24, 48, 72 and 96 hours and the absorbance measured at 665 nm using a Jenway 6100 spectrophotmeter. The cell densities of the control cultures, at initiation and at termination, were determined by direct counting with the aid of a haemocytometer.

5. Statistics/Data evaluation

Percentage inhibition of growth at each test concentration was calculated by comparing the area under the test curve with that under the solvent control curve, and the average maximum growth rate for each test concentration with that for the solvent control. Inhibition values were plotted against test

concentration, a line fitted by eye and the EC_{50} values with respect to the area under the growth curve, E_bC_{50} (96 h), and growth rate, E_rC_{50} (0 – 24 h) read from the graph.

The No Observed Effect Concentration (NOEC) was estimated by visual comparison of the measured and and calculated growth curves of the treated algal suspensions with those of the control.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Verification of test concentrations showed that the measured test concentrations, when viewed as an overall mean (results from 0 and 96 hours) were within the desired limits (i.e. >80% of nominal values) and therefore, the nominal test concentrations were used for the calculation of EC_{50} values.

Table: Measured test concentrations from the exposure of *Scenedesmus subspicatus* to Aclonifen

Nominal concentration (mg/L)	Measured concentration				Mean measured concentration	
	0 Hours		72 Hours			
	mg/L	% nominal	mg/L	% nominal	mg/L	% nominal
Control	<LOQ	-	<LOQ	-		-
0.00125	0.00153	122.4	0.00126	100.8	0.00140	111.6
0.0025	0.00310	124.0	0.00227	90.8	0.00269	107.4
0.0050	0.00521	104.2	0.00499	99.8	0.00510	102.0
0.010	0.00973	97.3	0.0109	109.0	0.0103	103.2
0.020	0.0214	107.0	0.0214	107.0	0.0214	107.0

LOQ = Limit of Quantitation = 0.00001 mg/L

The validated method is summarised in Document M-CA4 (CA 4.1.2/64).

B. BIOLOGICAL DATA

All test and control cultures were inspected microscopically at 96 hours. There were no abnormalities detected in any of the control or test cultures except at the highest test concentration of 0.020 mg/L where the cells were observed to be clumped and colourless.

The measured pH in the test cultures increased slightly over the test period form pH 7.9 at initation to pH 8.0 – 8.9 at termination.

Table: Summary of effects from the exposure of *Scenedesmus subspicatus* to Aclonifen

Nominal concentration (mg/L)	Area under curve (96 h)	Growth inhibition (%)	Growth rate (0 – 24 h)	Reduction in growth rate (%)
Control	15.516	-	0.0451	-
Solvent control	15.648	-	0.0458	-
0.00125	15.556	1	0.0453	1
0.0025	15.084	4	0.0418	9
0.0050	11.152	29	0.0348	24
0.010	4.332	72	0.0149	68
0.020	1.168	93	0.0078	83

C. VALIDITY CRITERIA

Validity criterion	Required[1] (OECD 201, 2011)	Achieved[2]	
		Control	Solvent Control
Increase in control biomass	16	56	59
Mean coefficient of variation for section-by-section specific growth rates in the control cultures	≤ 35%	54%	55%
Coefficient of variation of average specific growth rates in replicate control cultures	≤ 7%	1%	2%

[1]: After 72 hours
[2]: After 96 hours

The study was conducted in accordance with OECD Guideline for Testing of Chemicals No. 201 "Alga, Growth Inhibition Test" (1984) and all relevant validity criteria for the guidelines that were in force at the time of performing the study were satisfied.

In terms of the current version of OECD 201 (2011), the control growth rate and the coefficient of variation of average specific growth rates in control cultures satisfied current validity criteria. However, the coefficient of variation of sectional growth rates in control cultures was greater than 35% and hence did not meet the current validity criterion. Overall, as the test fulfilled only two of three validity criteria; with regards to the OECD Guideline 201 (2011) the study is not valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Response variable	Nominal Concentration (mg/L)	
	EC_{50}	NOEC
Growth Rate (0 – 24 h)	0.0069	0.0025
Area Under Curve (0 – 96 h)	0.0067	0.0025

III. CONCLUSION

Exposure of *Scenedesmus subspicatus* to Aclonifen resulted in an E_rC_{50} (0 – 24 h) value of 0.0069 mg/L and an E_bC_{50} (0 – 96 h) value of 0.0067 mg/L based on nominal test concentrations. The No Observed Effect Concentration (NOEC) after 96 hours was 0.0025 mg/L.

[redacted] *(1990)*

Assessment and conclusion by applicant:

In the previous submission (DAR, 2006), this study was evaluated and accepted as valid for risk assessment purposes. The study was conducted in accordance with OECD Guideline for Testing of Chemicals No. 201 "Alga, Growth Inhibition Test" (1984) and all relevant validity criteria for the guidelines that were in force at the time of performing the study were satisfied.

In terms of the current version of OECD 201 (2011), the control growth rate and the coefficient of variation of average specific growth rates in control cultures satisfied current validity criteria.

However, the coefficient of variation of sectional growth rates in control cultures was greater than 35% and hence did not meet the current validity criterion. Overall, as the test fulfilled only two of three validity criteria; with regards to the OECD Guideline 201 (2011) the study is not valid.

As this study does not meet current OECD guideline validity criteria, it should be considered as supportive only.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.6.1/02
Report Author:	[redacted]
Report Year:	2001
Report Title:	Aclonifen freshwater algal growth inhibition study in a sediment water system (Scenedesmus subspicatus)
Report No:	C015751
Document No:	M-201114-01-1
Guideline(s) followed in study:	EU (=EEC): 92/69, C3; OECD: 201
Deviations from current test guideline:	Current Guideline: OECD 201, 2011 Initial cell density higher than recommended concentration of 2 – 5 x 103 cells/mL. Validity criteria not satisfied.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Offisially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

A study was performed to assess the inhibitory effect of Aclonifen on the growth of the unicellular freshwater green alga *Scenedesmus subspicatus* in a static sediment-water system during an exposure period of 96 hours. The test was run with a control (6 replicates), solvent control (3 replicates) and nominal test concentrations of 5.5, 9.4, 15.9, 27.0 and 46.0 µg/L (3 replicates). Observations of cell growth were recorded daily (24, 48, 72 and 96 hours) to determine the potential effect on growth rate and biomass (area under the curve) relative to the control.

The nominal concentrations of aclonifen in the overlying dilution water of an extra replicate of each test group was verified by chemical analysis shortly after test initiation (T0 + 30 min) and from pooled samples of each test level at the end of the 96-hour exposure period. The analytical recoveries from the test solutions showed the initial measured concentrations of aclonifen were all close to the nominal values (85 -114 % recovery). At termination of the 96-hour exposure period, recoveries from the overlying dilution water in the four highest test concentrations were significantly lower than the initial

measured values (5 - 8 % recovery). The recovery at the lowest nominal concentration of 5.5 µg/L was below the limit of quantification (0.5 µg/L) for this study. The results of this test are presented in terms of the nominal test concentrations.

Following 96 hours exposure to the test substance, the cell culture densities observed were used to calculate the percentage inhibitions, I_A based on the area under the growth curve and I_μ based on growth rate.

The percentage inhibition values I_A were used to calculate the 96-hour E_bC_{50} value at 21.5 µg/L using linear regression. Based on the inhibition of growth rate I_μ the 96-hour E_rC_{50} was empirically estimated to be in excess of the highest nominal tested concentration of 46.0 µg/L.

Based on the cell culture densities observed following 96 hours exposure and on the percentage inhibitions I_A and I_μ, the 96-hour NOEC of the test substance to *Scenedesmus subspicatus* under the conditions of this test was empirically estimated to be 5.5 µg/L of aclonifen.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen
   Batch no.: 97013/03
   Purity: 992 g/kg
   Appearance: Medium yellow fine powder
   Date received: Not provided
   Storage: In the dark in an airtight container at room temperature (approximately 20°C)
   Expiry date: 20 September 2002

2. **Test Organism:** *Scenedesmus subspicatus*
   Strain: 86.81 SAG
   Source: [redacted]
   Pre-culture: The algal cells used for the test cultures were taken from a pre-culture which was initiated 96 hours prior to the test under the same conditions as in the test

3. **Test water:** Nutrient medium as per guideline

4. **Sediment:** An artificial sediment (OECD 207, 1984, adapted) was prepared as follows (on the basis of dry weights):
   - 5% sphagnum peat (as close to pH 5.5 to 6.0 as possible, no visible plant remains, air dried and finely ground)
   - 20% kaolin clay (kaolinite content preferably above 30%)
   - 75% industrial sand (fine sand should predominate with more than 50% of the particles between 50 and 200 microns)

- pH of the final mixture of the sediment is adjusted to 6.0 ± 0.5 by addition of calcium carbonate (chemically pure quality).

The dry constituents were blended in the correct proportions and mixed thoroughly, in a TURBULA mixer (model T50A) for one hour.

B. STUDY DESIGN AND METHODS

1. In-life phase: 21 - 29 June 2001

2. Exposure conditions

Test vessels:	300 mL Erlenmeyer flasks fitted with stainless steel caps which permitted gas exchange. Each test vessel contained 200 mL of test solution
Experimental design:	5 test concentrations (5.5, 9.4, 15.9, 27.0 and 46.0 mg/L) plus 1 control and 1 solvent control (100 µL dimethylformamide/litre)
Replicates:	Three replicate vessels were prepared for the solvent control and each treatment group. Six replicate vessels were prepared for the dilution water control
Initial cell density:	Approximately 2 x 10^4 cells/mL
Temperature:	23 ± 2 °C
pH:	7.28 – 7.63
Aeration:	None. Gaseous exchange and suspension of algal cells maintained by orbital shaker at 85 ± 10 rpm
Photoperiod:	Continuous
Light intensity:	Approximately 8000 lux

3. Administration of the test item

A primary stock solution of 0.46 mg/mL was prepared for the highest test substance concentration by dissolution of 9.2 mg of test substance in 20 mL of the solvent (DMF). Stock solutions for the four lower concentration levels were prepared by serial dilution of the first stock solution in the solvent. The nominal concentrations of these stock solutions were 0.270, 0.158, 0.093 and 0.054 mg/mL.

Each stock solution was manually agitated and submitted to magnetic agitation. Test solutions were prepared by adding known volumes (0.02 ml) of the appropriate stock solution to each test vessel which already contained appropriate quantities/volumes of sediment, dilution water and algal inoculum.

4. Test organism assignment and treatment

Approximately 16 g of dry artificial sediment was filled into each test vessel and humidified with approximately 8 mL of dilution water. A volume of 190 mL of dilution water was added to each test vessel. The preparation was then left to stand at 4°C for a period of a minimum 2 to 3 days.

24 hours prior to test initiation, the test vessels were placed on the agitation table. Agitation was provided at a rate of approximately 85 rpm. This advance was to allow the sediment to settle and therefore minimize turbidity in the water column at test initiation.

Approximately one hour prior to test initiation, the appropriate volume of algal inoculum was added to each test vessel. This brought the total volume of overlying water per test vessel to 200 mL.

The exposure phase was initiated by addition of the test substance to the test vessels. Agitation of the test vessels was not stopped during introduction of the test substance. The duration of exposure phase was 96 hours.

5. Measurements and observations

Measurements of culture density were made after 24, 48, 72 and 96 hours of exposure to the treatment levels.

Cell counts were performed using a Malassez haemocytometer and a microscope. The culture density measurements were used to calculate the percentages of inhibition I_x (based on growth curve area) and I_μ (based on the growth rate).

6. Statistics/Data evaluation

Percentage inhibition of growth at each test concentration was calculated by comparing the area under the test curve with that under the solvent control curve, and the average maximum growth rate for each test concentration with that for the solvent control.

Statistical tests were performed using SAS package (version 6.12).

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The nominal concentrations of aclonifen in the overlying dilution water of an extra replicate of each test group was verified by chemical analysis shortly after test initiation (T0 + 30 min) and from pooled samples of each test level at the end of the 96-hour exposure period. The analytical recoveries from the test solutions showed the initial measured concentrations of aclonifen were all close to the nominal values (85 -114 % recovery). At termination of the 96-hour exposure period, recoveries from the overlying dilution water in the four highest test concentrations were significantly lower than the initial measured values (5 - 8 % recovery). The recovery at the lowest nominal concentration of 5.5 µg/L was below the limit of quantification (0.5 µg/L) for this study. The results of this test are presented in terms of the nominal test concentrations.

Table: Measured test concentrations from the exposure of *Scenedesmus subspicatus* to Aclonifen in a sediment water system

Nominal concentration (µg/L)	Measured concentration			
	0 Hours		96 Hours	
	µg/L	% nominal	µg/L	% nominal
Control	<LOQ	-	<LOQ	-
Solvent Control	<LOQ	-	<LOQ	-
5.5	4.7	85	<LOQ	-
9.4	10.7	114	0.8	9
15.9	15.8	99	1.2	8
27.0	24.3	90	1.3	5

46.0	44.6	97	2.7	6

LOQ = Limit of Quantitation = 0.5 µg/L

The validated method is summarised in Document M-CA4 (CA 4.1.2/84).

B. BIOLOGICAL DATA

The cell culture densities recorded at each observation time during the exposure period were used to calculate the percentage inhibition of growth I_A (based on the area under the growth curve) and I_μ (based on growth rate) after 24, 48, 72 and 96 of exposure compared to the dilution water control group.

Based on percentage inhibition I_A (inhibition of cell culture density) and I_μ (inhibition of growth rate), the majority of the inhibition of algal growth in this study was already observed following 24 hours exposure to the test substance. From T-48h onwards, some recovery of algal growth was observed at all of the nominal concentrations of the test substance. By test termination, no significant inhibition of algal growth rate (reflected by I_μ) was observed up to the nominal concentration of 15.9 µg/L of aclonifen.

Table: Summary of effects from the exposure of *Scenedesmus subspicatus* to Aclonifen after 96 hours in a water sediment system

Nominal concentration (µg/L)	Inhibition (%)	
	Area under curve	Growth rate
5.5	6.6	0
9.4	11.3	0
15.9	39.0	8.7
27.0	68.6	29.6
46.0	71.4	31.0

The percentage inhibition values I_A were used to calculate the 96-hour E_bC_{50} value at 21.5 µg/L using linear regression.

Based on the inhibition of growth rate I_μ, the 96-hour E_rC_{50} was empirically estimated to be in excess of the highest nominal tested concentration of 46.0 µg/L.

Based on the cell culture densities observed following 96 hours exposure and on the percentage inhibitions I_A and I_μ, the NOEC of the test substance to *Scenedesmus subspicatus* under the conditions of this test was empirically estimated to be 5.5 µg/L of aclonifen.

C. VALIDITY CRITERIA

Validity criterion	Required* (OECD 201, 2011)	Achieved	
		Control	Solvent Control
Increase in control biomass	16	18	17
Mean coefficient of variation for section-by-section specific growth rates in the control cultures	≤ 35%	59%	58%
Coefficient of variation of average specific growth rates in replicate control cultures	≤ 7%	1%	1%

* Based on 0 – 72 hours

The study was conducted in accordance with OECD Guideline for Testing of Chemicals No. 201 "Alga, Growth Inhibition Test" (1984) and all relevant validity criteria for the guidelines that were in force at the time of performing the study were satisfied.

In terms of the current version of OECD 201 (2011), the control growth rate and the coefficient of variation of average specific growth rates in control cultures satisfied current validity criteria. However, the coefficient of variation of sectional growth rates in control cultures was greater than 35% and hence did not meet the current validity criterion. Overall, as the test fulfilled only two of three validity criteria with regards to the OECD Guideline 201 (2011) the study is not valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Response variable	Nominal Concentration (µg/L)	
	EC_{50}	NOEC
Growth Rate (0 – 96 h)	> 46.0	
Area Under Curve (0 – 96 h)	21.5	5.5

III. CONCLUSION

Exposure of *Scenedesmus subspicatus* to Aclonifen in a water sediment system resulted in an E_rC_{50} (0 – 96 h) value of greater than 46.0 µg/L and an E_bC_{50} (0 – 96 h) value of 21.5 µg/L based on nominal test concentrations. The No Observed Effect Concentration (NOEC) after 96 hours was 5.5 µg/L.

[redacted] *(2001)*

Assessment and conclusion by applicant:
In the previous submission (DAR, 2006), this study was evaluated and accepted as valid for risk assessment purposes. The study was conducted in accordance with OECD Guideline for Testing of Chemicals No. 201 "Alga, Growth Inhibition Test" (1984) and all relevant validity criteria for the guidelines that were in force at the time of performing the study were satisfied.

In terms of the current version of OECD 201 (2011), the control growth rate and the coefficient of variation of average specific growth rates in control cultures satisfied current validity criteria. However, the coefficient of variation of sectional growth rates in control cultures was greater than 35% and hence did not meet the current validity criterion.

Therefore, as this study does not meet current OECD guideline validity criteria, it should be considered as supportive only.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.6.1/03
Report Author:	[redacted]
Report Year:	2016
Report Title:	Amendment no. 1 - Desmodesmus subspicatus growth inhibition test with aclonifen tech. (BCS-AG74518)
Report No:	EBCL0001
Document No:	M-574872-02-1
Guideline(s) followed in study:	EU Directive 91/414/EEC Regulation (EC) No. 1107/2009 U.S. EPA Pesticide Assessment Guidelines, Subdivision J, §122-2, 123-2 OCSPP Guideline 850.4500 (January 2012)
Deviations from current test guideline:	Current Guideline: OECD 201, 2011 None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to assess the influence of the test item on exponentially growing populations of Desmodesmus subspicatus expressed as NOEC, LOEC and EC_x for growth rate and further endpoints of algal biomass (cells per volume). The test was run with a control, solvent control and nominal test concentrations of 0.0894, 0.286, 0.916, 2.93, 9.38 and 30.0 µg a.s./L over a test period of 96 hours. Observations of cell growth were recorded daily (0, 24, 48, 72 and 96 hours) to determine the potential effect on growth rate and biomass relative to the control.

The analytical findings of aclonifen tech. (BCS-AG74518) in the treatment levels found on Day 0 were 90.0% to 104% of nominal (average 98.1%). After 72 hours analytical findings of 84.8% to 93.7% of nominal (average 90.2%) were found and after 96 hours analytical findings of 67.9% to 87.3% of nominal (average 74.9%) were found. Results after 72 hours are based on nominal test concentrations and after 96 hours on mean measured test concentration.

The E_rC_{50} (0 – 72h) was calculated to be 24.6 µg a.s./L based on nominal test concentrations, the NOEC after 72 hours was 9.38 µg a.s./L. After 96 hours exposure, the E_rC_{50} (0 – 96 h) was 20.3 µg a.s./L with a NOEC of 0.0811 µg a.s./L based on mean measured test concentrations.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen tech. (BCS-AG74518)
   Batch no.: AE F068300-01-14
   Purity: 99.5% w/w
   Appearance: Yellow powder
   Date received: Not provided
   Storage: +10 to +30° C

Expiry date:	29 November 2016
2. Test Organism:	*Desmodesmus subspicatus* formerly named *Scenedesmus subspicatus,*
Strain:	SAG 86.81 ESP
Source:	[redacted]
Pre-culture:	Pre cultures were prepared from stock cultures 3 days before the start of the test using OECD medium
3. Test water:	OECD medium as per guideline

B. STUDY DESIGN AND METHODS

1. In-life phase: 06 June – 12 July 2016

2. Exposure conditions

Test vessels:	300 mL conical flasks containing 150 mL test solution sealed with cellulose plugs
Experimental design:	6 test concentrations (0.0894, 0.286, 0.916, 2.93, 9.38 and 30.0 µg a.s./L) plus 1 control and 1 solvent control (100 µL DMF/litre)
Replicates:	Four replicate vessels were prepared for each control and treatment group
Initial cell density:	1 x 10^4 cells/mL
Temperature:	22.4 – 23.3 °C
pH:	7.8 – 8.1
Aeration:	None. Gaseous exchange and suspension of algal cells maintained by orbital shaker at 100 rpm
Photoperiod:	Continuous
Light intensity:	4.62 – 4.89 klux

3. Administration of the test item

Prior to the test the stock solution was prepared by dissolving 30.6 mg of the test item in 100 mL dimethylformamide (DMF) by intense stirring for 5 minutes. An adequate amount of the stock solution was transferred to a dilution series to obtain the concentration levels used in the study.

4. Test organism assignment and treatment

The test item was applied into the test medium on Day 0.

5. Measurements and observations

Morphological examination of cells using a microscope were made after 0, 24, 48 72 and 96 hours.

Cell numbers per volume (as a surrogate for biomass per volume) were estimated photometrically. For this purpose, small samples of treated, inoculated test medium were placed in 5 cm cuvettes after 24,

48,72 and 96 hours of the exposure period (without replacing after measurement). The extinctions were determined at a wave length of 578 nm using a single-beamphotometer. The photometer was calibrated using untreated and treated culture medium of each concentration level without algae. Cell numbers were computed from extinction values using the conversion formula (Statistical Software „ToxRat Professional", version 3.2.1):

log10 (cell no.) = 6.433 + 1.089 x log10 (extinction)

To detect possible alterations in algae cells that might influence extinction measurements, such as unusual cell size, pooled samples of all test concentrations and control were examined under a microscope at a magnification of 400 times. Cell numbers were estimated photometrically only if alterations that might influence extinction were not detected.

The pH was measured at the start of the study and additionally after 72 and after 96 hours in all test levels and the controls by an electronic pH meter.

Samples were analysed for the actual concentration of aclonifen tech. (BCS-AG74518) present in the test medium of all treatment levels and the control after 0, 72 and 96 hours.

6. Statistics/Data evaluation

EC_x values (e.g. x = 50) and confidence intervals were calculated for the stated exposure period.

The LOEC and NOEC determinations from the appropriate parameter (inhibition) were done, using the ANOVA procedure (p = 0.05, one sided) and properly selected multiple t-tests.

All calculations were carried out using Microsoft Excel® spreadsheets and shown are rounded values. All further statistical evaluations were done using the commercial program ToxRat Professional.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The test concentrations measured after 0 and 72 h ranged between 80 and 120% of nominal therefore all statistical evaluations for the time period 0 - 72 h were based on nominal test concentrations.

The test concentrations measured after 96 h were found to be below 80% of nominal therefore all statistical evaluations for the time period 0 – 96 h were based on mean measured test concentrations.

Table: Measured test concentrations from the exposure of *Desmodesmus subspicatus* to Aclonifen

Nominal concentration (µg a.s./L)	Measured Concentration							
	0 Hours		72 Hours		96 Hours		0 - 96 Hours[1]	
	µg a.s./L	% nom	µg a.s./L	% nom	µg a.s./L	% nom	µg a.s./L	% nom
Control	<LOQ		<LOQ	-	<LOQ	<LOQ	<LOQ	-
Solvent Control	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
0.0894	0.0929	104	0.0838	93.7	0.0667	74.6	0.0811	90.7
0.286	0.289	101	0.263	92.0	0.207	72.4	0.253	88.5
0.916	0.914	99.8	0.828	90.4	0.655	71.5	0.800	87.3
2.93	2.96	101	2.62	89.4	1.99	67.9	2.52	86.0
9.38	8.44	90.0	7.95	84.8	7.07	75.4	7.82	83.4

30.0	27.8	92.7	27.2	90.7	26.2	87.3	27.1	90.3

[1]: Mean measured concentration of Day 0, Day 3 and Day 4
Nom: Nominal concentration
LOQ: Limit of Quantitation = 0.0150 µg a.s./L

The validated method is summarised in Document M-CA4 (CA 4.1.2/90).

B. BIOLOGICAL DATA

No morphological change in algae was observed in any test concentration.

There was no significant change in measured pH values in the control and test cultures over the test period.

Table: Summary of effects from the exposure of *Scenedesmus subspicatus* to Aclonifen

Nominal concentration (µg a.s./L)	Growth rate (0 – 72 h)	Inhibition (%)	Growth rate (0 – 96 h)	Inhibition (%)
Pooled control	1.137		1.166	
0.0894	1.126	0.9	1.159	0.6
0.286	1.120	1.5	1.121	3.8[1]
0.916	1.122	1.3	1.128	3.3[1]
2.93	1.141	-0.4	1.127	3.3[1]
9.38	1.065	6.3[1]	1.127	3.3[1]
30.0	0.428	62.3[1]	0.339	70.9[1]

[1]: significantly reduced based on Williams' multiple sequential t-test procedure (α=0.05, one-sided smaller)

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 201, 2011)	Achieved*
Increase in control biomass	16	30.3
Mean coefficient of variation for section-by-section specific growth rates in the control cultures	≤ 35%	27.1%
Coefficient of variation of average specific growth rates in replicate control cultures	≤ 7%	1.8%

*Based on pooled controls

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Parameter	µg a.s./L				
	EC_{50}	EC_{20}	EC_{10}	LOEC	NOEC
72-hour test duration[1]					
Growth rate (r)	24.6 [23.5 – 25..7]	14.5 [13.1 – 15.7]	11.0 [9.59 – 12.2]	9.38	2.93
Yield (y)	14.7 [12.8 – 17.3]	9.37 [7.74 – 10.8]	7.41 [5.75 – 8.78]	9.38	2.93
96-hour test duration[2]					

Growth rate (r)	20.3 [19.9 – 20.8]	13.1 [12.5 – 13.7]	10.4 [9.77 – 11.0]	0.253	0.0811
Yield (y)	10.7 [9.93 – 11.7]	4.06 [3.67 – 4.45]	2.44 [2.14 – 2.76]	0.253	0.0811

[1]: Results based on nominal test concentrations
[2]: Results based on mean measured test concentrations
[95% confidence limits]

III. CONCLUSION

Exposure of *Desmodesmus subspicatus* to Aclonifen resulted in an E_rC_{50} (0 – 72h) value of 24.6 µg a.s./L based on nominal test concentrations, the NOEC after 72 hours was 9.38 µg a.s./L. After 96 hours exposure, the E_rC_{50} (0 – 96 h) was 20.3 µg a.s./L with a NOEC of 0.0811 µg a.s./L, based on mean measured test concentrations.

[redacted] *(2016)*

Assessment and conclusion by applicant:
All validity criteria were satisfied and therefore this study can be considered to be valid.

Exposure of *Desmodesmus subspicatus* to Aclonifen resulted in an E_rC_{50} (0 – 72h) value of 24.6 µg a.s./L based on nominal test concentrations, the NOEC after 72 hours was 9.38 µg a.s./L. After 96 hours exposure, the E_rC_{50} (0 – 96 h) was 20.3 µg a.s./L with a NOEC of 0.0811 µg a.s./L, based on mean measured test concentrations.

The 96-hour results were based on the arithmetic mean measured test concentrations. EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2015)[7] recommends that mean measured concentrations are calculated using the geometric mean rather than the arithmetic mean. The geometric mean measured concentrations calculated from the 0, 72 and 96-Hour analysis results were 0.0804, 0.251, 0.791, 2.49, 7.80 and 27.1 µg a.s./L. Given that the geometric mean measured concentrations differed from the arithmetic mean measured concentrations only at the second decimal place, it was considered that recalculation of the study endpoints based on the geometric mean measured concentrations was not necessary.

Assessment and conclusion by RMS:

CA 8.2.6.2 Effects on growth of an additional algal species

[7] EFSA (European Food Safety Authority), 2015. Technical report on the outcome of the pesticides peer review meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2015:EN-924. 62 pp.

Data Point:	KCA 8.2.6.2/01
Report Author:	[redacted]
Report Year:	1998
Report Title:	ACLONIFEN - Toxicity to the freshwater diatom, Navicula pelliculosa
Report No:	R005692
Document No:	M-171422-01-1
Guideline(s) followed in study:	EU (=EEC): L383A-C.3.; OECD: 201
Deviations from current test guideline:	Current Guideline: OECD 201, 2011 Validity criteria not satisfied
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

A study was performed to assess the inhibitory effect of Aclonifen on the growth of the freshwater diatom, *Navicula pelliculosa* during an exposure period of 72 hours. The test was run with a control, solvent control and nominal test concentrations of 0.063, 0.13, 0.25, 0.50, 1.0 and 2.0 mg a.s./L. Observations of cell growth were recorded daily (24, 48 and 72 hours) to determine the potential effect on growth rate and biomass (area under the curve) relative to the control.

Measured concentrations for the test solutions at test initiation and termination were generally similar and established the desired concentration gradient. Mean measured test concentrations ranged from 92 to 110% of the nominal concentrations and defined the treatment levels tested as 0.068, 0.12, 0.23, 0.47, 1.0 and 1.9 mg a.i./L. All results were based on the mean measured test concentrations.

Exposure of *Navicula pelliculosa* to Aclonifen resulted in an E_rC_{50} (0 – 72 h) value of 1.2 mg a.s./L (95% confidence limits: 0.72 to 2.1 mg a.s./L) and an E_bC_{50} (0 – 72 h) value of 0.47 mg a.s./L (95% confidence limits: 0.31 to 0.72 mg a.s./L) based on the mean measured test concentrations. The No Observed Effect Concentration (NOEC) after 72 hours based on growth rate was 0.23 mg a.s./L, and 0.068 mg a.s./L based on cell biomass.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen
   Batch no.: 97013/03
   Purity: 994 g/kg
   Appearance: Yellow powder
   Date received: 29 May 1998
   Storage: Room temperature in the dark
   Expiry date: 18 April 1999

2. **Test Organism:** *Navicula pelliculosa*

Strain:	15 3045, Class Bacillariophyceae
Source:	[redacted]
Pre-culture:	The inoculum used to initiate the toxicity test was taken from a stock culture that had been transferred to fresh medium four days before testing
3. Test water:	AAP medium

B. STUDY DESIGN AND METHODS

1. In-life phase:	23 - 26 June 1998
2. Exposure conditions	
Test vessels:	250 mL Erlenmeyer flasks fitted with stainless steel caps which permitted gas exchange. Each test vessel contained 100 mL of test solution
Experimental design:	6 test concentrations (0.063, 0.13, 0.25, 0.50, 1.0 and 2.0 mg a.s./L) plus 1 control and 1 solvent control (100 µL dimethylformamide/litre)
Replicates:	Three replicate vessels were prepared for the control, solvent control and each treatment group
Initial cell density:	Approximately 1.0 x 10^4 cells/mL
Temperature:	24 ± 1 °C
pH:	7.36 - 8.9
Aeration:	None. Gaseous exchange and suspension of algal cells maintained by orbital shaker at 100 ± 10 rpm
Photoperiod:	Continuous
Light intensity:	3200 - 5400 lux

3. Administration of the test item

A 20 mg a.s./mL stock solution was prepared by dissolving 0.5031 g (0.5001 g as active substance) of Aclonifen in 25 mL of dimethylformamide. Test solutions were prepared from dilutions of this primary stock solution.

Replicate flasks per treatment level and the controls were conditioned prior to use by rinsing with the appropriate exposure solution. One hundred milliliters of the appropriate exposure solution were then placed in each replicate flask. The control and solvent control flasks were prepared and maintained under the same conditions as the treatment vessels but contained no Aclonifen.

4. Test organism assignment and treatment

Approximately 30 minutes after the test solutions were prepared and added to the test flasks, 1.46 mL of an inoculum of *Navicula pelliculosa* cells, at a density of 68 x 10^4 cells/mL, was aseptically introduced into each flask containing 100 mL of test solution. This inoculum provided the required cell density of approximately 1.0 x 10^4 cells/mL.

5. Measurements and observations

At each daily interval, cell counts were conducted on each replicate vessel using a hemacytometer (Neubauer Improved) and a compound microscope. One sample was taken from each flask for counting. Observations of the health of the cells were made and recorded at each daily interval.

Temperature was measured continuously with a minimum/maximum thermometer located in a flask of water adjacent to the test flasks in the environmental chamber. Minimum and maximum temperatures and the shaking rate of the orbital shakers were recorded daily. The light intensity of the test area was measured at 0 hour and at each daily interval of the exposure period. Water quality parameters (pH and conductivity) were measured at test initiation and termination of the 72-hour exposure period.

At test initiation (0 hour) and test termination (72 hours), one sample from each exposure solution and the controls was analyzed for Aclonifen concentration.

6. Statistics/Data evaluation

A t-Test ([redacted] 1981) was used to compare the 72-hour control and solvent control growth rate and biomass data. If control and solvent control data were not significantly different ($p \leq 0.05$), these data were pooled for use in statistical evaluation of the data for treatment effects. If the data were found to be significantly different, the solvent control data was used to determine treatment effects.

Based on the results of statistical analysis, the No-Observed-Effect Concentration (NOEC), the highest test concentration which demonstrates no statistically adverse effect ($p \leq 0.05$) when compared to the pooled control data, was determined. The data were first checked for normality using Shapiro-Wilks' Test ([redacted] 1989) and for homogeneity of variance using Bartlett's Test ([redacted], 1985). If the data sets passed the test for homogeneity and normality, the Williams' Test ([redacted] 1971, 1972) was used to determine the NOEC. If the data did not pass the tests for homogeneity and normality, then Kruskal-Wallis' Test was used to determine the NOEC. All statistical determinations were made at the 95% level of certainty, except in the case of Shapiro-Wilks' and Bartlett's Tests, where the 99% level of certainty was applied.

The E_bC_{50} and E_rC_{50} values, the theoretical concentration of test substance which would cause a 50% reduction in biomass and growth rate, respectively, and the 95% confidence limits, were determined by linear regression of response (percent reduction of biomass or growth rate as compared with the pooled control) versus mean measured test concentration. The EC values were calculated using four linear regression curves based on (a) untransformed data, (b) untransformed response versus logarithm-transformed concentration, (c) probit-transformed response versus untransformed concentration, and (d) probit-transformed response versus logarithm-transformed concentration. The regression line which provided the best fit of the untransformed or transformed data was selected based on the highest coefficient of determination, r^2. This regression equation was then applied to calculate each EC value and its 95% confidence limits, using the method of inverse prediction ([redacted] 1981). A computer program was used to assist in these computations.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Measured concentrations for the test solutions at test initiation and termination were generally similar and established the desired concentration gradient. Mean measured test concentrations ranged from 92

to 110% of the nominal concentrations and defined the treatment levels tested as 0.068, 0.12, 0.23, 0.47, 1.0 and 1.9 mg a.s./L. All results were based on the mean measured test concentrations.

Table: Measured test concentrations from the exposure of *Navicula pelliculosa* to Aclonifen

Nominal concentration (mg a.s./L)	Measured concentration				Mean measured concentration	
	0 Hours		72 Hours			
	mg a.s./L	% nominal	mg a.s./L	% nominal	mg a.s./L	% nominal
Control	<LOQ	-	<LOQ	-		
Solvent Control	<LOQ	-	<LOQ	-	-	-
0.063	0.075	119	0.061	97	0.068	108
0.13	0.13	100	0.11	85	0.12	92
0.25	0.24	96	0.22	88	0.23	92
0.50	0.49	98	0.44	88	0.47	93
1.0	1.1	110	0.9	91	1.0	101
2.0	2.0	100	1.8	90	1.9	95

LOQ = Limit of Quantitation = 0.026 mg a.s./L

The validated method is summarised in Document M-CA4 (CA 4.1.2/66).

B. BIOLOGICAL DATA

The pH of the test and control solutions ranged from 7.3 to 7.5 at test initiation and from 7.8 to 8.9 at test termination. The increase in pH observed between test initiation and test termination is common in static algal cultures and is due to photosynthesis by the algae.

At test termination, the 1.9 mg a.s./L treatment level was observed to contain cell fragments. Cells exposed to the remaining treatment levels (0.068 to 1.0 mg a.s./L) and the controls were observed to be normal.

Statistical analysis demonstrated no significant difference in total biomass or 0-72 h average growth rate at test termination (72 hours) between the control and solvent control, therefore, pooled control data was used for further statistical analysis to determine treatment level effects.

Table: Summary of effects from the exposure of *Navicula pelliculosa* to Aclonifen

Nominal concentration (mg a.s./L)	Area under curve (72 h)	Growth inhibition (%)	Growth rate (0 – 72 h)	Reduction in growth rate (%)
Control	37×10^4	-	1.28	-
Solvent control	35×10^4	-	1.29	-
Mean control	36×10^4	-	1.28	-
0.068	35×10^4	1.9	1.29	-0.23
0.12	33×10^4 [1]	7.2	1.26	1.7
0.23	27×10^4 [1]	24	1.25	2.8
0.47	18×10^4 [1]	50	1.03[1]	20
1.0	9.0×10^4 [1]	75	0.700[1]	45
1.9	4.0×10^4 [1]	89	0.462[1]	64

[1]: Significantly reduced as compared to the pooled control based on Williams' Test

Based on Williams' Test, the 0-72 hour No-Observed-Effect Concentration (NOEC) for biomass was determined to be 0.068 mg a.s./L. The 72-hour E_bC_{50} value (95% confidence limits) for cell biomass was calculated to be 0.47 mg a.s./L (0.31 to 0.72 mg a.s./L).

The 0-72-hour No-Observed-Effect Concentration (NOEC) for growth rate was determined to be 0.23 mg a.s./L. The 72-hour E_rC_{50} value (95% confidence limits) was calculated to be 1.2 mg a.s./L (0.72 to 2.1 mg a.s./L).

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 201, 2011)	Achieved	
		Control	Solvent Control
Increase in control biomass	16	46	46
Mean coefficient of variation for section-by-section specific growth rates in the control cultures	≤ 35%	41%	47%
Coefficient of variation of average specific growth rates in replicate control cultures	≤ 10%	2%	1%

The study was conducted in accordance with OECD Guideline for Testing of Chemicals No. 201 "Alga, Growth Inhibition Test" (1984) and all relevant validity criteria for the guidelines that were in force at the time of performing the study were satisfied.

In terms of the current version of OECD 201 (2011), the control growth rate and the coefficient of variation of average specific growth rates in control cultures satisfied current validity criteria. However, the coefficient of variation of sectional growth rates in control cultures was greater than 35% and hence did not meet the current validity criterion. Overall, as the test fulfilled only two of three validity criteria; with regards to the OECD Guideline 201 (2011) the study is not valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Response variable	Mean Measured Concentration (mg a.s./L)	
	EC_{50} [95% confidence limits]	NOEC
Growth Rate (0 – 72 h)	1.2 [0.72 – 2.1]	0.23
Area Under Curve (0 – 72 h)	0.47 [0.31 – 0.72]	0.068

III. CONCLUSION

Exposure of *Navicula pelliculosa* to Aclonifen resulted in an E_rC_{50} (0 – 72 h) value of 1.2 mg a.s./L (95% confidence limits: 0.72 to 2.1 mg a.s./L), and an E_bC_{50} (0 – 72 h) value of 0.47 mg a.s./L (95% confidence limits: 0.31 to 0.72 mg a.s./L) based on the mean measured test concentrations. The No Observed Effect Concentration (NOEC) after 72 hours based on growth rate was 0.23 mg a.s./L, and 0.068 mg a.s./L based on cell biomass.

[redacted] *(1998)*

Assessment and conclusion by applicant:

In the previous submission (DAR, 2006), this study was evaluated and accepted as valid for risk assessment purposes. The study was conducted in accordance with OECD Guideline for Testing of Chemicals No. 201 "Alga, Growth Inhibition Test" (1984) and all relevant validity criteria for the guidelines that were in force at the time of performing the study were satisfied.

In terms of the current version of OECD 201 (2011), the control growth rate and the coefficient of variation of average specific growth rates in control cultures satisfied current validity criteria. However, the coefficient of variation of sectional growth rates in control cultures was greater than 35% and hence did not meet the current validity criterion. Overall, as the test fulfilled only two of three validity criteria; with regards to the OECD Guideline 201 (2011) the study is not valid.

Therefore, as this study does not meet current OECD guideline validity criteria, it should be considered as supportive only.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.6.2/02
Report Author:	[redacted]; [redacted]
Report Year:	2019
Report Title:	Amendment no. 1: Alga, growth inhibition test (OECD 201), static exposure - Effect of aclonifen (AE F068300) on the growth of 6 algal species
Report No:	BAY-025/4-30
Document No:	M-278578-02-1
Guideline(s) followed in study:	OECD Guideline for Testing of Chemicals, Sect. 2: Effects on Biotic Systems, No. 201 "Alga, Growth Inhibition Test", Adopted June 1984. Paris: OECD (1981), under consideration of Draft revised guideline 201 "Freshwater alga and cyanobacteria, Growth Inhibition Test", October 2004.
Deviations from current test guideline:	Current Guideline: OECD 201, 2011 Validity criteria for some species not satisfied
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to determine the toxicity of the test item aclonifen (AE F068300) on six algal species of several taxonomic groups. The algae were exposed to various concentrations of the test item

over several generations under static conditions over a period of 72 hours or over a period to obtain at least a 16-fold growth in the control cultures.

Recovery of algal growth was investigated for a sensitive algal species to determine if the algistatic effect noted in the definite growth inhibition test is reversible.

Stock solutions of the test item were prepared in acetone. The exposure concentrations were assessed by chemical analysis. The nominal test concentrations were as follows:

Chlorella vulgaris	0, 62.5, 125, 250, 500, 1000 µg a.s./L
Chlamydomonas reinhardtii	0, 3.0, 10.0, 30.0, 100, 300 µg a.s./L
Xanthonema debile	0, 5.0, 15.8, 50.0, 158, 500 µg a.s./L
Closterium cornu	0, 25.6, 64.0, 160, 400, 1000 µg a.s./L
Synechococcus leopoliensis	0, 25.6, 64.0, 160, 400, 1000 µg a.s./L
Nannochloropsis limnetica	0, 125, 250, 500, 1000, 2000 µg a.s./L

For each concentration plot three replicates and for controls (test medium only) and acetone control six replicates each were exposed.

The test item was generally stable over the test periods (measured concentration at test end maximum ± 20% of initial concentration). The measured test concentrations deviated more than 20% from the nominal concentrations in several test concentrations. Therefore, the effect values were calculated based on the mean measured concentrations (arithmetic mean).

Concentration-effect relationships were observed for all algal species and were statistically analyzed to obtain effect concentrations. The effect concentrations regarding inhibition of growth rate are summarized as follows:

Growth Rate	E_rC_{50} (µg a.s./L)	E_rC_{10} (µg a.s./L)	NOEC (µg a.s./L)
Synechococcus leopoliensis	74.9	34.4	19.3
Chlamydomonas reinhardtii	75.3	5.1	3.42
Closterium cornu	112	47.8	111
Xanthonema debile	319	108	45.6
Chlorella vulgaris	450	129	85.0
Nannochloropsis limnetica	513	389	263

It could be shown that the algicidal properties of aclonifen were reversible. There were no statistically significant differences in the growth rates of *Chlamydomonas reinhardtii* pre-exposed to 99.4 and 260 µg a.s./L (inhibition of growth rate > 60%) or control medium after 48 h and 96 h after transfer to untreated growth medium.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen, technical
   Batch no.: OP2150250
   Purity: 98.6%
   Appearance: Yellow powder

	Date received:	Not provided
	Storage:	25 ± 5 °C
	Expiry date:	30 March 2007

2. Test Organisms:

a)	**Specification:**	*Chlorella vulgaris*, Chlorophyceae, Chlorophyta
	Strain:	CCAP No 211/11B
	Source:	[redacted]
b)	**Specification:**	*Chlamydomonas reinhardtii*, Chlorophyceae, Chlorophyta
	Strain:	SAG-No 11.32a
	Source:	[redacted]
c)	**Specification:**	*Xanthonema debile*, Xanthophyceae, Heterokontophyta
	Strain:	SAG-No 836-1
	Source:	[redacted]
d)	**Specification:**	*Closterium cornu*, Zygnematophycea, Streptophyta (Charophyta)
	Strain:	SAG-No 132.80
	Source:	[redacted]
e)	**Specification:**	*Synechococcus leopoliensis*, Chroococcales, Cyanobacteria
	Strain:	SAG-No 1402-1
	Source:	[redacted]
f)	**Specification:**	*Nannochloropsis limnetica*, Eustigmatophyceae, Heterokontophyta
	Strain:	SAG-No 18.99
	Source:	[redacted]
	Pre-culture:	Before onset of a test a pre-culture was established in growth medium to obtain exponentially growing algae for the test. The culture duration of the pre-cultures was dependent on the proliferation behaviour of the respective alga culture and was between 3-4 days

3. Test water: Culture media recommended by OECD 201 or the culture collections SAG and CCAP were used

B. STUDY DESIGN AND METHODS

1. In-life phase: 29 November 2005 – 24 May 2006

2. Exposure conditions

Test vessels:	250 mL conical glass flasks covered with silicone-sponge caps containing 100 mL test solution

Experimental design:	5 test concentrations plus 1 control and 1 solvent control (100 µL dimethylformamide/litre) per test species
Replicates:	Six replicate vessels were prepared for the control and solvent control and three replicate vessels for each treatment group.
Initial cell density:	10,000 cells/mL for *Chlorella vulgaris*, *Chlamydomonas reinhardtii* and *Xanthonema debile*. The cell sizes of these species are in the same range as the sizes of the standard species *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus* and the initial cell density recommended in the OECD guideline was used. For *Closterium cornu* 2000 cells/mL were used, due to the large size of the algae. For the picoplankton species *Synechococcus leopoliensis* and *Nannochloropsis limnetica* 1 × 10^5 cell/mL were used, due to the small size of the algae as recommended in the proposal for updating OECD guideline.
Temperature:	21.5 – 22.1 °C
pH:	7.80 – 9.67
Aeration:	None. Gaseous exchange and suspension of algal cells maintained by orbital shaker at 100 rpm
Photoperiod:	Continuous
Light intensity:	7365 - 8462 lux

3. Administration of the test item

Concentrated stock solutions were prepared by diluting the test item in acetone and diluted with acetone to application solutions. Acetone concentrations in the test cultures was 100 µL/L. The acetonic application solutions were added to the growth media to obtain the required test concentrations and the test media were treated with ultra sound (ultrasonic water bath) for 5 minutes. The test item concentrations in the water phase were chemically analyzed after separation of the algae by centrifugation at low g-force.

4. Test organism assignment and treatment

All work for the test preparation was performed under sterile conditions. The test vessels were filled with 100 mL test solution.

The cell density of the inoculum culture was determined and an adequate aliquot of the inoculum culture was added into the test culture to obtain the required cell density. Prior to the addition of the inoculum culture, the respective volume was removed from the 100 mL test solution. The initial cell density was exemplary checked by microscopical counting in one vessel at test start.

For the filamentous *Xanthonema debile* individual test vessels were prepared for each sampling point (9 replicates per test concentration, 18 replicates for controls). To break the filaments into shorter chains or single cells as far as possible, the whole test vessel was sonicated in an ultrasonic water bath for 8 minutes.

5. Measurements and observations

Cell density was determined in aliquots of every test vessel after 24, 48 and 72 or 96 hours.

The pH values were measured in an additionally prepared replicate at test start and directly in the test vessels at the end of the test. During the exposure the incubation temperature was measured once a day in an additionally prepared control vessel, which was continuously incubated.

The freshly prepared solutions at test start and the aged solutions after 72 h were analysed for the test item concentrations by HPLC measurement with an UV-detection of the analyte at 391 nm.

The algistatic property of the test item was determined using the algal species showing the highest sensitivity to aclonifen regarding biomass integral, *Chlamydomonas reinhardtii*.

6. Statistics/Data evaluation

- The evaluation of the concentration-effect-relationships and the calculations of effect concentrations were based on the arithmetic mean of the measured concentrations. The measured concentrations varies by < 20% from the initial measured.
- The mean value of the cell counts for each concentration plot was used for plotting growth curves.
- Calculation of the percent inhibition of growth rate [r] and biomass (B) was performed according to the guideline.
- The percent inhibition values were plotted as a function of the test item concentration.
- Where the test results showed an inhibition with levels around 50% they were statistically analyzed to determine EC_{10} and EC_{50} values together with 95% confidence intervals, if possible, using Probit-analysis assuming log-normal distribution of the values by using the computer programme ToxRat.
- The NOEC and LOEC were determined using the Williams t-test or the Welch t-test.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The concentrations of aclonifen were generally stable during the test (deviations of concentrations at test end from concentrations at test start < 20%). The measured test concentrations of aclonifen were between 47 and 140% of the nominal levels variable in the different tests:

Since the deviations of the measured concentrations from the nominal concentrations of aclonifen were mostly higher than 20% and the test item was stable over the test period, the effect values were calculated based on mean measured concentrations (arithmetic mean).

Table: Measured test concentrations from the exposure of six algal species to Aclonifen

Nominal concentration (µg a.s./L)	Measured Concentration					
	0 Hours		72 Hours		Mean	
	µg a.s./L	% nominal	µg a.s./L	% initial	µg a.s./L	% initial
Chlorella vulgaris						
Control	0	-	0	-	0	-
62.5	45.0	72.0	30.0	66.7	37.5	83.3
125	96.0	76.8	74.0	77.1	85.0	88.5
250	215	86.0	173	80.6	194	90.3
500	296	59.2	310	105	303	102

1000	611	61.1	608	99.5	610	99.8
Chlamydomonas reinhardtii						
Control	0	-	0	-	0	-
3	3.14	105	3.70	118	3.42	109
10	13.1	131	12.6	95.8	12.8	97.9
30	34.2	114	28.0	81.9	31.1	91.0
100	103	103	95.4	92.3	99.4	96.2
300	274	91.3	246	89.8	260	94.9
Xanthonema debile						
Control	0	-	0	-	0	-
5.0	6.99	140	6.20	88.7	6.6	94.1
15.8	16.0	102	15.4	96.3	15.7	98.0
50	48.9	97.8	42.3	86.5	46.6	93.2
158	137	86.7	140	102	139	101
500	431	86.2	430	99.8	430	99.9
Closterium cornu						
Control	0	-	0	-	0	-
25.6	17.5	68.4	15	85.7	16.3	92.9
64	49.0	76.6	45.5	92.9	47.3	96.4
160	124	77.5	97	78.2	111	89.1
400	304	76.0	255	83.9	280	92.0
1000	742	74.2	711	95.8	726	97.9
Synechococcus leopoliensis						
Control	0	-	0	-	0	-
25.6	17.5	68.4	21	120	19.3	110
64	42.0	65.6	41.7	99.2	41.8	99.6
160	96.0	60.0	111	116	104	108
400	230	57.6	265	115	248	108
1000	625	62.5	758	121	691	110
Nannochloropsis limnetica						
Control	0	-	0	-	0	-
125	93.0	74.4	68.3	73.5	80.7	86.7
250	212	84.8	152	71.5	182	85.8
500	252	50.4	273	108	263	104
1000	468	46.8	644	138	556	119
2000	1401	70.1	1236	88.2	1319	94

The validated method is summarised in Document M-CA4 (CA 4.1.2/67).

B. BIOLOGICAL DATA

Deviations of the pH values in the different control cultures were between 0.24 and 1.47.

A concentration dependent inhibition of algal growth could be observed in all six algae tests. Microscopic observation revealed normal appearances of the algae despite an increase in cell debris in the cultures with increasing growth inhibition.

The test results showed a clear dose response relationship. They were statistically analyzed to determine 72 hour EC_{50}-values together with 95% confidence intervals and an EC_{10}-value using Probit-analysis assuming log-normal distribution of the values. The NOEC and LOEC were determined using the [redacted] t-test or the [redacted] t-test. There were no statistically significant differences between the control and the acetone control. For the evaluation the acetone controls were used.

Table: Summary of effects from the exposure of six algal species to Aclonifen

Mean measured concentration (µg a.s./L)	Growth parameter			
	Mean specific growth rate (0 – 72h)	% Inhibition	Biomass (@72h)	% Inhibition
Chlorella vulgaris				
Solvent Control	2.217	-	465	-
37.5	2.102	5.2	304	34.7
85.0	2.110	4.8	273	41.4
194	1.933	12.8	166	64.4
303	1.302	41.3	31.9	93.1
610	0.889	59.9	6.92	98.5
Chlamydomonas rheinhardtii				
Pooled Control	1.385	-	59.5	-
3.42	1.380	0.4	65.3	-9.9
12.8	1.121	19.1	23.9	59.9
31.1	0.867	37.4	21.8	63.4
99.4	0.553	60.0	8.33	86.0
260	0.471	66.0	7.12	88.0
Xanthonema debileu				
Solvent Control	1.233	-	28.23	-
6.6	1.255	-1.8	29.25	-3.6
15.7	1.171	5.0	22.23	21.2
45.6	1.183	4.0	22.83	19.1
139	1.040	15.6	11.61	58.9
430	0.443	64.1	1.04	96.3
Closterium cornu				
Solvent Control	1.042	-	42.58	-
16.3	0.995	4.5	37.17	12.7
47.3	0.859	17.6	28.17	33.9
111	0.596	42.7	14.67	65.6
280	1.177	213.0	-0.83	102.0
726	-1.177	213.0	-1.50	103.5
Synechococcus leopoliensis				
Solvent Control	1.852	-	1.85	-
19.3	1.802	2.7	1.80	2.7
41.8	1.537	17.0	1.54	17.0
104	0.559	69.8	0.56	69.8
248	-0.012	100.7	-0.01	100.7
692	-0.258	113.9	-	-
Nannochloropsis limnetica				
Solvent Control	0.990	-	153.5	-
80.7	0.930	6.1	144.3	6.0
181	1.008	-1.8	167.5	-9.1
263	1.023	-3.3	170.0	-10.1
556	0.352	64.5	41.37	73.0
1319	-0.196	119.8	-	-

Algistatic property

The growth rate of *Chlamydomonas reinhardtii* exposed to 99.4 and 260 µg a.s./L was inhibited > 50%. After transfer into fresh medium without aclonifen, a fast recovery could be observed. There were no statistically significant differences in the growth rates after 48 h and 96 h, pre-exposed to 31.1, 99.4 and 260 µg a.s./L or control medium in the cultures with equal initial cell counts (960 cell/mL).

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 201, 2011)	Achieved					
		Chlorella vulgaris	*Chlamydomonas reinhardtii*	*Xanthonema debile*	*Closterium cornu*	*Synechococcus leopoliensis*	*Nannochloropsis limnetica*
Increase in control biomass	16	751	64	41	23	260	20
Mean coefficient of variation for section-by-section specific growth rates in the control cultures	≤ 35%	**43.7%**	**39.5%**	35.0%	29.0%	**59.5%**	23.8%
Coefficient of variation of average specific growth rates in replicate control cultures	≤ 10%	3.6%	1.5%	4.0%	4.2%	1.5%	2.1%

All validity criteria calculated against solvent controls
Values in **bold** fail the relevant validity criterion

With the exception of the tests performed on *C. vulgaris, C. reinhardtii* and *S. leopoliensis,* all validity criteria were satisfied in all other tests and therefore these tests can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Parameter	Mean measured concentration (µg a.s./L)			
	EC_{10} (0 – 72 h)	EC_{50} (0 – 72 h)	LOEC	NOEC
Chlorella vulgaris				
Growth rate (r)	129 [18.8 – 207]	450 [323 – 889]	194	85.0
Biomass (b)	16.2 [0.00 – 44.4]	86.8 [12.5 – 196.5]	<37.5	<37.5
Chlamydomonas reinhardtii				
Growth rate (r)	5.10 [0.33 – 13.4]	75.3 [40.1 – 171]	12.8	3.42
Biomass (b)	2.43 [n.d.]	15.8 [n.d]	12.8	3.42
Xanthonema debile				
Growth rate (r)	108 [54.7 – 151]	319 [258 – 400]	139	45.6
Biomass (b)	21.5 [0.001 – 52.6]	98.7 [24.9 – 472]	15.7	6.60
Closterium cornu				

Growth rate (r)	47.8 [0.82 – 76.7]	112 [61.0 – 229]	280	111
Biomass (b)	19.5 [4.92 – 32.5]	68.2 [46.1 – 98.3]	<16.3	<16.3
Synechococcus leopoliensis				
Growth rate (r)	34.4 [29.3 – 38.9]	74.9 [69.6 – 80.5]	41.8	19.3
Biomass (b)	20.1 [18.5 – 21.5]	37.0 [35.8 – 38.1]	41.8	19.3
Nannochloropsis limnetica				
Growth rate (r)	389 [n.d.]	513 [n.d.]	556	263
Biomass (b)	303 [222 – 357]	461 [402 – 524]	556	263

[95% confidence limits]
n.d.: not determined due to mathematical reasons

III. CONCLUSION

Concentration-effect relationships were observed for all algal species and were statistically analyzed to obtain effect concentrations. The effect concentrations regarding inhibition of growth rate are summarized as follows:

Growth Rate	**E_rC_{50} (µg a.s./L)**	**E_rC_{10} (µg a.s./L)**	**NOEC (µg a.s./L)**
Synechococcus leopoliensis	74.9	34.4	19.3
Chlamydomonas reinhardtii	75.3	5.1	3.42
Closterium cornu	112	47.8	111
Xanthonema debile	319	108	45.6
Chlorella vulgaris	450	129	85.0
Nannochloropsis limnetica	513	389	263

It could be shown that the algicidal properties of aclonifen were reversible.

[redacted] *(2006)*

Assessment and conclusion by applicant:

With the exception of the tests performed on *C. vulgaris, C. reinhardtii and S. leopoliensis,* all validity criteria were satisfied in all other tests and therefore these tests can be considered to be valid.

Concentration-effect relationships were observed for all algal species and were statistically analyzed to obtain effect concentrations. The effect concentrations regarding inhibition of growth rate are summarized as follows:

Growth Rate	**E_rC_{50} (µg a.s./L)**	**E_rC_{10} (µg a.s./L)**	**NOEC (µg a.s./L)**
Synechococcus leopoliensis	74.9	34.4	19.3
Chlamydomonas reinhardtii	75.3	5.1	3.42
Closterium cornu	112	47.8	111
Xanthonema debile	319	108	45.6
Chlorella vulgaris	450	129	85.0
Nannochloropsis limnetica	513	389	263

It could be shown that the algicidal properties of aclonifen were reversible.

The results were based on the arithmetic mean measured test concentrations. EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2015)[8] recommends that mean measured concentrations are calculated using the geometric mean rather than the arithmetic mean. The geometric mean measured concentrations are presented below.

Species	Geometric mean measured concentrations (µg a.s./L)
Synechococcus leopoliensis	19, 42, 103, 247 and 688
Chlamydomonas reinhardtii	3.4, 13, 31, 99 and 260
Closterium cornu	16, 47, 110, 278 and 726
Xanthonema debile	6.6, 16, 45, 138 and 430
Chlorella vulgaris	37, 84, 193, 303 and 609
Nannochloropsis limnetica	80, 180, 262, 549 and 1320

Given that the geometric mean measured concentrations were similar to those determined from the arithmetic mean, it was considered that recalculation of the study endpoints based on the geometric mean measured concentrations was not necessary.

Assessment and conclusion by RMS:

[8] EFSA (European Food Safety Authority), 2015. Technical report on the outcome of the pesticides peer review meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2015:EN-924. 62 pp.

Data Point:	KCA 8.2.6.2/03
Report Author:	[redacted]; [redacted]
Report Year:	2019
Report Title:	Freshwater alga, growth inhibition test (OECD 201) - Aclonifen: Effects on Synechococcus leopoliensis in a 96 hours growth test
Report No:	EBCL0021
Document No:	M-649614-01-1
Guideline(s) followed in study:	OECD Guideline 201: "Freshwater Alga and Cyanobacteria, Growth Inhibition Test" (March 23, 2006); Annex 5 corrected (July 28, 2011). EPA OCSPP 850.4550: Cyanobacteria (Anabaena flos-aquae) Toxicity (January 2012). Japanese MAFF: Algae Growth Inhibition Test. Data requirements for registration of agricultural chemicals: Notification No.12-Nousan 8147 (rev. 2014).
Deviations from current test guideline:	Current Guideline: OECD 201, 2011 None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to assess the inhibitory effect of Aclonifen on the growth of the uni-cellular freshwater cyanobacteria *Synechococcus leopoliensis* during an exposure period of 96 hours. The test was run with a control, solvent control and nominal test concentrations of 8.00, 25.3, 80.0, 253 and 800 µg a.s./L. Observations of cell growth were recorded daily (24, 48, 72 and 96 hours) to determine the potential effect on growth rate relative to the control.

To quantify substance-related effects, growth of the test species in the test solutions was compared to that of the controls. The concentrations causing a 10, 20 and 50% inhibition of growth after 72 and 96 hours were determined and expressed as the E_rC_{10}, E_rC_{20}, and E_rC_{50} values, respectively.

Additional response variables were yield and "area under the growth curve" (biomass), which may be needed to fulfil specific regulatory requirements in some countries. The 72 - and 96 - hour EC_{10}, EC_{20}, and EC_{50} values for these parameters were also determined (E_yC_x for yield and E_bC_x for biomass).

According to the guidelines, NOEC and LOEC values were additionally determined, if possible.

The concentrations of aclonifen in the test media were determined by chemical analysis using LC-MS/MS at the start of the test, after 72 and after 96 hours (LOQ = 1.5 µg a.s./L). At test start, the measured concentrations were in the range of 99.2 and 113% of the nominal values. After 72 hours the measured concentrations ranged between 81.8 and 103% of nominal. At the end of the 96-hour test, the concentrations were between 100 and 114% of nominal. Since the test concentrations did not deviate by more than 20% from the nominal concentrations, the nominal values were used for the evaluation.

There were concentration dependent effects on the growth of *Synechococcus leopoliensis* up to 32.6% inhibition at the highest test concentration of 800 µg a.s./L after 72 hours and up to 45.3% after 96 hours. The 72- and 96-hour E_rC_{50} for growth rate were calculated to be > 800 µg a.s./L and 644 µg a.s./L,

respectively. The NOEC values for growth rate after 72 and 96 hour were 25.3 and 8.00 µg a.s./L, respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen
	Batch no.:	AE F068300-01-15
	Purity:	> 99.5% aclonifen in dried material 1.81% water in undried material (> 97.7% a.s. in technical test item)
	Appearance:	Yellow crystalline solid powder
	Date received:	24 May 2018
	Storage:	Store at 25 ± 5 °C
	Expiry date:	02 May 2020
2.	**Test Organism:**	*Synechococcus leopoliensis*
	Strain:	UTEXIS 625
	Source:	[redacted]
	Pre-culture:	Prior to testing, a pre-culture was established in the growth medium 20xAAP to obtain exponentially-growing cyanobacteria for the test. The culture duration of the pre-culture was 3 days.
3.	**Test water:**	Sterilised synthetic 20-fold AAP medium according to OECD 201 was used as growth medium. This medium was selected since exponential growth of the cyanobacteria could be maintained during the test period

B. STUDY DESIGN AND METHODS

1. In-life phase: 29 October – 02 November 2018

2. Exposure conditions

Test vessels:	250 mL conical glass flasks covered with air-permeable silicone-sponge caps containing 100 mL test solution
Experimental design:	5 test concentrations (8.00, 25.3, 80.0, 253 and 800 µg a.s./L) plus 1 control and 1 solvent control (100 µL dimethylformamide/litre)
Replicates:	Eight replicate vessels were prepared for the control and solvent control and four replicate vessels for each treatment group.
Initial cell density:	Approximately 1.0 x 10^4 cells/mL

Temperature:	22.0 – 22.5 °C
pH:	7.83 – 8.63
Aeration:	None. Gaseous exchange and suspension of algal cells maintained by orbital shaker at 150 rpm
Photoperiod:	Continuous
Light intensity:	3216 - 3315 lux

3. Administration of the test item

For a stock solution of aclonifen in the solvent dimethylformamide (DMF) 40.96 mg test item (purity 97.7% a.s. in technical test item, equivalent to 40.0 mg pure aclonifen (a.s.)) were transferred from a teflon weighing boat using DMF to a glass flask with 5 mL DMF resulting in a yellowish, clear solution. 100 µL of this stock solution was given to 1 L growth medium and intensively stirred for 15 min using a magnetic stirrer. Subsequently, the other test concentrations were prepared by serial dilution from this stock solution. The concentration of the solvent was 0.1 milliliters per liter (mL/L) and was the same in all test treatments and the solvent control. In addition, a control with growth medium only was included in the test.

4. Test organism assignment and treatment

There were eight replicates of the control and solvent control and four replicates per treatment level. The test vessels were filled with 100 mL of the respective test solutions containing the test item and the controls.

The cell density of the inoculum culture (pre-culture) was determined and 257 µL of the inoculum culture (cell density 3.888 x 10^6 cell/mL) were added into the individual test vessels and filled up to 100 mL test solution or untreated growth medium to obtain the required cell density of 10 000 cells/mL.

5. Measurements and observations

The cell concentrations were determined in the pre-culture prior to the initiation of the test and daily during the 4-day growth test.

The cell numbers were counted microscopically after 24 hours, since the cell densities were too low for measuring the chlorophyll fluorescence. Five group squares of a Fuchs-Rosenthalchamber were counted per replicate and the mean value was multiplied with 500 to obtain the cell number/mL.

The cell density was determined by measurements of chlorophyll fluorescence (Synergy MX Multi Detection Reader) on the other days of the test. The excitation wavelength was 685 nm (gain 100) and the emission wavelength was 620 nm.

The following equation for relating cell count to fluorescence was used:

$y = 0.0013 x - 199.68$ $r^2 = 0.9967$ (y = fluorescence; x = cell counts/mL):

Microscopic observations were performed to verify a normal and healthy appearance of the inoculum culture and to observe any abnormal appearance of the cyanobacteria (as may be caused by the exposure to the test substance) and test media during the growth test.

Samples of freshly prepared test media were taken from all five test solutions, the control and the solvent control at the beginning of the test prior to distribution to the test vessels. After 72 hours and at test end (96 hours), analysis was performed in representative individual replicates per treatment level.

6. Statistics/Data evaluation

The evaluations of the concentration-effect-relationships and the calculations of effect concentrations were performed as outlined in the OECD guideline 201 and the EPA OCSPP:

- Due to the analytical recovery between 80 and 120% of the nominal concentrations, the nominal values according to OECD 23 were used for the evaluation.
- The mean value of the cell counts for each concentration plot were used for plotting growth curves.
- Mean growth rates, yield and "area under the growth curve" were calculated for the entire exposure period of 0 – 72 hours and 0 – 96 hours.
- Calculation of the percent inhibition compared to the control of growth rate [r], yield [y] and "area under the growth curve (cumulative biomass)" [B] were performed according to the guidelines.
- The percent inhibition values of the three parameters were plotted as a function of the concentrations of the test item.
- The test results were statistically analysed to determine an EC_{50}, EC_{20} and EC_{10} (growth rate, yield, "area under the growth curve") values together with 95% confidence intervals using linear Weibull regression analysis. Individual replicate responses were used for the regression analysis. Statistically significant difference was not observed between the control and the solvent control. The analysis was performed against the pooled controls.
- According to OECD 201 the LOEC and the NOEC were statistically determined. The computer program ToxRat® was used for statistical evaluations.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

At the start of the exposure, the recoveries of the measured concentrations were in the range of 99.2 and 113% of nominal. After 72 hours, the measured concentrations ranged between 81.8 and 103% of nominal. At the end of the 96-hour test, the concentrations were between 100 and 114% of nominal.

The test was evaluated using the nominal concentrations according to OECD 23, since the test item concentrations were within 80 and 120% of the nominal values.

Table: Measured test concentrations from the exposure of *Synechococcus leopoliensis* to Aclonifen

Nominal concentration (µg a.s./L)	Measured Concentration					
	0 Hours		72 Hours		96 Hours	
	µg a.s./L	% nom	µg a.s./L	% nom	µg a.s./L	% nom
Control	<LOQ	-	<LOQ	-	<LOQ	<LOQ
Solvent Control	<LOQ	-	<LOQ	-	<LOQ	-
8.00	7.94	99.2	6.55	81.8	8.00	100
25.3	27.4	108	25.0	98.8	26.5	105
80.0	90.4	113	82.2	103	88.2	110
253	287	113	250	98.9	265	105
800	882	110	769	96.2	916	114

Nom: Nominal concentration
LOQ: Limit of Quantitation = 1.5 µg a.s./L

The validated method is summarised in Document M-CA4 (CA 4.1.2/78).

B. BIOLOGICAL DATA

The pH of the controls and solvent controls was measured to be 7.85 and 7.90 in the fresh medium, respectively, at test start. The pH of the test media was between 7.83 and 7.92 at test start. At the termination of the growth test the pH of the aged control media ranged between 8.55 and 8.61 and between 8.60 and 8.63 for the solvent control. The pH of the test media was between 8.60 and 8.64. The pH of the control medium did not increase by more than 1.5 units during the test.

Table: Summary of effects from the exposure of *Synechococcus leopoliensis* to Aclonifen for 72 hours

Nominal concentration (µg a.s./L)	Growth parameter					
	Mean specific growth rate	% Inhibition	Yield	% Inhibition	Biomass	% Inhibition
Control	1.474	-	82	-	1519	-
Solvent Control	1.460	79		1471		-
8.00	1.474	-0.49	82	-2.08	1512	-1.16
25.3	1.452	1.01	77	4.19	1424	4.75
80.0	1.156[1]	21.21	34	61.40	834[1]	44.23
253	1.016[1]	30.71	20[2]	75.01	688[1]	54.00
800	0.989[1]	32.59	18[2]	77.10	653[1]	56.35

[1]: Significant difference to pooled control, Williams- t-test α = 0.05, one-sided smaller
[2]: Significant difference to pooled control, Welch -t-test α = 0.05, one-sided smaller

Exposure of *Synechococcus leopoliensis* to Aclonifen resulted in E_rC_{10}, E_rC_{20} and E_rC_{50} (0 – 72 h) values of 35.4, 148 and greater than 800 µg a.s./L respectively based on the geometric mean measured test concentrations. The No Observed Effect Concentration (NOEC) after 72 hours based on growth rate was 25.3 µg a.s./L, and the Lowest Observed Effect Concentration (LOEC) was 80 µg a.s./L.

Table: Summary of effects from the exposure of *Synechococcus leopoliensis* to Aclonifen for 96 hours

Nominal concentration (µg a.s./L)	Growth parameter					
	Mean specific growth rate	% Inhibition	Yield	% Inhibition	Biomass	% Inhibition
Control	1.385	-	254	-	5559	-
Solvent Control	1.367	-	237	-	5265	-
8.00	1.373	0.28	242	1.75	5398	0.26
25.3	1.290[1]	6.30	175[1]	29.00	4445[1]	17.87
80.0	0.968[1]	34.89	35.1[1]	85.71	1629[1]	69.91
253	0.768[1]	44.17	20.6[1]	91.61	1177[1]	78.25
800	0.753[1]	45.28	19.4[1]	92.12	1106[1]	79.56

[1]: Significant difference to pooled control, Welch-t-test α = 0.05, one-sided smaller

Exposure of *Synechococcus leopoliensis* to Aclonifen resulted in E_rC_{10}, E_rC_{20} and E_rC_{50} (0 – 96 h) values of 13.6, 51.1 and 644 µg a.s./L respectively based on the geometric mean measured test concentrations. The No Observed Effect Concentration (NOEC) after 96 hours based on growth rate was 8.00 µg a.s./L, and the Lowest Observed Effect Concentration (LOEC) was 25.3 µg a.s./L.

C. VALIDITY CRITERIA

Validity criterion	Required* (OECD 201, 2011)	Achieved	
		Control	Solvent Control
Increase in control biomass	16	83.3	79.9
Mean coefficient of variation for section-by-section specific growth rates in the control cultures	≤ 35%	5.1%	5.7%
Coefficient of variation of average specific growth rates in replicate control cultures	≤ 10%	1.3%	1.1%

*: After 72 hours

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Parameter	Geometric mean measured concentration (µg a.s./L)				
	EC_{50}	EC_{20}	EC_{10}	LOEC	NOEC
72-hour test duration					
Growth rate (r)	>800 [-]	148 [90 – 214]	35.4 [13.2 – 62.7]	80	25.3
Yield (y)	93.5 [62.7 – 133]	27.5 [13.3 – 42.1]	14.5 [5.31 – 25.1]	80	25.3
Biomass (b)	288 [192 – 483]	85.9 [15.2 – 60.4]	12.1 [3.30 – 25.0]	25.3	8.0
96-hour test duration					
Growth rate (r)	644 [412 – 1268]	51.1 [24.4 – 81.9]	13.6 [3.95 – 27.6]	25.3	8.0
Yield (y)	37.6 [32.9 – 43.2]	20.2 [16.0 – 23.7]	14.5 [10.6 – 17.9]	25.3	8.0
Biomass (b)	65.8 [47.9 – 90.3]	19.5 [10.3 – 28.9]	10.3 [4.28 – 17.1]	25.3	8.0

[95% confidence limits]

III. CONCLUSION

Exposure of *Synechococcus leopoliensis* to Aclonifen resulted in E_rC_{10}, E_rC_{20} and E_rC_{50} (0 – 96 h) values of 13.6, 51.1 and 644 µg a.s./L respectively based on the geometric mean measured test concentrations. The No Observed Effect Concentration (NOEC) after 96 hours based on growth rate was 8.0 µg a.s./L, and the Lowest Observed Effect Concentration (LOEC) was 25.3 µg a.s./L.

[redacted] *(2018)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

Exposure of *Synechococcus leopoliensis* to Aclonifen resulted in E_rC_{10}, E_rC_{20} and E_rC_{50} (0 - 96 h) values of 13.6, 51.1 and 644 µg a.s./L respectively based on the geometric mean measured test concentrations. The No Observed Effect Concentration (NOEC) after 96 hours based on growth rate was 8.0 µg a.s./L, and the Lowest Observed Effect Concentration (LOEC) was 25.3 µg a.s./L.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.6.2/04
Report Author:	[redacted]
Report Year:	2019
Report Title:	Freshwater alga, growth inhibition test (OECD 201) aclonifen: Effects on Navicula pelliculosa in a 96 hours growth test
Report No:	EBCL0020
Document No:	M-648378-01-1
Guideline(s) followed in study:	OECD Guideline 201, Freshwater Alga and Cyanobacteria, Growth Inhibition Test (March 23, 2006); Annex 5 corrected (July 28, 2011). EPA OCSPP 850.4500: Algal Toxicity. (January 2012). Japanese MAFF, Algae Growth Inhibition Test. Data requirements for registration of agricultural chemicals, Notification No.12-Nousan 8147 (rev. 2014).
Deviations from current test guideline:	Current Guideline: OECD 201, 2011 None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to assess the inhibitory effect of Aclonifen on the growth of the uni-cellular freshwater algae *Navicula pelliculosa* during an exposure period of 96 hours. The test was run with a control, solvent control and nominal test concentrations of 17.2, 51.6, 155, 466 and 1399 µg a.s./L. Observations of cell growth were recorded daily (24, 48, 72 and 96 hours) to determine the potential effect on growth rate relative to the control.

To quantify substance-related effects, growth of the test species in the test solutions was compared to that of the controls. The concentrations causing a 10, 20 and 50% inhibition of growth after 72 and 96 hours were determined and expressed as the E_rC_{10}, E_rC_{20}, and E_rC_{50} values, respectively.

Additional response variables were yield and "area under the growth curve" (biomass), which may be needed to fulfil specific regulatory requirements in some countries. The 72 - and 96 - hour EC_{10}, EC_{20}, and EC_{50} values for these parameters were also determined (E_yC_x for yield and E_bC_x for biomass).

According to the guidelines, NOEC and LOEC values were additionally determined, if possible.

The concentrations of aclonifen in the test media were determined by chemical analysis using LC-MS/MS at the start of the test, after 72 and after 96 hours (LOQ = 1.5 µg a.s./L). At test start, the measured concentrations were in the range of 83.8 and 112% of the nominal values. After 72 hours the measured concentrations were between 71.2 and 104% and after 96 hours between 69.7 and 108% of initial measured concentrations. Since the test concentrations decreased by more than 20% during the test, the geometric mean exposure concentrations were used for the evaluation (72 hour geometric mean: 13.4, 41.2, 134, 392 and 1505 µg a.s./L; 96 hour geometric mean: 13.2, 39.9, 132, 385 and 1499 µg a.s./L).

There were concentration dependent effects on the growth of the *Chlorella vulgaris* up to 73.7% inhibition at the highest test concentration after 72 hours and up to 82.8% after 96 hours, hence the 72- and 96-hour E_rC_{50} for growth rate were 803 and 672.6 µg a.s./L respectively. The NOEC values for growth rate after 72 and 96 hours were 134 and 132 µg a.s./L respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen
	Batch no.:	AE F068300-01-15
	Purity:	> 99.5% aclonifen in dried material 1.81% water in undried material (> 97.7% a.s. in technical test item)
	Appearance:	Yellow crystalline solid, powder
	Date received:	24 May 2018
	Storage:	Store at 25 ± 5 °C
	Expiry date:	02 May 2020
2.	**Test Organism:**	*Navicula pelliculosa*
	Strain:	UTEX IS 625
	Source:	[redacted]
	Pre-culture:	Prior to testing, a pre-culture was established in standard OECD growth medium in 20-fold AAP growth medium supplemented with silicate to obtain exponentially-growing algae for the test. The culture duration of the pre-cultures was 3 days
3.	**Test water:**	Sterilised synthetic 20-fold AAP medium according to OECD 221 was used as growth medium. The medium was supplemented with sodium metasilicate pentahydrate (Na_2SiO_3 x 5 H_2O) to obtain a concentration of 20 mg Si/L according to

EPA OCSPP 850.4500. This medium was selected since exponential growth of the algae could be maintained during the test period

B. STUDY DESIGN AND METHODS

1. In-life phase: 10 – 14 September 2018

2. Exposure conditions

Test vessels:	250 mL conical glass flasks covered with air-permeable silicone-sponge caps containing 100 mL test solution
Experimental design:	5 test concentrations (17.2, 51.6, 155, 466, and 1399 µg a.s./L) plus 1 control and 1 solvent control (100 µL dimethylformamide/litre)
Replicates:	Eight replicate vessels were prepared for the control and solvent control and four replicate vessels for each treatment group.
Initial cell density:	Approximately 1.0 x 10^4 cells/mL
Temperature:	22.0 – 23.5 °C
pH:	7.58 – 8.12
Aeration:	None. Gaseous exchange and suspension of algal cells maintained by orbital shaker at 150 rpm
Photoperiod:	Continuous
Light intensity:	4609 - 4709 lux

3. Administration of the test item

For a stock solution of aclonifen in the solvent dimethylformamide (DMF) 71.58 mg test item (purity 97.7% a.s. in technical test item, equivalent to 69.934 µg pure aclonifen (a.s.)) were transferred from a teflon weighing boat using DMF to a 5 mL glass volumetric flask and filled up to 5 mL, resulting in a yellowish, clear solution. 100 µL of this stock solution was given to 1 L OECD growth medium and intensively stirred for 15 min using a magnetic stirrer. Subsequently, the other test concentrations were prepared by serial dilution from this stock solution. The concentration of the solvent was 0.1 milliliters per liter (mL/L) and was the same in all test treatments and the solvent control. In addition a control with growth medium only was included in the test.

4. Test organism assignment and treatment

There were eight replicates of the control and solvent control and four replicates per treatment level. The test vessels were filled with 100 mL of the respective test solutions containing the test item and the controls.

The cell density of the inoculum culture (pre-culture) was determined and 1091 µL of the inoculum culture (cell density 9.1664 x 10^5 cell/mL) were added into the individual test vessels and filled up to 100 mL test solution or untreated growth medium to obtain the required cell density of 10 000 cells/mL.

5. Measurements and observations

The cell concentrations were determined in the pre-culture prior to the initiation of the test and daily during the 4-day growth test. The cell density was determined by measurements of chlorophyll

fluorescence (Synergy MX Multi Detection Reader). The excitation wavelength was 690 nm (gain 80), the emission wavelength was 438 nm).

A calibration curve for relating cell count to fluorescence was used:

y = 0.0312 x + 107.57 (y = fluorescence; x = cell counts/mL)

Microscopic observations were performed to verify a normal and healthy appearance of the inoculum culture and to observe any abnormal appearance of the algae (as may be caused by the exposure to the test substance) and test media during the growth test.

Samples of freshly prepared test media were taken from all five test solutions, the control and the solvent control at the beginning of the test prior to distribution to the test vessels. After 72 hours and at test end (96 hours), analysis was performed in representative individual replicates per treatment level.

6. Statistics/Data evaluation

The evaluations of the concentration-effect-relationships and the calculations of effect concentrations were performed as outlined in the OECD guideline 201 and the EPA OCSPP:

- Due to the analytical recovery < 80% of the initial concentrations of the two lowest test concentrations after 72 h, the geometric mean measured values according to OECD 23 were used (values from 0 and 72 hours).
- Due to the analytical recoveries < 80% of the nominal concentrations after 96 h, the geometric mean measured values (time weighted mean) according to OECD 23 were used (values from 0, 72 and 96 hours).
- The mean value of the cell counts for each concentration plot were used for plotting growth curves.
- Mean growth rates, yield and "area under the growth curve" were calculated for the entire exposure period of 0 – 72 hours and 0 – 96 hours.
- Calculation of the percent inhibition compared to the control of growth rate [r], yield [y] and "area under the growth curve (cumulative biomass)" [B] were performed according to the guidelines.
- The percent inhibition values of the three parameters were plotted as a function of the concentrations of the test item.
- The test results were statistically analysed to determine an EC_{50}, EC_{20} and EC_{10} (growth rate, yield, "area under the growth curve") values together with 95% confidence intervals using linear [redacted] regression analysis. Individual replicate responses were used for the regression analysis. Statistically significant difference was not observed between the control and the solvent control. The analysis was performed against the pooled controls.
- According to OECD 201 the LOEC and the NOEC were statistically determined. The computer program ToxRat® was used for statistical evaluations.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

At the start of the exposure, the recoveries of the measured concentrations were in the range of 83.8 and 112% of nominal. After 72 hours, the measured concentrations ranged between 71.2 and 104% of nominal. At the end of the 96-hour test, the concentrations were between 69.7 and 108% of nominal.

The test was evaluated using the geometric means of the measured concentrations (time weighted means) according to OECD 23. For the 72-hour test, the mean measured values were calculated from the 0 and 72 hour concentrations (13.4, 41.2, 134, 392 and 1505 µg a.s./L). For the 96-hour test, the mean measured values were calculated from the 0, 72 and 96 hour concentrations (13.2, 39.9, 132, 385 and 1499 µg a.s./L).

Table: Measured test concentrations from the exposure of *Navicula pelliculosa* to Aclonifen

Nominal concentration (µg a.s./L)	Measured Concentration									
	0 Hours		72 Hours		0 - 72 Hours[1]		96 Hours		0 - 96 Hours[2]	
	µg a.s./L	% nom	µg a.s./L	% nom	µg a.s./L	% nom	µg a.s./L	% nom	µg a.s./L	% nom
Control	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	<LOQ	<LOQ	-
Solvent Control	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
17.2	14.4	83.8	12.4	71.9	13.4	77.6	12.9	75.3	13.2	76.6
51.6	46.2	89.5	36.7	71.2	41.2	79.8	36.0	69.7	39.9	77.3
155	151	97.7	119	76.6	134	86.5	133	85.6	132	85.1
466	468	100	329	70.6	392	84.2	404	86.6	385	82.6
1399	1564	112	1448	104	1505	108	1515	108	1499	107

[1]: Geometric mean measured concentration of day 0 and day 3
[2]: Geometric mean measured concentration of day 0, day 3 and day 4
Nom: Nominal concentration
LOQ: Limit of Quantitation = 1.5 µg a.s./L

The validated method is summarised in Document M-CA4 (CA 4.1.2/79).

B. BIOLOGICAL DATA

The pH of the control was measured to be 7.61 in the fresh medium (solvent control: 7.56) and the pH of the test media was between 7.57 and 7.65 at test start. At the termination of the growth test the pH of the aged control media ranged between 9.31 and 9.46 (for solvent control: between 9.23 and 9.47) and between 8.61 and 9.46 for the test media.

The pH of the control and solvent control media increased by 1.8 units during the 96-hour test, slightly higher than 1.5 units recommended for a 72-hour test according to OECD 201 for metals and compounds which are hydrolytically unstable.

Since aclonifen did not hydrolyse under alkaline conditions, the pH of the test media is without influence on the outcome of the test.

Table: Summary of effects from the exposure of *Navicula pelliculosa* to Aclonifen for 72 hours

Geometric mean measured concentration (µg a.s./L)	Growth parameter					
	Mean specific growth rate	% Inhibition	Yield	% Inhibition	Biomass	% Inhibition
Control	1.543	-	102	-	1743	-

Solvent Control	1.515	-	93	-	1671	-
13.4	1.529	0.04	97	0.38	1711	-0.25
41.2	1.552	-1.46	104	-6.49	2006	-17.51
134	1.490	2.57	86	11.50[2]	1659	2.78
392	1.167	23.72[1]	32	66.58[2]	884	48.19[2]
1505	0.403	73.67[1]	2.6	97.37[2]	95	94.44[2]

[1]: Significant difference to pooled control, Welch-t-test α = 0.05, one-sided smaller
[2]: Significant difference to pooled control, Williams-t-test α = 0.05, one-sided smaller

Exposure of *Navicula pelliculosa* to Aclonifen resulted in E_rC_{10}, E_rC_{20} and E_rC_{50} (0 – 72 h) values of 227, 351 and 803 µg a.s./L respectively based on the geometric mean measured test concentrations. The No Observed Effect Concentration (NOEC) after 72 hours based on growth rate was 134 µg a.s./L, and the Lowest Observed Effect Concentration (LOEC) was 392 µg a.s./L.

Table: Summary of effects from the exposure of *Navicula pelliculosa* to Aclonifen for 96 hours

Geometric mean measured concentration (µg a.s./L)	Growth parameter					
	Mean specific growth rate	% Inhibition	Yield	% Inhibition	Biomass	% Inhibition
Control	1.291	-	172	-	5557	-
Solvent Control	1.288	-	172	-	4859	-
13.2	1.298	-0.64	179	-3.32	5027	-1.39
39.9	1.295	-0.44	177	-2.52	5381	-8.54
132	1.287	0.16	171	0.94	4756	4.07
385	0.955[1]	25.96	45[2]	73.88	1817[2]	63.35
1499	0.222[1]	82.81	1.6[2]	99.07	145[2]	97.07

[1]: Significant difference to pooled control, Welch-t-test α = 0.05, one-sided smaller
[2]: Significant difference to pooled control, Williams-t-test α = 0.05, one-sided smaller

Exposure of *Navicula pelliculosa* to Aclonifen resulted in E_rC_{10}, E_rC_{20} and E_rC_{50} (0 – 96 h) values of 231, 333 and 672 µg a.s./L respectively based on the geometric mean measured test concentrations. The No Observed Effect Concentration (NOEC) after 96 hours based on growth rate was 132 µg a.s./L, and the Lowest Observed Effect Concentration (LOEC) was 385 µg a.s./L.

C. VALIDITY CRITERIA

Validity criterion	Required* (OECD 201, 2011)	Achieved	
		Control	Solvent Control
Increase in control biomass	16	103	94.5
Mean coefficient of variation for section-by-section specific growth rates in the control cultures	≤ 35%	12.5%	14.2%
Coefficient of variation of average specific growth rates in replicate control cultures	≤ 10%	2.7%	1.6%

*: After 72 hours

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Parameter	Geometric mean measured concentration (µg a.s./L)				
	EC_{50}	EC_{20}	EC_{10}	LOEC	NOEC
72-hour test duration					
Growth rate (r)	803 [724 – 893]	351 [296 – 403]	227 [181 – 272]	392	134
Yield (y)	296 [270 – 324]	169 [142 – 193]	126 [99 – 149]	134	41.2
Biomass (b)	412 [375 – 456]	224 [185 – 255]	163 [124 – 195]	392	134
96-hour test duration					
Growth rate (r)	672 [614 – 736]	333 [292 – 372]	231 [194 – 266]	385	132
Yield (y)	305 [296 – 315]	204 [193 – 214]	157 [145 – 167]	385	132
Biomass (b)	330 [315 – 345]	202 [186 – 216]	146 [130 – 161]	385	132

[95% confidence limits]

III. CONCLUSION

Exposure of *Navicula pelliculosa* to Aclonifen resulted in E_rC_{10}, E_rC_{20} and E_rC_{50} (0 – 96 h) values of 231, 333 and 672 µg a.s./L respectively based on the geometric mean measured test concentrations. The No Observed Effect Concentration (NOEC) after 96 hours based on growth rate was 132 µg a.s./L, and the Lowest Observed Effect Concentration (LOEC) was 385 µg a.s./L.

[redacted] *(2018)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

Exposure of *Navicula pelliculosa* to Aclonifen resulted in E_rC_{10}, E_rC_{20} and E_rC_{50} (0 – 96 h) values of 231, 333 and 672 µg a.s./L respectively based on the geometric mean measured test concentrations. The No Observed Effect Concentration (NOEC) after 96 hours based on growth rate was 132 µg a.s./L, and the Lowest Observed Effect Concentration (LOEC) was 385 µg a.s./L.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.6.2/05
Report Author:	[redacted]; [redacted]
Report Year:	2018
Report Title:	Freshwater alga, growth inhibition test (OECD 201) aclonifen: Effects on Chlorella vulgaris in a 96 hours growth test
Report No:	BAY-025/4-10/J
Document No:	M-646486-01-1
Guideline(s) followed in study:	OECD Guideline 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (March 23, 2006); Annex 5 corrected (July 28, 2011). EPA OCSPP 850.4500: Algal Toxicity. (January 2012). Japanese MAFF: Algae Growth Inhibition Test. Data requirements for registration of agricultural chemicals: Notification No.12-Nousan 8147 (rev. 2014).
Deviations from current test guideline:	Current Guideline: OECD 201, 2011 None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to assess the inhibitory effect of Aclonifen on the growth of the uni-cellular freshwater algae *Chlorella vulgaris* during an exposure period of 96 hours. The test was run with a control, solvent control and nominal test concentrations of 87.5, 175, 350, 700 and 1400 µg a.s./L. Observations of cell growth were recorded daily (24, 48, 72 and 96 hours) to determine the potential effect on growth rate relative to the control.

To quantify substance-related effects, growth of the test species in the test solutions was compared to that of the controls. The concentrations causing a 10, 20 and 50% inhibition of growth after 72 and 96 hours were determined and expressed as the E_rC_{10}, E_rC_{20}, and E_rC_{50} values, respectively.

Additional response variables were yield and "area under the growth curve" (biomass), which may be needed to fulfil specific regulatory requirements in some countries. The 72 - and 96 - hour EC_{10}, EC_{20}, and EC_{50} values for these parameters were also determined (E_yC_x for yield and E_bC_x for biomass).

According to the guidelines, NOEC and LOEC values were additionally determined, if possible.

The concentrations of aclonifen in the test media were determined by chemical analysis using LC-MS/MS at the start of the test, after 72 and after 96 hours (LOQ = 1.5 µg a.s./L). At test start, the measured concentrations were in the range of 118 and 137% of the nominal values. After 72 hours the measured concentrations were between 77.3 and 97.1% and after 96 hours between 77.9 and 94.6% of initial measured concentrations. Since the test concentrations decreased by more than 20% during the test, the geometric mean exposure concentrations were used for the evaluation (72 hour geometric mean: 96.5, 210, 404, 830 and 1594 µg a.s./L; 96 hour geometric mean: 93.5, 205, 400, 821, 1583 µg a.s./L).

There were concentration dependent effects on the growth of the *Chlorella vulgaris* up to 42.0% inhibition at the highest test concentration after 72 hours and up to 46.3% after 96 hours, hence the 72-

and 96-hour E_rC_{50} for growth rate was > 1594 and > 1583 µg a.s./L, respectively. The NOEC values for growth rate after 72 and 96 hour were 96.5 and 93.5 µg a.s./L, respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen
	Batch no.:	AE F068300-01-15
	Purity:	> 99.5% aclonifen in dried material 1.81% water in undried material (> 97.7% a.s. in technical test item)
	Appearance:	Yellow crystalline solid powder
	Date received:	24 May 2018
	Storage:	Store at 25 ± 5 °C
	Expiry date:	02 May 2020
2.	**Test Organism:**	*Chlorella vulgaris*
	Strain:	211-11b SAG
	Source:	[redacted]
	Pre-culture:	Prior to testing, a preculture was established in standard OECD growth medium to obtain exponentially-growing algae for the test. The culture duration of the pre-culture was 3 days
3.	**Test water:**	Sterilised synthetic growth medium according to OECD 201

B. STUDY DESIGN AND METHODS

1. In-life phase: 29 October – 02 November 2018

2. Exposure conditions

Test vessels:	250 mL conical glass flasks covered with air-permeable silicone-sponge caps containing 100 mL test solution
Experimental design:	5 test concentrations (87.5, 175, 350, 700 and 1400 µg a.s./L) plus 1 control and 1 solvent control (50 µL dimethylformamide/litre)
Replicates:	Eight replicate vessels were prepared for the control and solvent control and four replicate vessels for each treatment group.
Initial cell density:	Approximately 1.0 x 10^4 cells/mL
Temperature:	22.0 – 23.5 °C
pH:	7.58 – 8.12
Aeration:	None. Gaseous exchange and suspension of algal cells maintained by orbital shaker at 150 rpm
Photoperiod:	Continuous

Light intensity: 4609 - 4709 lux

3. Administration of the test item

For a stock solution of aclonifen in the solvent dimethylformamide (DMF) 143.31 mg test item (purity 97.7% a.s. in technical test item, equivalent to 140 mg pure aclonifen (a.s.)) were transferred from a teflon weighing boat using DMF to a 5 mL volumetric flask and filled up with DMF resulting in a yellowish, clear solution. 50 µL of this stock solution was given to 1 L OECD growth medium and intensively stirred for about 15 min using a magnetic stirrer. Subsequently, the other test concentrations were prepared by serial dilution from this stock solution. The concentration of the solvent was 0.05 milliliters per liter (mL/L) and was the same in all test treatments and the solvent control. In addition a control with growth medium only was included in the test.

4. Test organism assignment and treatment

There were eight replicates of the control and solvent control and four replicates per treatment level. The test vessels were filled with 100 mL of the respective test solutions containing the test item and the controls.

The cell density of the inoculum culture (pre-culture) was determined and 178 µL of the inoculum culture (cell density 5.623 x 10^6 cell/mL) were added into the individual test vessels and filled up to 100 mL test solution or untreated growth medium to obtain the required cell density of 10 000 cells/mL.

5. Measurements and observations

The cell concentrations were determined in the pre-culture prior to the initiation of the test and daily during the 4-day growth test. The cell density was determined by measurements of chlorophyll fluorescence (Synergy MX Multi Detection Reader). The emission wavelength was 440 nm and the excitation wavelength was 690 nm (gain 100).

A analysis function for relating cell count to fluorescence was used:

y = 75.366 x (y = cell counts/mL; x = fluorescence).

Microscopic observations were performed to verify a normal and healthy appearance of the inoculum culture and to observe any abnormal appearance of the algae (as may be caused by the exposure to the test substance) and test media during the growth test.

Samples of freshly prepared test media were taken from all five test solutions, the control and the solvent control at the beginning of the test prior to distribution to the test vessels. After 72 hours and at test end (96 hours), analysis was performed in representative individual replicates per treatment level.

6. Statistics/Data evaluation

The evaluations of the concentration-effect-relationships and the calculations of effect concentrations were performed as outlined in the OECD guideline 201 and the EPA OCSPP:

- Due to the analytical recovery < 80% of the initial concentrations of the two lowest test concentrations after 72 h, the geometric mean measured values according to OECD 23 were used (values from 0 and 72 hours).

- Due to the analytical recoveries < 80% of the initial concentrations of one of the two lowest test concentrations after 72 and 96 h, the geometric mean measured values (time weighted mean) according to OECD 23 were used (values from 0, 72 and 96 hours).
- The mean value of the cell counts for each concentration plot were used for plotting growth curves.
- Mean growth rates, yield and "area under the growth curve" were calculated for the entire exposure period of 0 – 72 hours and 0 – 96 hours.
- Calculation of the percent inhibition compared to the control of growth rate [r], yield [y] and "area under the growth curve (cumulative biomass)" [B] were performed according to the guidelines.
- The percent inhibition values of the three parameters were plotted as a function of the concentrations of the test item.
- The test results were statistically analysed to determine an EC_{50}, EC_{20} and EC_{10} (growth rate, yield, "area under the growth curve") values together with 95% confidence intervals using linear Probit or Weibull regression analysis. Individual replicate responses were used for the regression analysis. Statistically significant difference was observed between the control and the solvent control so the analysis was performed against the solvent control.
- According to OECD 204 the LOEC and the NOEC were statistically determined. The computer program ToxRat® was used for statistical evaluations.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

At the start of the exposure, the recoveries of the measured concentrations were in the range of 118 and 137% of nominal. After 72 hours, the measured concentrations ranged between 103 and 117% of nominal (77.3 - 97.1% of initial). At the end of the 96 hour test, the concentrations were between 91.6 and 117% of nominal (77.9 - 94.6% of initial). The test item concentrations decreased by more than 20% from the initial concentrations in the two lowest concentrations during the entire test period.

The test was evaluated using the geometric means of the measured concentrations (time weighted means) according to OECD 23. For the 72-hour test, the mean measured values were calculated from the 0 and 72 hour concentrations (96.5, 210, 404, 830 and 1594 µg a.s./L). For the 96-hour test, the geometric mean measured values were calculated from the 0, 72 and 96 hour concentrations (93.5, 205, 400, 824 and 1583 µg a.s./L).

Table: Measured test concentrations from the exposure of *Chlorella vulgaris* to Aclonifen

Nominal concentration (µg a.s./L)	Measured Concentration									
	0 Hours		72 Hours		0- 72 Hours[1]		96 Hours		0 - 96 Hours[2]	
	µg a.s./L	% nom	µg a.s./L	% nom	µg a.s./L	% nom	µg a.s./L	% nom	µg a.s./L	% nom
Control	<LOQ		<LOQ	-	<LOQ	-	<LOQ	<LOQ	<LOQ	-
Solvent Control	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
87.5	103	118	90.3	103	96.5	110	80.2	91.6	93.5	107
175	239	137	185	105	210	120	196	112	205	117
350	426	122	384	110	404	116	391	112	400	114

700	842	120	817	117	830	119	772	110	821	117
1400	1729	124	1470	105	1594	114	1636	117	1583	113

[1]: Geometric mean measured concentration of day 0 and day 3
[2]: Geometric mean measured concentration of day 0, day 3 and day 4
Nom: Nominal concentration
LOQ: Limit of Quantitation = 1.5 µg a.s./L

The validated method is summarised in Document M-CA4 (CA 4.1.2/80).

B. BIOLOGICAL DATA

The pH of the fresh control and solvent control media was measured to be 7.70 and 7.65, respectively and the pH of the test media was between 7.58 and 7.65 at test start. At the termination of the growth test the pH of the aged control and solvent control media ranged between 7.94 and 8.12 and between 7.90 and 8.10 for the test media.

Table: Summary of effects from the exposure of *Chlorella vulgaris* to Aclonifen for 72 hours

Geometric mean measured concentration (µg a.s./L)	Growth parameter					
	Mean specific growth rate	% Inhibition	Yield	% Inhibition	Biomass	% Inhibition
Control	1.731	-	180	-	3387	-
Solvent Control	1.633	-	134	-	2308	-
96.50	1.640	-0.42	137	-2.75	2813	-21.90
210	1.370[1]	16.09	60[1]	54.97	1399[2]	39.39
404	1.206[1]	26.12	36[1]	72.46	1107[2]	52.02
830	1.168[1]	28.47	34[1]	74.47	759[2]	67.08
1594	0.948[1]	41.96	16[1]	87.90	283[2]	87.72

[1]: Significant difference to solvent control, Williams $\alpha = 0.05$, one-sided smaller
[2]: Significant difference to solvent control, Step-down Jonckheere-Terpstra $\alpha = 0.05$, one-sided smaller

Exposure of *Chlorella vulgaris* to Aclonifen resulted in E_rC_{10}, E_rC_{20} and E_rC_{50} (0 – 72 h) values of 139, 373 and greater than 1594 µg a.s./L respectively based on the geometric mean measured test concentrations. The No Observed Effect Concentration (NOEC) after 72 hours based on growth rate was 96.5 µg a.s./L, and the Lowest Observed Effect Concentration (LOEC) was 210 µg a.s./L.

Table: Summary of effects from the exposure of *Chlorella vulgaris* to Aclonifen for 96 hours

Geometric mean measured concentration (µg a.s./L)	Growth parameter					
	Mean specific growth rate	% Inhibition	Yield	% Inhibition	Biomass	% Inhibition
Control	1.572	-	540	-	12038	-
Solvent Control	1.504	-	410	-	8845	-
93.5	1.468	2.40	356[1]	13.29	8740	1.19
205	1.250[1]	16.90	151[1]	63.05	3944[1]	55.41
400	1.108[1]	26.29	84[1]	79.52	2560[1]	71.06
821	1.063[1]	29.33	72[1]	82.35	2040[1]	76.93

1583	0.808[1]	46.29	25[1]	93.71	787[1]	91.09

[1]: Significant difference to solvent control, Williams α = 0.05, one-sided smaller

Exposure of *Chlorella vulgaris* to Aclonifen resulted in E_rC_{10}, E_rC_{20} and E_rC_{50} (0 – 96 h) values of 132, 338 and greater than 1583 µg a.s./L respectively based on the geometric mean measured test concentrations. The No Observed Effect Concentration (NOEC) after 96 hours based on growth rate was 93.5 µg a.s./L, and the Lowest Observed Effect Concentration (LOEC) was 205 µg a.s./L.

C. VALIDITY CRITERIA

Validity criterion	Required* (OECD 201, 2011)	Achieved	
		Control	Solvent Control
Increase in control biomass	16	182	135
Mean coefficient of variation for section-by-section specific growth rates in the control cultures	≤ 35%	19.5%	24.4%
Coefficient of variation of average specific growth rates in replicate control cultures	≤ 10%	2.7%	2.6%

*: After 72 hours

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Parameter	Geometric mean measured concentration (µg a.s./L)				
	EC_{50}	EC_{20}	EC_{10}	LOEC	NOEC
72-hour test duration					
Growth rate (r)	>1594 [-]	373 [268 – 472]	139 [74.1 – 204]	210	96.5
Yield (y)	254 [197 – 320]	95.5 [54.0 – 133]	57.2 [26.1 – 88.2]	210	96.5
Biomass (b)	402 [336 – 459]	145 [101 – 186]	85.2 [51.6 – 119]	404	210
96-hour test duration					
Growth rate (r)	>1583 [-]	338 [246 – 425]	132 [74.7 – 191]	205	93.5
Yield (y)	190 [154 – 228]	85.4 [54.2 – 113]	56.3 [30.2 – 79.8]	≤93.5	<93.5
Biomass (b)	246 [198 – 301]	98.2 [61.3 – 131.0]	60.8 [31.8 – 88.7]	205	93.5

[95% confidence limits]

III. CONCLUSION

Exposure of *Chlorella vulgaris* to Aclonifen resulted in E_rC_{10}, E_rC_{20} and E_rC_{50} (0 – 96 h) values of 132, 338 and greater than 1583 µg a.s./L respectively based on the geometric mean measured test concentrations. The No Observed Effect Concentration (NOEC) after 96 hours based on growth rate was 93.5 µg a.s./L, and the Lowest Observed Effect Concentration (LOEC) was 205 µg a.s./L.

[redacted] *(2018)*

Assessment and conclusion by applicant:
All validity criteria were satisfied and therefore this study can be considered to be valid. Exposure of *Chlorella vulgaris* to Aclonifen resulted in E_rC_{10}, E_rC_{20} and E_rC_{50} (0 – 96 h) values of 132, 338 and greater than 1583 µg a.s./L respectively based on the geometric mean measured test concentrations. The No Observed Effect Concentration (NOEC) after 96 hours based on growth rate was 93.5 µg a.s./L, and the Lowest Observed Effect Concentration (LOEC) was 205 µg a.s./L.

Assessment and conclusion by RMS:

CA 8.2.7 Effects on aquatic macrophytes

Data Point:	KCA 8.2.7/01
Report Author:	[redacted]
Report Year:	1998
Report Title:	ACLONIFEN - Toxicity to the duckweed, Lemna gibba
Report No:	R005693
Document No:	M-171423-01-1
Guideline(s) followed in study:	FIFRA Guideline Reference #: 122-2 and 123-2
Deviations from current test guideline:	Current Guideline: OECD 221, 2006 Study conducted over 14 days, instead of 7. Day 0 inoculum was 15 fronds (5 plants) not 9-12 fronds. Deviations not considered to affect study integrity.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

The effects of Aclonifen, on the growth and reproduction of the aquatic monocotyledonous plant, *Lemna gibba,* were investigated in an exposure to nominal concentrations of 0.00078, 0.0016, 0.0030, 0.0063, 0.013 and 0.025 mg a.s./L.

Fronds of *Lemna gibba* were exposed to Aclonifen for fourteen days in a semi-static system with test medium renewal on Days 3, 6 9 and 12. The effect was expressed in terms of percent inhibition in frond number and biomass relative to the blank control on day seven of the study.

The mean measured concentrations were 0.00070, 0.0012, 0.0020, 0.0049, 0.011 and 0.020 mg a.s./L. Since the determined test concentrations were below the nominal concentrations, all reported results are related to mean measured concentrations, calculated as the average over all measurements per test

concentration. Therefore, the mean measured test concentration was used to calculate the study endpoints.

The 14-Day EC_{50} value for frond density (with corresponding 95% confidence limits) was calculated to be 0.012 (0.010 to 0.014) mg a.s./L. The 14-Day No-Observed-Effect Concentration (NOEC) was determined to be 0.0012 mg a.s./L.

The 14-Day EC_{50} value for biomass (with corresponding 95% confidence limits) was calculated to be 0.0060 (0.0022 to 0.017) mg a.s./L. The 14-Day NOEC was empirically estimated to be 0.0012 mg a.s./L.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test material:**	Aclonifen technical
	Batch no.:	97013/03
	Purity:	994 g/kg
	Expiry:	April 2006 (retest)
2.	**Test organism:**	*Lemna gibba*
	Strain:	G3
	Source:	[redacted]
3.	**Treatment:**	Nominal test concentrations of 0.00078, 0.0016, 0.0030, 0.0063, 0.013 and 0.025 mg a.s./L
4.	**Test vessels:**	270 mL crystallising dishes with 100 mL test solution, covered with inverted glass petri dishes
	Test water:	Hoaglands medium
5.	**Environmental conditions:**	
	Temperature:	24 – 25°C
	pH:	4.9 – 6.6 (new and aged exposure solutions)
	Photoperiod:	Continuous illumination, range 3200 - 5400 lux

B. STUDY DESIGN AND METHODS

1. **In-life phase:** 7 to 24 July 1998

2. **Test organism assignment and treatment**

Colonies consisting of 3 fronds were transferred from the inoculum culture to each test vessel. Each test vessel contained 5 plants, a total of 15 fronds, with 3 replicates per treatment. An additional three replicates for the control, solvent control and 0.00078 mg a.s./L were set up for analysis. The test vessels were placed in a random order and were repositioned each day of measurement to minimize differences in light intensity. A semi-static test procedure was used and the test media were renewed on Days 3, 6, 9 and 12. Test vessels were re-positioned each working day.

3. **Dose preparation**

A 2.5 mg a.s./mL primary stock solution was prepared by dissolving 0.0629 g (0.0625 g as active ingredient) of Aclonifen to volume in a 25-mL volumetric flask with dimethyl formamide (DMF). The resulting stock solution was observed to be clear and amber in colour, with no visible undissolved test substance (e.g., precipitate). The nominal test solutions were prepared from dilutions of the 2.5 mg a.s./mL primary stock solution as follows:

Stock solution concn (mg a.s./L)	Volume of stock used (mL)	DMF dilution (mL)	Secondary stock (mg a.s./L)	Volume of secondary stock used (mL)	Hoagland's medium dilution (mL)	Nominal exposure concn (mg a.s./L)
2.5	2.5	25	0.25	0.100	1000	0.025
0.25	5.2	10	0.13	0.100	1000	0.013
0.25	2.5	10	0.063	0.100	1000	0.0063
0.25	1.2	10	0.030	0.100	1000	0.0030
0.25	0.64	10	0.016	0.100	1000	0.0016
0.25	0.31	10	0.0078	0.200	2000	0.00078

A solvent control solution was also prepared by diluting 0.200 mL of DMF to 2000 mL in Hoagland's medium. The concentration of DMF in the solvent control solution was equal to the concentration present in each test solution (i.e., 0.100 mL/L). Additional untreated Hoagland's medium was used to culture the control population. The renewal treatment and control solutions prepared on Days 3, 6 ,9 and 12 were prepared according to the procedures described above from the secondary stock solutions that had been refrigerated at 4 ± 1°C since preparation.

4. Measurements and observations

Frond counts were made on Days 0, 3, 6, 9, 12 and 14. Following Day 3, 6, 9 and 12 observations, the fronds were transferred to newly prepared solutions. At test termination (Day 14) frond densities for each treatment, control and solvent control replicate vessel were determined. Fronds were counted, then removed, blotted dry and transferred to pre-weighed aluminium pans. Fronds were dried for 3 days prior to dry weight determination.

Temperature was measured continuously with a minimum/maximum thermometer located in a flask of water adjacent to the test vessels within the environmental chamber. Temperature readings were recorded daily. Light intensity was measured at test initiation and daily during the exposure period. The pH of the all exposure solutions was measured at test initiation, at each 3-Day interval, and at test termination. Test solution remaining after filling the crystallizing dishes was used for initial pH measurements. Measurements recorded at the 3-Day intervals were measured in both old and new exposure solutions. At test termination, after frond counts were completed, the three replicate vessels of the treatment levels, the control and the solvent control were combined, a portion of each composite solution was transferred to a 100-mL beaker for pH measurement.

At the beginning and end of one renewal period (i.e., Day 0 and Day 3), one sample was removed from each treatment, control and solvent control solution to be analysed for Aclonifen concentration. Samples analysed on Day 0 were removed from the newly prepared test solutions subsequent to division into replicate test vessels. Samples analysed at the end of the renewal period (Day 3) were removed from the individual composite solutions after the replicate solutions of each test concentration, the control and solvent control had been respectively combined. Samples were analysed by GC using an electron capture detector (GC-ECD).

5. Statistics

Means and standard deviations of frond densities were calculated for each treatment level, the control and the solvent control at each observation interval. Means and standard deviations for biomass were also calculated for each treatment level, the control and the solvent control and were based on the dry plant weight determined at test termination. A t-Test ([redacted]1981) was used to compare the 14-Day control and solvent control growth rate and biomass data. If control and solvent control data

were not significantly different ($p < 0.05$), these data were pooled for use in statistical evaluation of the data for treatment effects. If a significant difference was determined, solvent control data was used for further statistical evaluation of treatment level effects. Additionally, percent inhibition of the 14-Day mean frond density and biomass of the treatment data were calculated relative to the pooled control data.

The EC_{25} and EC_{50} values and the 95% confidence limits, were determined by linear regression of response (percent reduction of frond density or biomass as compared with the pooled control) versus mean measured test concentration. The EC values were calculated using four linear regression curves based on (a) untransformed data, (b) untransformed response versus logarithm-transformed concentration, (c) probit-transformed response versus untransformed concentration, and (d) probit-transformed response versus logarithm-transformed concentration. The regression line which provided the best fit of the untransformed or transformed data was selected based on the highest coefficient of determination, r2. This regression equation was then applied to calculate each EC value and its 95% confidence limits, using the method of inverse prediction ([redacted] 1981). A computer program was used to assist in these computations.

The highest test concentration that caused no significant adverse effect on the 14-Day frond density or biomass when compared to the pooled control, the No-Observed-Effect Concentration (NOEC), was determined. The data were first checked for normality using [redacted] Test ([redacted] 1989) and for homogeneity of variance using [redacted] Test ([redacted] 1985). If the data sets passed the test for homogeneity and normality, the [redacted] Test ([redacted] 1971, 1972) was used to determine the NOEC. If the data did not pass the tests for homogeneity and normality, then [redacted] Test was used to determine the NOEC. All statistical determinations were made at the 95% level of certainty, except in the case of [redacted] Tests, where the 99% level of certainty was applied.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

At test initiation, the measured concentrations approximated the desired nominal concentrations. Measured concentrations decreased slightly over the 3-Day renewal period. Mean measured test concentrations ranged from 66 to 90% of the nominal concentrations and defined the treatment levels tested as 0.00070, 0.0012, 0.0020, 0.0049, 0.011 and 0.020 mg a.s./L. Therefore, the mean measured test concentration was used to calculate the study endpoints.

Table: Mean measured concentrations (mg/L) of Aclonifen in the exposure solutions

Nominal concn (mg a.s./L)	Measured concn (mg a.s./L)			% nominal
	Day 0	Day 3	Mean	
Control	<0.00015	<0.00016	n.a.	n.a.
Solvent control	<0.00015	<0.00016	n.a.	n.a.
0.00078	0.00082	0.00058	0.00070	90
0.0016	0.0016	0.00080	0.0012	74
0.0030	0.0022	0.0017	0.0020	66
0.00063	0.0056	0.0043	0.0049	78
0.013	0.012	0.010	0.011	84
0.025	0.022	0.019	0.020	82

n.a. = not applicable

The validated method is summarised in Document M-CA4 (CA 4.1.2/68).

B. BIOLOGICAL DATA

Frond numbers

Frond production (density) and observations of the fronds recorded during the 14-Day exposure to Aclonifen are presented below. At test termination, the control and solvent control solutions averaged 581 and 557 fronds per replicate, respectively. Statistical analysis (t-Test) determined no significant difference between control and solvent control data, therefore, control data were pooled for further statistical analysis (569 fronds/replicate). Frond production in the 0.00070, 0.0012, 0.0020, 0.0049, 0.011 and 0.020 mg a.s./L treatment levels averaged 577, 592, 530, 466, 285 and 81 fronds per replicate, respectively. Based on the results of [redacted] Tests, this data set passed the requirements for normality and homogeneity of variance, therefore, [redacted] Test was used to determine treatment-related effects.

A significant reduction in frond density in treatment levels 0.0020 mg a.s./L as compared to the pooled control was detected. Therefore, the NOEC for frond density was determined to be 0.0012 mg a.s./L. The 14-Day EC_{50} (corresponding 95% confidence limits) for frond density was calculated to be 0.012 mg a.s./L (0.010 to 0.014 mg a.s./L).

Mean frond numbers are presented in the following table:

Table: Mean frond numbers over 14-Day exposure to aclonifen technical

Mean measured concn (mg/L)	Day 3	Day 6	Day 9	Day 12	Day 14	% inhibition
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	
Control	33 (3.5)	104 (13)	225 (12)	419 (3.8)	581 (12)	-
Solvent control	35 (4.4)	103 (11)	220 (4.2)	421 (4.5)	557 (20)	-
Pooled control	-	-	-	-	569 (20)	-
0.00070	36 (2.1)	106 (7.2)	210 (4.0)	432 (15)	577 (15)	-1.5
0.0012	34 (4.6)	93 (16)	228 (4.2)	435 (12)	592 (8.1)	-4.1
0.0020	34 (12)	112 (4.0)	220 (5.6)	415 (5.5)	530 (9.1)*	6.8
0.0049	33 (1.7)	91 (5.7)	207 (6.7)	407 (4.0)	466 (10)*	18
0.011	31 (1.2)	75 (2.5)	158 (11)	214 (9.5)	285 (6.0)*	50
0.020	29 (3.8)	50 (5.8)	62 (8.0)	69 (9.5)	81 (11)*	86

Day 0 = 15 fronds (5 plants) per test flask, 3 replicates
SD = Standard deviation
Negative % inhibition indicates growth relative to pooled control
* Statistically significant compared to pooled control (Williams' Test)

Biomass

The 14-Day biomass (dry weight) for the control and solvent control averaged 0.1380 and 0.1685 g, respectively. Statistical analysis (t-Test) determined no significant difference between control and solvent control data, therefore, control data were pooled for further statistical analysis (0.1533 g). Frond biomass in the 0.00070, 0.0012, 0.0020, 0.0049, 0.011 and 0.020 mg a.s./L treatment levels averaged 0.1895, 0.2276, 0.1246, 0.0668, 0.0205 and 0.0158 g, respectively.

Based on the results of [redacted] and [redacted] Tests, this data set did not pass the requirements for homogeneity of variance, therefore, [redacted] Test was used to determine treatment-related effects. [redacted] Test did not detect a statistically significant reduction in frond biomass in any

treatment level as compared to the pooled control. Since the reduction at the 0.0020, 0.0049, 0.011 and 0.020 mg a.s./L concentrations represented a percent inhibition of 19, 56, 87 and 90%, respectively, as compared to the pooled control, the 14-Day NOEC for frond biomass was empirically estimated to be 0.0012 mg a.s./L. The 14-Day EC_{50} value (corresponding 95% confidence limits) for biomass was calculated to be 0.0060 mg a.s./L (0.0022 to 0.017 mg a.s./L).

Table: Biomass (frond dry weight) after 14-Day exposure to aclonifen technical

Mean measured concn (mg a.s./L)	Mean 14 -day biomass (g)	SD	% inhibition
Control	0.1380	0.023	-
Solvent control	0.1685	0.056	-
Pooled control	0.1533	0.042	-
0.00070	0.1895	0.055	-24
0.0012	0.2276	0.033	-48
0.0020	0.1246	0.028*	19
0.0049	0.0668	0.002*	56
0.011	0.0205	0.0002*	87
0.020	0.0148	0.0158*	90

SD = Standard deviation

Negative % inhibition indicates growth relative to pooled control

* [redacted] Test did not distinguish any treatment levels to be significantly reduced as compared to the pooled control data. Therefore, this treatment was empirically estimated to be reduced as compared to the pooled control

Shape of fronds

At test termination, slightly chlorotic to chlorotic, small fronds and fronds with less root formation were observed in the 0.011 and 0.020 mg a.s./L treatment solutions. Small fronds were observed in the 0.0049 mg a.s./L treatment level. Fronds exposed to the remaining treatment levels, the control and the solvent control were observed to be normal.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 221, 2006)	Achieved
Doubling time of frond number in the control <2.5 days (60 h), corresponding to approximately 7-fold increase in 7 days	<2.5 days	≤2.31 days

The US EPA guideline followed in this study did not specify validity criteria (US EPA 122-2 and 123-2, 1982).

Validity of the study was assessed against the current version of OECD Test Guideline 221: *Lemna* sp. Growth Inhibition Test (2006) which requires a doubling time of less than 2.5 days over a test period of 7 days. In this test, frond number observations were not made on Day 7 and hence the doubling time was determined using the frond number data from Days 6 and 9. The doubling time using the Day 0 – 6 data was 2.16 days and 2.31 days for the Day 0 – 9 period. The validity criterion was satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Parameter	Mean measured concn (mg/L)			
	Frond density	95% confidence limit	Biomass (dry weight)	95% confidence limit
EC_{50}	0.012	0.010 – 0.014	0.0060	0.0022 – 0.017
EC_{25}	0.0065	0.0047 – 0.0082	0.0038	0.0014 – 0.010
NOEC	0.0012	-	0.0012	-

III. CONCLUSION

A significant reduction in frond density (frond number) in treatment levels ≥0.0020 mg a.s./L as compared to the pooled control was detected. Therefore, the NOEC for frond density was determined to be 0.0012 mg a.s./L. The 14-Day EC_{50} (corresponding 95% confidence limits) for frond density was calculated to be 0.012 mg a.s./L (0.010 to 0.014 mg a.s./L).

Frond biomass (dry weight was reduced at the 0.0020, 0.0049, 0.011 and 0.020 mg a.s./L concentrations representing a percent inhibition of 19, 56, 87 and 90%, respectively, as compared to the pooled control. The 14-day NOEC for frond biomass was empirically estimated to be 0.0012 mg a.s./L. The 14-Day EC_{50} value (corresponding 95% confidence limits) for biomass was calculated to be 0.0060 mg a.s./L (0.0022 to 0.017 mg a.s./L).

[redacted] *(1998)*

Data Point:	KCA 8.2.7/02
Report Author:	[redacted]
Report Year:	2005
Report Title:	Non-GLP recalculation report - Lemna gibba G3 - growth inhibition test with Aclonifen (tech.)
Report No:	DOM 25010
Document No:	M-255537-01-1
Guideline(s) followed in study:	Originally reported under US-EPA FIFRA § 122-2 and 123-2 Recent recalculation is based on Revised Proposal for a New Guideline 221 Lemna sp. Growth Inhibition Test, Draft Document (October 2004)
Deviations from current test guideline:	Not applicable. Report is a re-evaluation of previously generated study data
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

The report for 'Aclonifen - Toxicity to the duckweed *Lemna gibba'* (study number 98-7-7411, [redacted] 1998) did not provide estimates of the EC_{50} values based on growth rate. Consequently, the data generated in this study have been re-analysed in an attempt to provide these values.

The mean measured concentrations were 0.00070, 0.0012, 0.0020, 0.0049, 0.011 and 0.020 mg a.s./L, equivalent to 0.70, 1.20, 2.00, 4.90, 11.0 and 20.0 µg a.s./L. All reported results are related to these mean measured concentrations.

All computations were carried out in ToxRat Professional version 2.09 (ToxRat Solutions GmbH, 2005).

Table: Frond numbers and dry weights, average growth rates and % inhibition

Mean measured concn (µg a.s./L)	Final frond no. (replicate means, day 14)	Final dry weight (replicate means, day 14) (mg)	% inhibition	
			Average growth rate for frond no.	Average growth rate for final dry weight
Control	581	138.0	-	-
Solvent control	587	168.5	-	-
Pooled control	569	153.3	-	-
0.70	577	189.8	-0.4	-5.3
1.20	592	227.6	-1.1	-10.4
2.00	530	124.6	1.9	5.0
4.90	466	66.8	5.5	20.2
11.0	285	20.5	19.0	50.0
20.0	81	15.8	53.7	57.2

Negative value means growth stimulation

The 0-14 Day E_rC_{50} figures are usable as substitutes for 0-7 Day E_rC_{50} values because of time-independency of such growth data. Endpoints based on OECD 221 (2004) are outlined below:

Table: Summary of endpoints

Endpoint (Day 0-14)	Effect on frond no. (µg a.s./L)	Effect on final dry weight (µg a.s./L)
E_rC_{10}	8.16 [6.24 – 9.59]	2.65 [1.14 – 4.04]
E_rC_{20}	10.9 [9.19 – 12.2]	4.65 [2.68 – 6.30]
E_rC_{50}	19.0 [17.5 – 21.2]	13.6 [10.6 – 18.8]
LOE_rC	4.90	4.90
NOE_rC	2.00	2.00

[95% confidence interval]

III. CONCLUSION

The original study was conducted according to US EPA test guidelines (FIFRA 1222-2 and 123-2, 1982), consequently statistical re-analysis of the study data was undertaken to determine E_rC_{50} values for frond number and dry weight of plants in accordance with OECD 221 requirements.

The E_rC_{50} determined for frond number was calculated to be 19.0 µg a.s./L (95% CI 17.5 – 21.2 µg a.s./L). The corresponding NOEC was determined to be 2.00 µg a.s./L.

The E_rC_{50} for plant dry weight was calculated to be 13.6 µg a.s./L (95% CI 10.6 – 18.8 µg a.s./L). The corresponding NOEC was determined to be 2.00 µg a.s./L.

[redacted] *(2005)*

Assessment and conclusion by applicant:

For the original study, it was not possible to determine frond number doubling time over 7 days, in accordance with current guideline due to frond counts not being performed on Day 7. The doubling time was therefore determined using the frond number data from Days 6 and 9 as a surrogate. The doubling time was 2.16 days and 2.31 days for the day 0 – 6 and day 0 – 9 period, respectively. It was considered that these data provided sufficient evidence that the validity criterion for doubling time over 7 days was satisfied and therefore this study can be considered to be valid.

In the original study, the 14-Day NOEC for Aclonifen technical was determined to be 0.0012 mg a.s./L, based on mean measured concentrations. The EC_{50} value for frond number density after 14 days was determined to be 0.012 mg a.s./L. The EC_{50} value for frond biomass after 14 days was determined to be 0.0060 mg a.s./L. Results were reported based on mean measured concentrations

The original study was conducted according to US EPA test guidelines (EFRA 1222-2 and 123-2, 1982), consequently statistical re-analysis of the study data was undertaken to determine E_rC_{50} (growth rate) values for frond number and dry weight of plants in accordance with OECD 221 requirements.

The E_rC_{50} determined for frond number was calculated to be 19.0 µg a.s./L (95% CI 17.5 – 21.2 µg a.s./L). The corresponding NOEC was determined to be 2.00 µg a.s./L.

The E_rC_{50} for plant dry weight was calculated to be 13.6 µg a.s./L (95% CI 10.6 – 18.8 µg a.s./L). The corresponding NOEC was determined to be 2.00 µg a.s./L.

EFSA's Outcome of the Pesticides Peer Review Meeting on general recurring issues in ecotoxicology (EFSA, 2015)[9] recommends that measured concentrations are calculated using the geometric mean. A summary of the arithmetic and geometric mean measured concentrations is provided in the following table:

Table: Measured concentrations from the exposure of *Lemna gibba* to Aclonifen

Nominal concentration (µg a.s./L)	Measured concentration (µg a.s./L)			
	Arithmetic mean	% Nominal	Geometric mean	% Nominal
0.78	0.70	90	0.69	88
1.6	1.20	75	1.13	71
3	2.00	67	1.93	64
6.3	4.90	78	4.91	78
13	11.00	85	10.95	84
25	20.00	80	20.45	82

Given that the geometric mean measured test concentrations were within 0.5 µg a.s./L of the arithmetic mean measured test concentrations it was considered that recalculation of the study

[9] EFSA (European Food Safety Authority), 2015. Technical report on the outcome of the pesticides peer review meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2015:EN-924. 62 pp.

endpoints based on the geometric mean measured concentrations was not necessary. Consequently, the E_rC_{50} for plant dry weight of 13.6 µg a.s./L is used for risk assessment.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.7/03
Report Author:	[redacted]
Report Year:	2018
Report Title:	1st amendment to the study report - Effect of aclonifen technical on the growth of Ceratophyllum demersum in the presence of sediment
Report No:	BAY-025/4-80/B
Document No:	M-408091-02-1
Guideline(s) followed in study:	draft guidance document of the SETAC AMRAP (Aquatic Macrophyte Risk Assessment for Pesticides) working group 2 (1)
Deviations from current test guideline:	Not applicable – no current applicable test guideline
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to determine the toxicity of the test item Aclonifen technical on the growth of the rootless *Ceratophyllum demersum* under static conditions over 14 days. The test was conducted following the draft guidance document of the SETAC AMRAP (Aquatic Macrophyte Risk Assessment for Pesticides) working group 2. The macrophytes were exposed to nominal concentrations of 1.0, 3.0, 9.0, 27.0 and 81.0 µg a.s./L.

The test item was dissolved in growth medium (Smart & Barko medium). For the growth tests three replicates for each test concentration and six replicates for controls (test medium only) were used with five plants per replicate. The plants were exposed to the test item in the aqueous phase of the test system in the presence of sediment.

Following parameters were measured: shoot length, fresh weight and dry weight. Plant length was recorded at test start and after 4, 7, 11 and 14 days. At test start, fresh weight was determined of all plants and dry weight was determined using 25 additional plants. At the end of the test all plants were harvested and their wet and dry weights were recorded. During the 14 days growth test the shoot length increased more than 50%.

Effective concentrations were calculated for relative increase and growth rate of the measured parameters.

At test start the measured concentrations were between 85.9 and 96.9% of nominal. During the 14-Day growth test the test item was not stable in the test media of all treatments leading to a lower concentration at the end of the test (27.4-32.5% of nominal). The test was evaluated using the mean measured concentrations of 0.56, 1.47, 4.35, 13.2 and 41.3 µg a.s./L.

In the static growth inhibition test with the rootless macrophyte *Ceratophyllum demersum* exposed to the test item (Aclonifen technical) over 14 days the EC_{50} values for increase in shoot length, fresh and dry weight were 11.5, 6.94 and 17.6 µg a.s./L, respectively. The EC_{50} values for growth rate of shoot length, fresh and dry weight were 22.6, 10.8 and greater than 41.3 µg a.s./L.

The NOEC value for all measured parameters was 0.56 µg/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen technical
   Batch no.: AE F068300-01-10
   Purity: 99.2% w/w
   Appearance: Yellow brown powder
   Date received: 30 September 2010
   Storage: Store at 25 ± 5 °C. Keep in tightly closed containers in a dry, cool, well ventilated place
   Expiry date: 27 February 2011

2. **Test Organism:** *Ceratophyllum demersum*, Haloragaceae, Dicotyledones
   Source: [redacted]
   Acclimatization: The plants are held submersed for at least 10 days prior to the test start in water and sediment of the same quality as used in the test

3. **Test water:** Smart & Barko medium (1985)

4. **Sediment:** Formulated sediment, based on the artificial soil used in OECD Guideline 219 was used:
   - 4-5% peat (dry weight, according to 2 ± 0.5% organic carbon) as close to pH 5.5 to 6.0 as possible; it is important to use peat in powder form, finely ground (particle size < 1 mm) and only air dried.
   - 20% (dry weight) kaolin clay (kaolinite content preferably above 30%).
   - 75-76% (dry weight) quartz sand (fine sand should predominate with more than 50 per cent of the particles between 50 and 200 µm).

- Deionised water was added to obtain moisture of the final mixture of about 30%.
- If needed, calcium carbonate of chemically pure quality ($CaCO_3$) was added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5.

For the batch of sediment containing nutrients instead of deionised water, an aqueous nutrient medium (with 300 mg/L sediment of both ammonium chloride and sodium phosphate in the appropriate amount of water) was added to obtain moisture of the final mixture of about 30%.

B. STUDY DESIGN AND METHODS

1. In-life phase: 03 – 17 December 2010

2. Exposure conditions

Test vessels: 2-L glass beakers (approximately 24 cm high and 11 cm diameter). Small plant pots (approx. 9 cm diameter and 8 cm high and around 330 mL volume, plastic, commercially available) were used as containers for the sediment. The sediment surface coverage was about 70% of the test vessel surface, the minimum overlaying water depth was 12 cm

Experimental design: 5 test concentrations (1.0, 3.0, 9.0, 27.0 and 81.0 µg a.s./L) plus a control

Replicates: 6 replicates for the control and 3 replicates per treatment group. Each replicate contained 5 plants.

Temperature: 18 – 19.5 °C

pH: 7.9 – 9.91

Aeration: None

Photoperiod: 16 hours light : 8 hours dark

Light intensity: 7472 - 7807 lux

3. Administration of the test item

A stock solution was prepared in acetone with a concentration of 16.33 mg test item/1 mL (stock solution 1). 50 µL of stock solution 1 were dispensed into 1 L graduated glass flask and the acetone was left to evaporate. The flask was filled up to 1 L to achieve a concentration of 816.5 µg test item/L equivalent to 810 µg a.s./L (stock solution 2). The stock solution 2 was stirred at room temperature for 24 hours and then the 1 litre was added to 9 L growth medium to obtain the highest test concentration of 81.0 µg a.s./L from which serial dilutions in growth medium were made to prepare the remainder of the test concentrations. The test media were stirred vigorously for 30 minutes at room temperature. The pH-values of the test media were not adjusted.

4. Preparation of test vessels

For the test, the sediment was filled into standard planting pots. Since the standard planting pots have wholes at the bottom, first a filter paper was put on the bottom of the vessel. Afterwards an

approximately 1 cm layer of the standard sediment was added. On top of this a 4 cm layer of sediment was added which had been supplemented with a nitrate and phosphate fertilizer (nutrient supplemented sediment). This was covered again with 1 cm of standard sediment without fertilizer (in order to provide sufficient nutrients to the plants via the sediment without enriching the water with further nutrients). A fine/very thin layer (approximately 2 mm) of coarse quartz sand was added on the top of the sediment in order to reduce suspension of sediment into the water.

5. Test organism assignment and treatment

After the pre-culture, the plants were removed from the pre-culture and cleaned of surplus water. Plants that were apparently not healthy were discarded at this stage. The plants were weighed and the length of the plants was recorded. The shoots were not potted into sediment since *Ceratophyllum* is a rootless macrophyte.

25 plants of the pre-culture were additionally harvested at this stage (only using the most homogenous individuals) and plant dry weight were determined to obtain the respective data for Day 0.

The pots with sediment were placed into the glass beakers. Afterwards, the test vessels were carefully filled up with 2 L of the respective amount of test medium containing the relevant amounts of the test item and the plants were placed into the medium above the sediment.

6. Measurements and observations

The correct application of the test item was confirmed by analytical measurements of aclonifen concentrations in the test media at test initiation and after the 14-Day exposure period.

During the 14-Day exposure period, shoot lengths were recorded at test start and on Days 7, 11, and 14.

Total plant fresh weight was determined after carefully blotting off remaining test medium of the plant. Dry weight was determined subsequently after weighing the fresh plants. The five plants per replicate were combined and the plants were dried in aluminium weighing boats at 105 °C for 24 hours.

Light intensities at the water surface were measured on Day 0 and 14. Oxygen contents and pH values of the test medium were recorded on Days 0, 9 and 14. Test media temperature was recorded using a data logger four times a day (Thermo Data Logger EL-USB-TC).

At the end of the growth test, all plants were harvested. Any symptoms (such as chlorosis or necrosis) or other observations were recorded. Total plant wet weight (after carefully blotting off remaining test medium) and subsequently, total plant dry weight was determined.

7. Statistics/Data evaluation

Statistical calculations were made on the results obtained for individual vessels, not for individual plants. Since *Ceratophyllum* plants were not planted into the sediment individual plants could not marked. Therefore, the total length and total weight per test vessel was used for evaluation.

Data evaluation was done for shoot length increase, fresh and dry weight as well as for growth rate of the respective parameters at test termination. The relative values (in percent of test start) of the parameters were used for the evaluation.

EC_{50} values were calculated by probit analysis modified for continuous data using the computer program ToxRat Professional (ToxRat Solutions, Alsdorf, Germany). No Observed Effect Concentrations (NOEC) were calculated, using ANOVA, followed by [redacted] test.

The evaluation was performed using mean measured concentrations. The replicates of each concentration plot were used for fitting concentration-response curves of the measured parameters.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Freshly prepared test solutions at test start and pooled samples of the test media at the end of the growth test were analysed for the test item using HPLC UV-VIS (LOQ 0.25 µg a.s./L) after sample concentration. The measured concentrations in the test media were between 85.9 and 96.9% of nominal at test start and between 27.2 and 32.5% at test end. Due to deviations from the nominal concentrations > 20% at test end, the analysis of the results is based on the geometric mean measured test concentrations.

Table: Measured test concentrations of Aclonifen during the exposure to *Ceratophyllum demersum*

Nominal concentration (µg a.s./L)	Measured concentration					
	Day 0		Day 14		Geometric mean measured	
	µg a.s./L	% nominal	µg a.s./L	% nominal	µg a.s./L	% nominal
Control	<LOQ	-	<LOQ	-	<LOQ	-
1.0	0.970	96.9	0.325	32.5	0.56	56.2
3.0	2.60	86.7	0.830	27.7	1.47	49.0
9.0	7.73	85.9	2.44	27.2	4.35	48.3
27.0	23.6	87.3	7.39	27.4	13.2	48.9
81.0	74.2	91.6	23.1	28.5	41.3	51.0

LOQ: Limit of Quantitation = 0.25 µg a.s./L

The validated method is summarised in Document M-CA4 (CA 4.1.2/09).

B. BIOLOGICAL DATA

There was a concentration dependent effect on the increase in shoot length and the fresh and dry weight of *Ceratophyllum demersum* over the 14-Day exposure period.

Table: Percentage inhibition for plant shoot length, fresh weight and dry weight during the exposure of *Ceratophyllum demersum* to Aclonifen

Geometric mean measured concentration (µg a.s./L)	% Inhibition after 14 days					
	Shoot length		Fresh weight		Dry weight	
	Increase	Growth rate	Increase	Growth rate	Increase	Growth rate
0.56	3.9	2.51	-2.00	-1.20	6.30	4.30
1.47	33.4	27.5	27.4	22.0	40.8	30.8
4.35	55.4	48.6	44.4	37.3	51.8	40.3
13.2	43.0	36.3	65.4	58.4	38.5	28.1
41.3	61.6	55.0	71.6	65.1	55.8	44.1

- negative values indicate increase in the observed parameter compared to control

For the assessment of effects on fresh weight the increase in weight of each individual plant was used. For dry weight, the increase was calculated using the dry weight of a separate set of plants regarded as

representative for the test plants. Due to the possible dry weight variability of plants with the same length, a higher variability of data can be expected. Therefore, the data of fresh weight are considered more reliable.

The test results were statistically analysed to determine the 14 day EC_{50} values together with 95% confidence intervals for parameters showing concentration dependent effects as well as NOEC values.

C. VALIDITY CRITERIA

Specific criteria for macrophyte growth tests using *Ceratophyllum* have not been set yet. At the time of performing the study, validity criteria proposed by the AMRAP working group were used to assess the validity of the study.

Validity criterion	Required (AMRAP working group)	Achieved
Increase in biomass (shoot length) in controls	≥50%	69.23%
Continuous growth throughout the test duration	Required	Yes
Temperature	20 ± 2°C	18 – 19.5°C

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint		Geometric mean measured concentration (µg a.s./L)			
		EC_{10}	EC_{20}	EC_{50}	NOEC
Shoot length	Relative increase	n.d.	0.51 [0.02 – 1.50]	11.5 [5.26 – 43.1]	0.56
	Growth rate	n.d.	0.84 [0.05 – 2.29]	22.6 [9.94 – 148]	0.56
Fresh weight	Relative increase	0.35 [0.10 – 0.73]	0.98 [0.41 – 1.67]	6.94 [4.69 – 10.7]	0.56
	Growth rate	0.46 [0.14 – 0.92]	1.35 [0.61 – 2.24]	10.8 [7.46 – 16.9]	0.56
Dry weight	Relative increase	n.d	0.21 [n.d.]	17.6 [n.d.]	0.56
	Growth rate	n.d.	0.70 [n.d.]	>41.3 [n.d.]	0.56

[95% confidence limits]
n.d.: not determined due to mathematical reasons or inappropriate data

III. CONCLUSION

In the static growth inhibition test with the rootless macrophyte *Ceratophyllum demersum* exposed to the test item (Aclonifen technical) over 14 days the EC_{50} values for increase in shoot length, fresh and dry weight were 11.5, 6.94 and 17.6 µg a.s./L, respectively. The EC_{50} values for growth rate of shoot length, fresh and dry weight were 22.6, 10.8 and greater than 41.3 µg a.s./L.

The NOEC value for all measured parameters was 0.56 µg/L.

[redacted] *(2018)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The most sensitive endpoint in the 14-Day exposure of Aclonifen Technical to the rootless aquatic macrophyte *Ceratophyllum demersum* was fresh weight growth rate. The statistical NOEC and E_rC_{50} for this endpoint were 0.56 and 10.8 µg a.s./L, respectively.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.7/04
Report Author:	[redacted]
Report Year:	2019
Report Title:	1st amendment to study report - Macrophytes, growth inhibition test - Effect of aclonifen technical on the growth of Elodea canadensis in the presence of sediment, static conditions
Report No:	BAY-025/4-80/C
Document No:	M-408117-02-1
Guideline(s) followed in study:	SETAC AMRAP (Aquatic Macrophyte Risk Assessment for Pesticides) working group 2 (n)
Deviations from current test guideline:	Not applicable – no current applicable test guideline
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to determine the toxicity of the test item Aclonifen technical on the growth of *Elodea canadensis* under static conditions over 14 days. The test was conducted following the draft guidance document of the SETAC AMRAP (Aquatic Macrophyte Risk Assessment for Pesticides) working group 2. The macrophytes were exposed to nominal concentrations of 5.0, 15.8, 50, 158, 500 and 1000 µg a.s./L.

The test item was dissolved in growth medium (Smart & Barko medium). For the growth tests three replicates for each test concentration and six replicates for controls (test medium only) were used with five plants per replicate. The plants were exposed to the test item in the aqueous phase of the test system in the presence of sediment.

Following parameters were measured: shoot length, fresh weight and dry weight. Plant length was recorded at test start and after 4, 7, 11 and 14 days. Prior to the onset of the test (-3 days), the fresh

weight and dry weight of the test plants were determined. At the end of the test all plants were harvested and their fresh and dry weights were recorded. During the 14 days growth test the shoot length increased more than 100%.

Effective concentrations were calculated for relative increase and growth rate of the measured parameters.

At test start the measured concentrations were between 35.6 and 41.3% of nominal. During the 14-Day growth test the test item was not stable in the test media of all treatments leading to lower concentrations at the end of the test (15.0 – 26.3% of nominal). Therefore, the test was evaluated using the geometric mean of the test concentrations measured in the different treatments, i.e. 4.84, 11.8, 38.3, 154 and 306 µg a.s./L.

There was no concentration-dependent significant effect on shoot length and the inhibition was below 50% up to the highest test concentration. Therefore, the respective EC_{50} values for length increase and growth rate were higher than the highest test concentration (> 306 µg a.s./L). Accordingly, the NOEC for shoot length increase and growth rate were ≥306 µg a.s./L.

In contrast, fresh weights and dry weights differed significantly from the controls in the two or four highest treatment groups, respectively, and the respective NOEC values were determined to be 38.3 µg a.s./L (fresh weight increase and growth rate) and 4.81 µg a.s./L (dry weight increase and growth rate). No meaningful concentration-dependencies were observed and effect concentrations could therefore not be calculated.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen technical
   Batch no.: AE F068300-01-10
   Purity: 99.2% w/w
   Appearance: Yellow brown powder
   Date received: 30 September 2010
   Storage: Store at 25 ± 5 °C. Keep in tightly closed containers in a dry, cool, well ventilated place
   Expiry date: 27 February 2011

2. **Test Organism:** *Elodea canadensis* (Canadian waterweed), Hydrocharitaceae, Alismatales, Monocotyledonous
   Source: [redacted]
   Acclimatization: The plants were held immersed for at least 5 days prior to the test start in water and sediment of the same quality as used in the test.

3. **Test water:** Smart & Barko medium (1985)

4. Sediment: Formulated sediment, based on the artificial soil used in OECD Guideline 219 was used:

- 4-5% peat (dry weight, according to 2 ± 0.5% organic carbon) as close to pH 5.5 to 6.0 as possible it is important to use peat in powder form, finely ground (particle size < 1 mm) and only air dried.
- 20% (dry weight) kaolin clay (kaolinite content preferably above 30%).
- 75-76% (dry weight) quartz sand (fine sand should predominate with more than 50 per cent of the particles between 50 and 200 µm).
- Deionised water was added to obtain moisture of the final mixture of about 30%.
- If needed, calcium carbonate of chemically pure quality ($CaCO_3$) was added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5.

For the batch of sediment containing nutrients, instead of deionised water, an aqueous nutrient medium (with 300 mg/L sediment of both ammonium chloride and sodium phosphate in the appropriate amount of water) was added to obtain moisture of the final mixture of about 30%.

B. STUDY DESIGN AND METHODS

1. In-life phase: 10 – 24 March 2011

2. Exposure conditions

Test vessels:	2-L glass beakers (approximately 24 cm high and 11 cm diameter). Small plant pots (approx. 9 cm diameter and 8 cm high and around 350 mL volume, plastic, commercially available) were used as containers for potting the plants into the sediment. The sediment surface coverage was about 70% of the test vessel surface; the minimum overlaying water depth was 12 cm
Experimental design:	5 test concentrations (5.0, 15.8, 50, 158, 500 and 1000 µg a.s./L) plus a control
Replicates:	6 replicates for the control and 3 replicates per treatment group. Each replicate contained 5 plants.
Temperature:	18 – 19.5 °C
pH:	7.83 – 9.50
Aeration:	None
Photoperiod:	16 hours light : 8 hours dark
Light intensity:	7897 - 8201 lux

3. Administration of the test item

The nominal concentrations up to 500 µg a.s./L were spaced by a factor of 3.16, due to the flat concentration-effect curve observed in the pre-test. The highest nominal test concentration, however, was 1000 µg a.s./L (and thus just a factor of 2 higher than the second highest) because of the limited solubility of the test item. The test media were prepared using different stock solutions. First, 100 mg of the test item were dissolved in 1 mL acetone (stock 1). Based on this stock solution, further stock solutions were prepared: stock 2: 100 µL stock 1 + 1 litre test medium; stock 3: 50 µL stock 1 + 1 litre of test medium; stock 4: 15.8 µL stock 1 + 1 litre of test medium. These stock solutions were stirred for 24 hours prior to further dilution to achieve the respective test concentration. The pH-values of the test media were not adjusted.

4. Preparation of test vessels

For the test, the sediment was filled into standard planting pots. Since the standard planting pots have wholes at the bottom, first a filter paper was put on the bottom of the vessel. Afterwards an approximately 1 cm layer of the standard sediment was added. On top of this a 4 cm layer of sediment was added which had been supplemented with a nitrate and phosphate fertilizer (nutrient supplemented sediment). This was covered again with 1 cm of standard sediment without fertilizer (in order to provide sufficient nutrients to the plants via the sediment without enriching the water with further nutrients). A fine/very thin layer (approximately 2 mm) of coarse quartz sand was added on the top of the sediment in order to reduce suspension of sediment into the water.

5. Test organism assignment and treatment

After the pre-culture, the plants were removed from the pre-culture and cleaned of sediment and surplus water; plants that were apparently not healthy were discarded at this stage. The plants were weighed (to reduce variability, the weight of the shoot tips used in the study should not differ by more than 30% from the mean). Shoots were then potted into the sediment and shoot length above sediment was measured. If required, the length of the plants above sediment was adjusted to 3 cm.

25 plants of the pre-culture were additionally harvested at this stage (only using the most homogenous individuals) and plant dry weight were determined to obtain the respective data for Day 0.

For the growth inhibition test, five plants were used per pot and test vessel and three replicates were prepared for each of the five treatments testing one of the five

The pots with sediment and plants were placed into the glass beakers. Afterwards, the test vessels were carefully filled up with 2 L of the respective test media.

6. Measurements and observations

The correct application of the test item was confirmed by analytical measurements of aclonifen concentrations in the test media at test initiation and after the 14-Day exposure period.

During the 14-Day exposure period, shoot lengths were recorded at test start and on Days 4, 7, 11, and 14.

Total plant fresh weight was determined after absorbing remaining test medium attached to the plants by means of tissue paper. Dry weight was determined subsequently after weighing the fresh plants. The five plants per replicate were combined and the plants were dried in aluminium weighing boats at 105 °C for 24 hours.

Light intensities at the water surface were measured on Day 0 and 14. Oxygen contents and pH values of the test medium were recorded on Days 0, 7 and 14. Test media temperature was recorded using a data logger four times a day (Thermo Data Logger EL-USB-TC).

At the end of the growth test, all plants were harvested. Any symptoms (such as chlorosis or necrosis, roots) or other observations were recorded. Total plant wet weight (after careful absorption of attached test medium) was determined followed by the estimation of total plant dry weight.

7. Statistics/Data evaluation

Statistical calculations were made on the results obtained for individual vessels, not for individual plants. Data evaluation was done for shoot length increase, fresh and dry weight as well as for growth rate of the respective parameters at test termination. The relative values (in percent of test start) of the parameters were used for the evaluation.

Growth rates were calculated for the fresh and dry weight and increase in shoot length (including side shoots) per vessel.

EC_{50} values were calculated by probit analysis modified for continuous data using the computer program ToxRat Professional (ToxRat Solutions, Alsdorf, Germany). No Observed Effect Concentrations (NOEC) were calculated, using ANOVA, followed by [redacted] test, [redacted] or [redacted] t-test.

The evaluation was performed using mean measured concentrations. The replicates of each concentration plot were used for fitting concentration-response curves of the measured parameters, if meaningful concentration-effect relationships were observed.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Freshly prepared test solutions at test start and pooled samples of the test media at the end of the growth test were analysed for the test item using HPLC-UV-VIS (LOQ 0.25 µg a.s./L) after sample concentration. The measured concentrations in the test media were between 35.6 and 41.3% of nominal at test start and between 15.0 and 26.3% at test end. Due to deviations from the nominal concentrations >20%, the analysis of the results is based on the geometric mean measured test concentrations.

Table: Measured test concentrations of Aclonifen during the exposure to *Elodea Canadensis*

Nominal concentration (µg a.s./L)	Measured concentration					
	Day 0		Day 14		Geometric mean measured	
	µg a.s./L	% nominal	µg a.s./L	% nominal	µg a.s./L	% nominal
Control	<LOQ	-	<LOQ	-	<LOQ	-
15.85	6.00	37.9	3.85	24.3	4.81	30.3
50.07	18.46	36.9	7.56	15.1	11.81	23.6
158.22	61.76	39.0	23.77	15.0	38.31	24.2
500.0	206.32	41.3	114.33	22.9	153.59	30.7
1000	356.26	35.6	263.25	26.3	306.25	30.6

LOQ: Limit of Quantitation = 0.25 µg a.s./L

The validated method is summarised in Document M-CA4 (CA 4.1.2/70).

B. BIOLOGICAL DATA

A significant effect on the increase of shoot length of *Elodea canadensis* after 14 days was observed only at the second highest test concentration (154 µg a.s./L) but not at the highest. No meaningful concentration-dependency was noticeable. Effect concentrations were not calculated because there was no concentration-dependency of effects ($p(F) > 0.05$; i.e. slope of the relationship was not significant different from zero).

Data on effect on fresh and dry weight of *Elodea canadensis* based on weight increase and weight growth rate were quite variable. Obviously the test item inhibited growth. Significant effects on fresh weight were detected at the two highest test concentrations (154 and 306 µg a.s./L) while dry weight was already significantly affected at 11.8 µg a.s./L. Nevertheless, no meaningful concentration-dependencies were noticeable and strongest effects were observed in the second highest test concentration (154 µg a.s./L).

Table: Percentage inhibition for plant shoot length, fresh weight and dry weight during the exposure of *Elodea canadensis* to Aclonifen

Geometric mean measured concentration (µg a.s./L)	% Inhibition after 14 days					
	Shoot length		Fresh weight		Dry weight	
	Increase	Growth rate	Increase	Growth rate	Increase	Growth rate
4.81	-5.1	-3.9	-13.2	-8.1	-16.2	-11.6
11.8	40.6	27.1	41.6	30.5	82.7	79.7
38.3	14.8	11.8	21.0	16.0	46.5	35.1
154	47.5	32.8	54.3	39.7	108.8	117.7
306	52.2	20.7	49.9	38.5	68.5	67.8

- negative values indicate increase in the observed parameter compared to control

For the assessment of effects on fresh and dry weights initial values at test start were determined on a different subset of plants which were regarded as representative. This procedure which is normally used only for dry weight determination at test start was necessary also for initial fresh weight determination because *Elodea* is very sensitive to blotting dry. Plants with the same length usually show variability in weight resulting in variability of the data for the assessment of weight increase during the test period.

The test results were statistically analysed to determine the 14-Day EC_{50} values together with 95% confidence intervals for parameters showing concentration dependent effects as well as NOEC values.

Effects of the test item could be observed but no meaningful concentration-dependency was noticeable. This led to unsatisfactory fitting of the respective concentration- effect curves ($p(F) > 0.05$; i.e. slope of the relationship was not significant different from zero) and the resulting effect concentrations are regarded as 'not valid'.

C. VALIDITY CRITERIA

Specific criteria for macrophyte growth tests using *Elodea* have not been set yet. At the time of performing the study, validity criteria proposed by the AMRAP working group were used to assess the validity of the study.

Validity criterion	Required (AMRAP working group)	Achieved

Increase in biomass (shoot length) in controls	>50%	253%
Continuous growth throughout the test duration	Required	Yes
Temperature	20 ± 2°C	18 – 19.5 °C

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint		Geometric mean measured concentration (µg a.s./L)	
		EC_{50}	NOEC
Shoot length	Relative increase	>306	≥306
	Growth rate	>306	≥306
Fresh weight	Relative increase	n.d.	38.3
	Growth rate	n.d.	38.3
Dry weight	Relative increase	n.d.	4.81
	Growth rate	n.d.	4.81

n.d.: not determined due to mathematical reasons or inappropriate data

III. CONCLUSION

In a static growth inhibition test with the rooted macrophyte *Elodea Canadensis* exposed to Aclonifen over 14 days the EC_{50} value for increase in shoot length was above the highest test concentration. No EC_{50} values were calculated for fresh weight and dry weight because there were no meaningful concentration-dependencies of the observed effects on these two parameters.

The NOEC for increase in shoot length based on the geometric mean measured test concentrations was greater than 306 µg a.s./L. For increase in fresh and dry weight NOEC values of 38.3 and 4.81 µg a.s./L respectively were determined.

[redacted] *(2019)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

In a static growth inhibition test with the rooted macrophyte *Elodea Canadensis* exposed to Aclonifen over 14 days the EC_{50} value for increase in shoot length was above the highest test concentration. No EC_{50} values were calculated for fresh weight and dry weight because there were no meaningful concentration-dependencies of the observed effects on these two parameters.

The NOEC for increase in shoot length based on the geometric mean measured test concentrations was greater than 306 µg a.s./L. For increase in fresh and dry weight NOEC values of 38.3 and 4.81 µg a.s./L respectively were determined.

Due to the lack of concentration-dependency of effects ($p(F) > 0.05$; i.e. slope of the relationship was not significant different from zero), EC_{10} and EC_{20} values could not be calculated, however as the E_rC_{50} value is the endpoint required for aquatic risk assessment this was not considered to affect the interpretation of the study results.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.7/05
Report Author:	[redacted]
Report Year:	2011
Report Title:	Effect of aclonifen technical on the growth of Cabomba caroliniana in the presence of sediment, static conditions
Report No:	BAY-025/4-80/O
Document No:	M-408124-01-1
Guideline(s) followed in study:	SETAC AMRAP (Aquatic Macrophyte Risk Assessment for Pesticides) working group 2 (1)
Deviations from current test guideline:	Not applicable, no current applicable test guideline
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to determine the toxicity of the test item Aclonifen technical on the growth of *Cabomba caroliniana* under static conditions over 21 days. The test was conducted following the draft guidance document of the SETAC AMRAP (Aquatic Macrophyte Risk Assessment for Pesticides) working group 2. The macrophytes were exposed to nominal concentrations of 2.00, 6.33, 20.0, 63.3 and 200 µg a.s./L.

The test item was dissolved in growth medium (Smart & Barko medium). For the growth tests three replicates for each test concentration and six replicates for controls (test medium only) were used with five plants per replicate. The plants were exposed to the test item in the aqueous phase of the test system in the presence of sediment.

Following parameters were measured: shoot length, fresh weight and dry weight. Plant length was recorded at test start and after 4, 7, 11, 14, 18 and 21 days. Prior to the onset of the test (-3 days), the fresh weight of the test plants were determined. Dry weight was determined using a set of representative plants. At the end of the test all plants were harvested and their fresh and dry weights were recorded. During the 21 days growth test the biomass of controls and the lowest treatment increased by 86 ± 36% and 107 ± 41% based on dry weight.

Effective concentrations were calculated for relative increase and growth rate of the measured parameters.

At test start the measured concentrations were between 67.4 and 74.4% of nominal. During the 21 day growth test the test item was not stable in the test media of all treatments leading to a lower concentration

at the end of the test (11.5-21.2% of nominal). Therefore, the test was evaluated using the geometric mean of the test concentrations measured in the different treatments with 0.62, 1.83, 5.56, 22.2, and 79.5 µg a.s./L.

There was no inhibition above 50% of the observed parameters (shoot length, fresh weight and dry weight) and EC-values could not be calculated. EC_{50} values were therefore considered to be greater than 79.5 µg a.s./L, the highest concentration tested.

The observed parameters were not significantly different from the controls up to the highest test concentration and the NOEC was determined to be ≥79.5 µg a.s./L.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen technical
   Batch no.: AE-L068300-01-10
   Purity: 99.2% w/w
   Appearance: Yellow brown powder
   Date received: 30 September 2010
   Storage: Store at 25 ± 5 °C. Keep in tightly closed containers in a dry, cool, well ventilated place.
   Expiry date: 27 February 2011

2. **Test Organism:** *Cabomba caroliniana,* Hydrocharitaceae, Alismatales, Monocotyledonous
   Source: [redacted]
   Acclimatization: The plants were held immersed for at least 10 days prior to the test start in water and sediment of the same quality as used in the test.

3. **Test water:** Smart & Barko medium (1985)

4. **Sediment:** Formulated sediment, based on the artificial soil used in OECD Guideline 219 was used:
   - 4-5% peat (dry weight, according to 2 ± 0.5% organic carbon) as close to pH 5.5 to 6.0 as possible; it is important to use peat in powder form, finely ground (particle size < 1 mm) and only air dried.
   - 20% (dry weight) kaolin clay (kaolinite content preferably above 30%).
   - 75-76% (dry weight) quartz sand (fine sand should predominate with more than 50 per cent of the particles between 50 and 200 µm).
   - Deionised water was added to obtain moisture of the final mixture of about 30%.

- If needed, calcium carbonate of chemically pure quality ($CaCO_3$) was added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5.

For the batch of sediment containing nutrients, instead of deionised water, an aqueous nutrient medium (with 300 mg/L sediment of both ammonium chloride and sodium phosphate in the appropriate amount of water) was added to obtain moisture of the final mixture of about 30%.

B. STUDY DESIGN AND METHODS

1. In-life phase: 07 – 28 January 2011

2. Exposure conditions

Test vessels:	2-L glass beakers (approximately 24 cm high and 11 cm diameter). Small plant pots (approx. 9 cm diameter and 8 cm high, and around 350 mL volume, plastic, commercially available) were used as containers for potting the plants into the sediment. The sediment surface coverage was about 70% of the test vessel surface; the minimum overlaying water depth was 12 cm
Experimental design:	5 test concentrations (2.00, 6.33, 20.0, 63.3 and 200 µg a.s./L) plus a control
Replicates:	6 replicates for the control and 3 replicates per treatment group. Each replicate contained 5 plants.
Temperature:	19.0 – 20.0 °C
pH:	7.74 – 8.72
Aeration:	None
Photoperiod:	16 hours light : 8 hours dark
Light intensity:	7614 – 7805 lux

3. Administration of the test item

The nominal concentrations were spaced by a factor of 3.16, due to the flat concentration-effect curve observed in the pre-test. A stock solution was prepared in acetone with a concentration of 40.32 mg test item/1 mL (stock solution 1). 50 µL of stock solution 1 were dispensed into 1 L graduated glass flask and the acetone was left to evaporate. The flask was filled up to 1 L to achieve a concentration of 2.016 mg test item/L equivalent to 2.00 mg a.s./L (stock solution 2). The stock solution 2 was stirred at room temperature for 24 hours and then the 1 litre was added to 9 L growth medium to obtain the highest test concentration of 200.0 µg a.s./L from which serial dilutions were made using growth medium to give the remaining test concentrations. The test media were stirred vigorously for 30 minutes at room temperature. The pH values of the test media were not adjusted.

4. Preparation of test vessels

For the test, the sediment was filled into standard planting pots. Since the standard planting pots have wholes at the bottom, first a filter paper was put on the bottom of the vessel. Afterwards, an approximately 1 cm layer of the standard sediment was added. On top of this a 4 cm layer of sediment was added which had been supplemented with a nitrate and phosphate fertilizer (nutrient supplemented sediment). This was covered again with 1 cm of standard sediment without fertilizer (in order to provide sufficient nutrients to the plants via the sediment without enriching the water with further nutrients). A fine/very thin layer (approximately 2 mm) of coarse quartz sand was added on the top of the sediment in order to reduce suspension of sediment into the water.

5. Test organism assignment and treatment

After the pre-culture, the plants were removed from the pre-culture and cleaned of sediment and surplus water; plants that were apparently not healthy were discarded at this stage. The plants were weighed. Shoots were then potted into the sediment and shoot length above sediment was measured. If required, the length of the plants above sediment was adjusted to 3 cm.

25 plants of the pre-culture were additionally harvested at this stage (only using the most homogenous individuals) and plant dry weight were determined to obtain the respective data for Day 0.

For the growth inhibition test, five plants were used per pot and test vessel and three replicates were prepared for each of the five treatments testing one of the five

The pots with sediment and plants were placed into the glass beakers. Afterwards, the test vessels were carefully filled up with 2 L of the respective test media.

6. Measurements and observations

The correct application of the test item was confirmed by analytical measurements of aclonifen concentrations in the test media at test initiation and after the 21-Day exposure period.

During the 21-Day exposure period, shoot lengths were recorded at test start and on Days 4, 7, 11, 14, 18, and 21.

Total plant fresh weight was determined after absorbing remaining test medium attached to the plants by means of tissue paper. Dry weight was determined subsequently after weighing the fresh plants. The five plants per replicate were combined and the plants were dried in aluminium weighing boats at 105 °C for 24 hours.

Light intensities at the water surface were measured on Day 0 and 14. Oxygen contents and pH values of the test medium were recorded on Days 0, 7, 14 and 21. Test media temperature was recorded using a data logger four times a day (Thermo Data Logger EL-USB-TC).

At the end of the growth test, all plants were harvested. Any symptoms (such as chlorosis or necrosis, roots) or other observations were recorded. Total plant wet weight (after careful absorption of attached test medium) was determined followed by the estimation of total plant dry weight.

7. Statistics/Data evaluation

Statistical calculations were made on the results obtained for individual vessels, not for individual plants. Data evaluation was done for shoot length increase, fresh and dry weight as well as for growth rate of the respective parameters at test termination. The relative values (in percent of test start) of the parameters were used for the evaluation.

EC_{50} values were calculated by probit analysis modified for continuous data using the computer program ToxRat Professional (ToxRat Solutions, Alsdorf, Germany). No Observed Effect Concentrations (NOEC) were calculated, using ANOVA, followed by [redacted] test, [redacted] or [redacted]-test.

The evaluation was performed using mean measured concentrations. The replicates of each concentration plot were used for fitting concentration-response curves of the measured parameters, if meaningful concentration-effect relationships were observed.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Freshly prepared test solutions at test start and pooled samples of the test media at the end of the growth test were analysed for the test item using HPLC UV-VIS (LOQ 0.25 µg a.s./L). The measured concentrations in the test media were between 67.4 and 74.4 % of nominal at test start and between 11.5 and 21.2% at test end. Due to deviations from the nominal concentrations >20%, the analysis of the results is based on the geometric mean measured test concentrations.

Table: Measured test concentrations of Aclonifen during the exposure to *Cabomba caroliniana*

Nominal concentration (µg a.s./L)	Measured concentration					
	Day 0		Day 21		Geometric mean measured	
	µg a.s./L	% nominal	µg a.s./L	% nominal	µg a.s./L	% nominal
Control	<LOD	-	<LOQ	-	<LOQ	-
2.0	1.45	72.7	0.27	13.3	0.62	31.1
6.33	4.32	68.3	0.78	12.3	1.83	29.0
20.0	13.48	67.4	2.29	11.5	5.56	27.8
63.3	45.00	71.1	10.96	17.3	22.21	35.1
200.0	148.82	74.4	42.46	21.2	79.50	39.7

LOQ: Limit of Quantitation = 0.25 µg a.s./L
LOD: Limit of Detection = 0.025 µg a.s./L (lowest calibration point and 100 mL work up volume)

The validated method is summarised in Document M-CA4 (CA 4.1.2/71).

B. BIOLOGICAL DATA

There was no concentration dependent effect on the increase in shoot length or the fresh and dry weight of *Cabomba caroliniana*.

Table: Percentage inhibition for plant shoot length, fresh weight and dry weight during the exposure of *Cabomba caroliniana* to Aclonifen

Geometric mean measured concentration (µg a.s./L)	% Inhibition after 21 days					
	Shoot length		Fresh weight		Dry weight	
	Increase	Growth rate	Increase	Growth rate	Increase	Growth rate
0.62	-10.8	-21.3	-79.2	-76.5	-25.2	-18.4
1.83	-1.6	-3.6	137.0	141.0	53.2	44.3
5.56	16.1	30.9	-92	-88.4	52.3	45.8
22.2	-29.8	-36.9	-23.4	-23.5	40.7	32.5
79.50	-32.0	14.5	39.2	41.4	23.8	18.6

- negative values indicate increase in the observed parameter compared to control

For the assessment of effects on fresh weight the increase in weight of each individual plant was used. For dry weight, the increase was calculated using the dry weight of a separate set of plants regarded as representative for the test plants. Plants with the same length usually show high dry weight variability. Therefore, the data of fresh weight are considered more reliable for assessing inhibitory effects.

The test results were statistically analysed to determine the 21-Day EC_{50} values together with 95% confidence intervals for parameters showing concentration dependent effects as well as NOEC values.

With shoot length, fresh weight and dry weight there was no clear concentration-effect relationship and no significant inhibition at the highest test concentration compared to the controls. Therefore, no EC-values were calculated.

C. VALIDITY CRITERIA

Specific criteria for macrophyte growth tests using *Cabomba caroliniana* have not been set yet. At the time of performing the study, validity criteria proposed by the AMRAP working group were used to assess the validity of the study.

Validity criterion	Required (AMRAP working group)	Achieved
Increase in biomass in controls	>50%	85.65%
Continuous growth throughout the test duration	Required	Yes
Temperature	20 ± 2°C	19.0 - 20 °C

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint		Geometric mean measured concentration (µg a.s./L)	
		EC_{50}	NOEC
Shoot length	Relative increase	>79.5	≥79.5
	Growth rate	>79.5	≥79.5
Fresh weight	Relative increase	>79.5	≥79.5
	Growth rate	>79.5	≥79.5
Dry weight	Relative increase	>79.5	≥79.5
	Growth rate	>79.5	≥79.5

III. CONCLUSION

In a static growth inhibition test with the rooted macrophyte *Cabomba caroliniana* exposed to Aclonifen over 21 days the EC_{50} values for increase in shoot length, fresh weight and dry weight were above the highest geometric mean measured test concentration of 79.5 µg a.s./L.

The observed parameters (shoot length, fresh weight and dry weight) were not significantly different from the controls up to the highest test concentration and the hence the NOEC was determined to be ≥79.5 µg a.s./L.

[redacted] *(2011)*

Assessment and conclusion by applicant:
All validity criteria were satisfied and therefore this study can be considered to be valid. In a static growth inhibition test with the rooted macrophyte *Cabomba caroliniana* exposed to Aclonifen over 21 days the EC_{50} values for increase in shoot length, fresh weight and dry weight were above the highest geometric mean measured test concentration of 79.5 µg a.s./L. The observed parameters (shoot length, fresh weight and dry weight) were not significantly different from the controls up to the highest test concentration and the hence the NOEC was determined to be ≥79.5 µg a.s./L. Due to the lack of concentration-dependency of effects ($p(F) > 0.05$; i.e. slope of the relationship was not significant different from zero), EC_{10} and EC_{20} values could not be calculated, however as the E_rC_{50} value is the endpoint required for aquatic risk assessment this was not considered to affect the interpretation of the study results.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.7/06
Report Author:	[redacted]
Report Year:	2011
Report Title:	Effect of aclonifen technical on the growth of Limnophila heterophylla in the presence of sediment, static conditions
Report No:	BAY-025/4-80/N
Document No:	M-408152-01-1
Guideline(s) followed in study:	SETAC AMRAP (Aquatic Macrophyte Risk Assessment for Pesticides) working group 2 (1)
Deviations from current test guideline:	Not applicable – no current applicable test guideline
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to determine the toxicity of the test item Aclonifen technical on the growth of *Limnophila heterophylla* under static conditions over 14 days. The test was conducted following the

draft guidance document of the SETAC AMRAP (Aquatic Macrophyte Risk Assessment for Pesticides) working group 2. The macrophytes were exposed to nominal concentrations of 5, 15.8, 50.0, 158.2 and 500 µg a.s./L.

The test item was dissolved in growth medium (Smart & Barko medium). For the growth tests three replicates for each test concentration and six replicates for controls (test medium only) were used with five plants per replicate. The plants were exposed to the test item in the aqueous phase of the test system in the presence of sediment.

Following parameters were measured: shoot length, fresh weight and dry weight. Plant length was recorded at test start and after 4, 7, 11 and 14 days. Prior to the onset of the test (-3 days), the fresh weight of the test plants were determined. Dry weight was determined using a set of representative plants. At the end of the test all plants were harvested and their fresh and dry weights were recorded. During the 14 days growth test the biomass based on dry weight increased more than 100%.

Effective concentrations were calculated for relative increase and growth rate of the measured parameters.

At test start the measured concentrations were between 23.0 and 39.0% of nominal. During the 14-Day growth test the test item was not stable in the test media of all treatments leading to a lower concentration at the end of the test (10.4–13.8% of nominal). Therefore, the test was evaluated using the geometric mean of the test concentrations measured in the different treatments with 1.01, 3.20, 9.68, 28.4 and 89.0 µg a.s./L.

The EC_{50} for increase in shoot length was 79.8 µg a.s./L and for growth rate of shoot length 122 µg a.s./L. For fresh weight and dry weight no meaningful concentration/response was found and since inhibition at the highest test concentration was below 50%, EC_{50} was estimated to be greater than 89.0 µg a.s./L.

NOEC values of 28.4 µg a.s./L could be calculated for increase of shoot length and both parameters of fresh weight. For dry weight and growth rate of plant shoots no meaningful concentration-effect relationship was found and there were no significant inhibitory effects at the highest test concentration (NOEC ≥89.0 µg a.s./L).

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen technical
	Batch no.:	AE F068300-01-10
	Purity:	99.2% w/w
	Appearance:	Yellow brown powder
	Date received:	30 September 2010
	Storage:	Store at 25 ± 5 °C. Keep in tightly closed containers in a dry, cool, well ventilated place
	Expiry date:	27 February 2011
2.	**Test Organism:**	*Limnophila heterophylla (Ambulia heterophylla)*, Scrophulariaceae, Scrophulariales, Dicotyledonous

	Source:	[redacted]
	Acclimatization:	The plants were held immersed for at least 5 days prior to the test start in water and sediment of the same quality as used in the test.
3.	**Test water:**	Smart & Barko medium (1985)
4.	**Sediment:**	Formulated sediment, based on the artificial soil used in OECD Guideline 219 was used: • 4-5% peat (dry weight, according to 2 ± 0.5% organic carbon) as close to pH 5.5 to 6.0 as possible; it is important to use peat in powder form, finely ground (particle size < 1 mm) and only air dried. • 20% (dry weight) kaolin clay (kaolinite content preferably above 30%). • 75-76% (dry weight) quartz sand (fine sand should predominate with more than 50 per cent of the particles between 50 and 200 µm). • Deionised water was added to obtain moisture of the final mixture of about 30%. • If needed, calcium carbonate of chemically pure quality ($CaCO_3$) was added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5. For the batch of sediment containing nutrients, instead of deionised water, an aqueous nutrient medium (with 300 mg/L sediment of both ammonium chloride and sodium phosphate in the appropriate amount of water) was added to obtain moisture of the final mixture of about 30%.

B. STUDY DESIGN AND METHODS

1. In-life phase: 13 – 27 January 2011

2. Exposure conditions

Test vessels:	2-L glass beakers (approximately 24 cm high and 11 cm diameter). Small plant pots (approx. 9 cm diameter and 8 cm high and around 350 mL volume, plastic, commercially available) were used as containers for potting the plants into the sediment. The sediment surface coverage was about 70% of the test vessel surface; the minimum overlaying water depth was 12 cm
Experimental design:	5 test concentrations (5, 15.8, 50.0, 158.2 and 500 µg a.s./L) plus a control

Replicates:	6 replicates for the control and 3 replicates per treatment group. Each replicate contained 5 plants.
Temperature:	18.0 – 21.0 °C
pH:	7.7 – 8.94
Aeration:	None
Photoperiod:	16 hours light : 8 hours dark
Light intensity:	7435 - 7584 lux

3. Administration of the test item

The nominal concentrations were spaced by a factor of 3.16, due to the flat concentration-effect curve observed in the pre-test. A stock solution was prepared in acetone with a concentration of 101 mg test item/500 µL (stock solution 1). 50 µL of stock solution 1 were dispensed into 1 L graduated glass flask and the acetone was left to evaporate. The flask was filled up to 1 L to achieve a concentration of 10.01 mg test item/L equivalent to 10.0 mg a.s./L (stock solution 2). The stock solution 2 was stirred at room temperature for 24 hours and then 0.5 litre was added to 9.5 L growth medium to obtain the highest test concentration of 500 µg a.s./L from which serial dilutions were made in growth medium to give the remainder of the test concentrations. The test media were stirred vigorously for 30 minutes at room temperature. The pH values of the test media were not adjusted.

4. Preparation of test vessels

For the test, the sediment was filled into standard planting pots. Since the standard planting pots have wholes at the bottom, first a filter paper was put on the bottom of the vessel. Afterwards an approximately 1 cm layer of the standard sediment was added. On top of this a 4 cm layer of sediment was added which had been supplemented with a nitrate and phosphate fertilizer (nutrient supplemented sediment). This was covered again with 1 cm of standard sediment without fertilizer (in order to provide sufficient nutrients to the plants via the sediment without enriching the water with further nutrients). A fine/very thin layer (approximately 2 mm) of coarse quartz sand was added on the top of the sediment in order to reduce suspension of sediment into the water.

5. Test organism assignment and treatment

After the pre-culture, the plants were removed from the pre-culture and cleaned of sediment and surplus water; plants that were apparently not healthy were discarded at this stage. The plants were weighed. Shoots were then potted into the sediment and shoot length above sediment was measured. If required, the length of the plants above sediment was adjusted to 3 cm.

25 plants of the pre-culture were additionally harvested at this stage (only using the most homogenous individuals) and plant dry weight were determined to obtain the respective data for Day 0.

For the growth inhibition test, five plants were used per pot and test vessel and three replicates were prepared for each of the five treatments.

The pots with sediment and plants were placed into the glass beakers. Afterwards, the test vessels were carefully filled up with 2 L of the respective test media.

6. Measurements and observations

The correct application of the test item was confirmed by analytical measurements of aclonifen concentrations in the test media at test initiation and after the 14-Day exposure period.

During the 14-Day exposure period, shoot lengths were recorded at test start and on Days 4, 7, 11, and 14.

Total plant fresh weight was determined after absorbing remaining test medium attached to the plants by means of tissue paper. Dry weight was determined subsequently after weighing the fresh plants. The five plants per replicate were combined and the plants were dried in aluminium weighing boats at 105 °C for 24 hours.

Light intensities at the water surface were measured on Days 0, 7 and 14. Oxygen contents and pH values of the test medium were recorded on Days 0, 7, and 14. Test media temperature was recorded using a data logger four times a day (Thermo Data Logger EL-USB-TC).

At the end of the growth test, all plants were harvested. Any symptoms (such as chlorosis or necrosis, roots) or other observations were recorded. Total plant wet weight (after careful absorption of attached test medium) was determined followed by the estimation of total plant dry weight.

7. Statistics/Data evaluation

Statistical calculations were made on the results obtained for individual vessels, not for individual plants. Data evaluation was done for shoot length increase, fresh and dry weight as well as for growth rate of the respective parameters at test termination. The relative values (in percent of test start) of the parameters were used for the evaluation.

EC_x values were calculated by probit analysis modified for continuous data using the computer program ToxRat Professional (ToxRat Solutions, Alsdorf, Germany). No Observed Effect Concentrations (NOEC) were calculated, using ANOVA, followed by [redacted] test, [redacted] or [redacted] t-test.

The evaluation was performed using mean measured concentrations. The replicates of each concentration plot were used for fitting concentration-response curves of the measured parameters, if meaningful concentration-effect relationships were observed.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Freshly prepared test solutions at test start and pooled samples of the test media at the end of the growth test were analysed for the test item using HPLC UV-VIS (LOQ 0.25 µg a.s./L). The measured concentrations in the test media were between 23.0 and 39.0% of nominal at test start and between 10.4 and 13.8% at test end. Due to deviations from the nominal concentrations >20%, the analysis of the results is based on the geometric mean measured test concentrations.

Table: Measured test concentrations of Aclonifen during the exposure to *Limnophila heterophylla*

Nominal concentration (µg a.s./L)	Measured concentration					
	Day 0		Day 14		Geometric mean measured	
	µg a.s./L	% nominal	µg a.s./L	% nominal	µg a.s./L	% nominal
Control	<LOD	-	<LOD	-	<LOD	-
5.0	1.95	39.0	0.52	10.4	1.01	20.1

15.8	5.81	36.8	1.76	11.1	3.20	20.2
50.0	15.93	31.9	5.88	11.8	9.68	19.4
158.2	40.31	25.5	19.98	12.6	28.38	17.9
500.0	115.0	23.0	68.86	13.8	88.99	17.8

LOD: Limit of Detection = 0.025 µg a.s./L (lowest calibration point and 100 mL work up volume)

The validated method is summarised in Document M-CA4 (CA 4.1.2/72).

B. BIOLOGICAL DATA

There was a concentration dependent effect on shoot length of *Limnophila heterophylla*. With fresh weight and dry weight there was no meaningful concentration effect.

Table: Percentage inhibition for plant shoot length, fresh weight and dry weight during the exposure of *Limnophila heterophylla* to Aclonifen

Geometric mean measured concentration (µg a.s./L)	% Inhibition after 14 days					
	Shoot length		Fresh weight		Dry weight	
	Increase	Growth rate	Increase	Growth rate	Increase	Growth rate
1.01	-17.6	-9	-22.5	-16.8	-40.5	-19.6
3.20	7.3	6.4	8.8	7.6	-4.7	-1
9.68	31.3	25.9	64.8	62.5	19.7	10.7
28.4	39.6	40.4	74.3	80.9	45.9	34.4
89.0	49.3	46.9	32.3	29.1	13.3	7.8

- negative values indicate increase in the observed parameter compared to control

The test results were statistically analysed to determine the 14-Day EC_{50} values together with 95% confidence intervals for parameters showing concentration dependent effects as well as NOEC values.

With fresh weight and dry weight there was no meaningful concentration effect curves and no EC-values could be calculated.

C. VALIDITY CRITERIA

Specific criteria for macrophyte growth tests using *Limnophila heterophylla* have not been set yet. At the time of performing the study, validity criteria proposed by the AMRAP working group were used to assess the validity of the study.

Validity criterion	Required (AMRAP working group)	Achieved
Increase in biomass in controls (dry weight)	>50%	286%
Continuous growth throughout the test duration	Required	Yes
Temperature	20 ± 2ºC	18.0 – 21.0 °C

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint		Geometric mean measured concentration (µg a.s./L)			
		EC_{10}	EC_{20}	EC_{50}	NOEC
Shoot length	Relative increase	0.51 [0.00 – 3.29]	2.88 [0.00 – 10.5]	79.8 [23.0 - >89.0]	28.4
	Growth rate	0.64 [n.d. – 3.93]	3.87 [0.00 – 13.6]	122* [32.4 - >89.0]	28.4
Fresh weight	Relative increase	n.d.	n.d.	>89.0	28.4
	Growth rate	n.d.	n.d.	>89.0	28.4
Dry weight	Relative increase	n.d.	n.d.	>89.0	≥89.0
	Growth rate	n.d.	n.d.	>89.0	≥89.0

[95% confidence limits]
n.d.: not determined due to mathematical reasons or inappropriate data
*extrapolated, highest test concentration was 89.0 µg/L showing inhibition of 45.3% ($p < 0.05$)

III. CONCLUSION

In a static growth inhibition test with the rooted macrophyte *Limnophila heterophylla* exposed to Aclonifen over 14 days the EC_{50} value for increase in shoot length was 79.8 µg a.s./L and for growth rate of shoot length 122 µg a.s./L. For fresh weight and dry weight no meaningful concentration-response was found and since inhibition at the highest test concentration was below 50%, EC_{50} was estimated to be greater than 89.0 µg a.s./L.

NOEC values of 28.4 µg a.s./L could be calculated for increase of shoot length and both parameters of fresh weight. For dry weight and growth rate of plant shoots no meaningful concentration-effect relationship was found and there were no significant inhibitory effects at the highest test concentration (NOEC ≥89.0 µg a.s./L).

[redacted] *(2011)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

In a static growth inhibition test with the rooted macrophyte *Limnophila heterophylla* exposed to Aclonifen over 14 days the EC_{50} value for increase in shoot length was 79.8 µg a.s./L, and for growth rate of shoot length the EC_{50} was 122 µg a.s./L. For fresh weight and dry weight no meaningful concentration-response was found and since inhibition at the highest test concentration was below 50%, EC_{50} values were estimated to be greater than 89.0 µg a.s./L.

NOEC values of 28.4 µg a.s./L were determined for increase of shoot length and both parameters of fresh weight. For dry weight and growth rate of plant shoots no meaningful concentration-effect relationship was found and there were no significant inhibitory effects at the highest test concentration (NOEC ≥89.0 µg a.s./L).

EC_{10} and EC_{20} values were determined for shoot length growth parameters, however due to the lack of concentration-dependency of effects, EC_{10} and EC_{20} values could not be calculated for fresh or dry weight growth parameters. However as the E_rC_{50} value is the endpoint required for aquatic risk assessment this was not considered to affect the interpretation of the study results.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.7/07
Report Author:	[redacted]
Report Year:	2011
Report Title:	Effect of aclonifen technical on the growth of Heteranthera zosterifolia in the presence of sediment, static conditions
Report No:	BAY-025/4-80/E
Document No:	M-408168-01-1
Guideline(s) followed in study:	SETAC AMRAP (Aquatic Macrophyte Risk Assessment for Pesticides) working group 2 (1)
Deviations from current test guideline:	Not applicable, no current applicable test guideline
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to determine the toxicity of the test item Aclonifen technical on the growth of *Heteranthera zosterifolia* under static conditions over 14 days. The test was conducted following the draft guidance document of the SETAC AMRAP (Aquatic Macrophyte Risk Assessment for Pesticides) working group 2. The macrophytes were exposed to nominal concentrations of 10.0, 31.6, 100.0, 316.0 and 1000 µg a.s./L.

The test item was dissolved in growth medium (Smart & Barko medium). For the growth tests three replicates for each test concentration and six replicates for controls (test medium only) were used with five plants per replicate. The plants were exposed to the test item in the aqueous phase of the test system in the presence of sediment.

Following parameters were measured: shoot length, fresh weight and dry weight. Plant length was recorded at test start and after 3, 7, 11 and 14 days. Prior to the onset of the test (-3 days), the fresh weight of the test plants were determined. Dry weight was determined using a set of representative plants. At the end of the test all plants were harvested and their fresh and dry weights were recorded. During the 14 day growth test the biomass based on dry weight increased by about 100% (119 ± 29%) in the controls.

Effective concentrations were calculated for relative increase and growth rate of the measured parameters.

At test start the measured concentrations were between 19.2 and 65.5% of nominal. During the 14-Day growth test the test item was not stable in the test media of all treatments leading to a lower concentration

at the end of the test (0.6 -13.4% of nominal). Therefore, the test was evaluated using the geometric mean of the test concentrations measured in the different treatments with 0.57, 3.46, 21.1, 93.8, 98.5 µg a.s./L.

In a static growth inhibition test with the rooted macrophyte *Heteranthera zosterifolia* exposed to Aclonifen over 14 days no meaningful concentration-responses and no inhibition higher than 50% were found for effects on length increase, fresh and dry weight and thus no EC_{50} values could be calculated. The respective EC_{50} values are assumed to be above the highest test concentration of 98.5 µg a.s./L.

Significant effects were observed on shoot length, growth rate of shoot length, and growth rate of fresh weight at the highest test concentration, the respective NOEC values were therefore determined to be 93.8 µg a.s./L. In contrast, increase of fresh weight, and increase and growth rate of dry weight were not significantly different from the controls and the respective NOECs were determined to be ≥98.5 µg a.s./L (geometric mean mean measured concentration).

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen technical
   Batch no.: AE F068300-01-10
   Purity: 99.2% w/w
   Appearance: Yellow brown powder
   Date received: 30 September 2010
   Storage: Store at 25 ± 5 °C. Keep in tightly closed containers in a dry, cool, well ventilated place
   Expiry date: 27 February 2011

2. **Test Organism:** *Heteranthera zosterifolia*, (stargrass), Pontederiaceae, Commelinales, Monocotyledonous
   Source: [redacted]
   Acclimatization: The plants were held immersed for at least 10 days prior to the test start in water and sediment of the same quality as used in the test.

3. **Test water:** Smart & Barko medium (1985)

4. **Sediment:** Formulated sediment, based on the artificial soil used in OECD Guideline 219 was used:
   - 4-5% peat (dry weight, according to 2 ± 0.5% organic carbon) as close to pH 5.5 to 6.0 as possible; it is important to use peat in powder form, finely ground (particle size < 1 mm) and only air dried.
   - 20% (dry weight) kaolin clay (kaolinite content preferably above 30%).

- 75-76% (dry weight) quartz sand (fine sand should predominate with more than 50 per cent of the particles between 50 and 200 µm).
- Deionised water was added to obtain moisture of the final mixture of about 30%.
- If needed, calcium carbonate of chemically pure quality ($CaCO_3$) was added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5.

For the batch of sediment containing nutrients, instead of deionised water, an aqueous nutrient medium (with 300 mg/L sediment of both ammonium chloride and sodium phosphate in the appropriate amount of water) was added to obtain moisture of the final mixture of about 30%.

B. STUDY DESIGN AND METHODS

1. In-life phase: 14 – 28 January 2011

2. Exposure conditions

Test vessels:	2-L glass beakers (approximately 24 cm high and 11 cm diameter). Small plant pots (approx. 9 cm diameter and 8 cm high and around 350 mL volume, plastic, commercially available) were used as containers for potting the plants into the sediment. The sediment surface coverage was about 70% of the test vessel surface; the minimum overlaying water depth was 12 cm
Experimental design:	5 test concentrations (10.0, 31.6, 100.0, 316.0 and 1000 µg a.s./L) plus a control
Replicates:	6 replicates for the control and 3 replicates per treatment group. Each replicate contained 5 plants.
Temperature:	18.0 - 21.0 °C
pH:	7.94 - 8.85
Aeration:	None
Photoperiod:	16 hours light : 8 hours dark
Light intensity:	7483 - 7597 lux

3. Administration of the test item

The nominal concentrations were spaced by a factor of 3.16, due to the flat concentration-effect curve observed in the pre-test. Three stock solutions were prepared in acetone by diluting 101 mg (stock 1a), 31.85 mg (stock 1b) and 10.08 mg (stock 1c) in 500 µL acetone each. 50 µL of these three stock solutions (stock 1a, 1b, 1c) were given into three 1 L graduated glass flasks and the acetone was left to evaporate. The flasks were filled up to 1 L to achieve the stock solutions 2, 3 and 4, respectively. The stock solutions were stirred at room temperature for 24 hours. Thereafter one litre of stock 2 was added to 9 litres growth medium to achieve the highest test concentration (conc. 5, nominal 1000 µg/L). Accordingly, 1 litre of

stock 3 and stock 4 were added to 9 litres of growth medium each to achieve the test concentrations 4 (316 µg/L) and 3 (100 µg/L). The two lowest test concentrations were prepared by diluting the test solutions 3 and 2. The test media were stirred vigorously for 30 minutes at room temperature. The pH-values of the test media were not adjusted.

4. Preparation of test vessels

For the test, the sediment was filled into standard planting pots. Since the standard planting pots have wholes at the bottom, first a filter paper was put on the bottom of the vessel. Afterwards an approximately 1 cm layer of the standard sediment was added. On top of this a 4 cm layer of sediment was added which had been supplemented with a nitrate and phosphate fertilizer (nutrient supplemented sediment). This was covered again with 1 cm of standard sediment without fertilizer (in order to provide sufficient nutrients to the plants via the sediment without enriching the water with further nutrients). A fine/very thin layer (approximately 2 mm) of coarse quartz sand was added on the top of the sediment in order to reduce suspension of sediment into the water.

5. Test organism assignment and treatment

After the pre-culture, the plants were removed from the pre-culture and cleaned of sediment and surplus water; plants that were apparently not healthy were discarded at this stage. The plants were weighed. Shoots were then potted into the sediment and shoot length above sediment was measured. If required, the length of the plants above sediment was adjusted to 3 cm.

25 plants of the pre-culture were additionally harvested at this stage (only using the most homogenous individuals) and plant dry weight were determined to obtain the respective data for Day 0.

For the growth inhibition test, five plants were used per pot and test vessel and three replicates were prepared for each of the five treatments.

The pots with sediment and plants were placed into the glass beakers. Afterwards, the test vessels were carefully filled up with 2 L of the respective test media.

6. Measurements and observations

The correct application of the test item was confirmed by analytical measurements of aclonifen concentrations in the test media at test initiation and after the 14-Day exposure period.

During the 14-Day exposure period, shoot lengths were recorded at test start and on Days 3, 7, 11, and 14.

Total plant fresh weight was determined after absorbing remaining test medium attached to the plants by means of tissue paper. Dry weight was determined subsequently after weighing the fresh plants. The five plants per replicate were combined and the plants were dried in aluminium weighing boats at 105 °C for 24 hours.

Light intensities at the water surface were measured on Days 0, 7 and 14. Oxygen contents and pH values of the test medium were recorded on Days 0, 7, and 14. Test media temperature was recorded using a data logger four times a day (Thermo Data Logger EL-USB-TC).

At the end of the growth test, all plants were harvested. Any symptoms (such as chlorosis or necrosis, roots) or other observations were recorded. Total plant wet weight (after careful absorption of attached test medium) was determined followed by the estimation of total plant dry weight.

7. Statistics/Data evaluation

Statistical calculations were made on the results obtained for individual vessels, not for individual plants. Data evaluation was done for shoot length increase, fresh and dry weight as well as for growth rate of the respective parameters at test termination. The relative values (in percent of test start) of the parameters were used for the evaluation.

EC_x values were calculated by probit analysis modified for continuous data using the computer program ToxRat Professional (ToxRat Solutions, Alsdorf, Germany). No Observed Effect Concentrations (NOEC) were calculated, using ANOVA, followed by [redacted] test, [redacted] or [redacted] t-test.

The evaluation was performed using mean measured concentrations. The replicates of each concentration plot were used for fitting concentration-response curves of the measured parameters, if meaningful concentration-effect relationships were observed.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Freshly prepared test solutions at test start and pooled samples of the test media at the end of the growth test were analysed for the test item using HPLC UV-VIS (LOQ 0.25 µg a.s./L). The measured concentrations in the test media were between 19.2 and 65.5% of nominal at test start and between 0.6 and 13.4% at test end. Due to deviations from the nominal concentrations >20%, the analysis of the results is based on the geometric mean measured test concentrations.

Table: Measured test concentrations of Aclonifen during the exposure to *Heteranthera zosterifolia*

Nominal concentration (µg a.s./L)	Measured concentration					
	Day 0		Day 14		Geometric mean measured	
	µg a.s./L	% nominal	µg a.s./L	% nominal	µg a.s./L	% nominal
Control	<LOQ		<LOD		<LOQ	-
10.0	5.87	58.7	0.06	0.6	0.57	5.7
31.60	18.59	58.8	0.65	2.0	3.46	11.0
100.0	58.44	58.4	7.63	7.6	21.11	21.1
316.0	207.12	65.5	42.49	13.4	93.82	29.7
1000.0	192.07	19.2	50.51	5.1	98.49	9.8

LOQ: Limit of Quantification = 0.25 µg/L
LOD: Limit of Detection = 0.025 µg a.s./L (lowest calibration point and 100 mL work up volume)

The validated method is summarised in Document M-CA4 (CA 4.1.2/73).

B. BIOLOGICAL DATA

There was no concentration dependent effect on the increase in shoot length, fresh weight or dry weight of *Heteranthera zosterifolia*.

Table: Percentage inhibition for plant shoot length, fresh weight and dry weight during the exposure of *Heteranthera zosterifolia* to Aclonifen

	% Inhibition after 14 days

Geometric mean measured concentration (µg a.s./L)	Shoot length		Fresh weight		Dry weight	
	Increase	Growth rate	Increase	Growth rate	Increase	Growth rate
0.57	31.1	25.9	4.8	3.3	23.6	-15.7
3.46	31.2	25.8	7.0	5.9	24.27	17.92
21.10	27.0	22.4	-7.7	-4.7	-11.62	-7.32
93.80	14.3	11.0	24.8	20.4	-3.32	-2.91
98.50	46.0	40.6	30.5	24.4	-20.39	-13.58

- negative values indicate increase in the observed parameter compared to control

The test results were statistically analysed to determine the 14-Day EC_{50} values together with 95% confidence intervals for parameters showing concentration dependent effects as well as NOEC values.

With shoot length, fresh weight and dry weight there was no clear concentration-effect relationship. Therefore, no EC-values were calculated.

C. VALIDITY CRITERIA

Specific criteria for macrophyte growth tests using *Heteranthera zosterifolia* have not been set yet. At the time of performing the study, validity criteria proposed by the AMRAP working group were used to assess the validity of the study.

Validity criterion	Required (AMRAP working group)	Achieved
Increase in biomass in controls (dry weight)	>50%	119%
Continuous growth throughout the test duration	Required	Yes
Temperature	20 ± 2°C	18.0 – 21.0 °C

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint		Geometric mean measured concentration (µg a.s./L)	
		EC_{50}	NOEC
Shoot length	Relative increase	>98.5	93.8
	Growth rate	>98.5	93.8
Fresh weight	Relative increase	>98.5	≥98.5
	Growth rate	>98.5	93.8
Dry weight	Relative increase	>98.5	≥98.5
	Growth rate	>98.5	≥98.5

III. CONCLUSION

In a static growth inhibition test with the rooted macrophyte *Heteranthera zosterifolia* exposed to Aclonifen over 14 days no meaningful concentration-responses and no inhibition higher than 50% were found for effects on length increase, fresh and dry weight and thus no EC_{50} values could be calculated. The respective EC_{50} values are assumed to be above the highest test concentration of 98.5 µg a.s./L.

Significant effects were observed on shoot length, growth rate of shoot length, and growth rate of fresh weight at the highest test concentration, the respective NOEC values were therefore determined to be 93.8 µg a.s./L. In contrast, increase of fresh weight, and increase and growth rate of dry weight were not significantly different from the controls and the respective NOECs were determined to be ≥98.5 µg a.s./L (geometric mean mean measured concentration).

[redacted] *(2011)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

In a static growth inhibition test with the rooted macrophyte *Heteranthera zosterifolia* exposed to Aclonifen over 14 days no meaningful concentration-responses and no inhibition higher than 50% were found for effects on length increase, fresh and dry weight and thus no EC_{50} values could be calculated. The respective EC_{50} values are therefore assumed to be greater than the highest geometric mean measured test concentration of 98.5 µg a.s./L.

Significant effects were observed on shoot length, growth rate of shoot length, and growth rate of fresh weight at the highest test concentration, the respective NOEC values were therefore determined to be 93.8 µg a.s./L. In contrast, increase of fresh weight, and increase and growth rate of dry weight were not significantly different from the controls and the respective NOECs were determined to be ≥98.5 µg a.s./L (geometric mean mean measured concentration).

Due to the lack of concentration-dependent effects, EC_{10} and EC_{20} values could not be calculated, however as the E_rC_{50} value is the endpoint required for aquatic risk assessment this was not considered to affect the interpretation of the study results.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.7/08
Report Author:	[redacted]
Report Year:	2011
Report Title:	Effect of aclonifen technical on the growth of Egeria densa in the presence of sediment, static conditions
Report No:	BAY-025/4-80/L
Document No:	M-408189-01-1
Guideline(s) followed in study:	SETAC AMRAP (Aquatic Macrophyte Risk Assessment for Pesticides) working group 2 (1)
Deviations from current test guideline:	Not applicable – no current applicable test guideline
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to determine the toxicity of the test item Aclonifen technical on the growth of *Egeria densa* under static conditions over 14 days. The test was conducted following the draft guidance document of the SETAC AMRAP (Aquatic Macrophyte Risk Assessment for Pesticides) working group 2. The macrophytes were exposed to nominal concentrations of 10, 31.6, 100, 316 and 1000 µg a.s./L.

The test item was dissolved in growth medium (Smart & Barko medium). For the growth tests three replicates for each test concentration and six replicates for controls (test medium only) were used with five plants per replicate. The plants were exposed to the test item in the aqueous phase of the test system in the presence of sediment.

Following parameters were measured: shoot length, fresh weight and dry weight. Plant length was recorded at test start and after 4, 7, 11 and 14 days. Prior to the onset of the test (-3 days), the fresh weight of the test plants were determined. Dry weight was determined using a set of representative plants. At the end of the test all plants were harvested and their fresh and dry weights were recorded. During the 14 day growth test the shoot length increased by more than 100% in the controls.

Effective concentrations were calculated for relative increase and growth rate of the measured parameters.

At test start the measured concentrations were between 33.9 and 38.2% of nominal. During the 14-Day growth test the test item was not stable in the test media of all treatments leading to a lower concentration at the end of the test (9.7-15.9% of nominal). Therefore, the test was evaluated using the geometric mean of the test concentrations measured in the different treatments with 1.88, 6.60, 23.0, 73.3, and 221 µg a.s./L.

In a static growth inhibition test with the rooted macrophyte *Egeria densa* exposed to Aclonifen over 14 days the EC_{50} values for increase in shoot length, fresh weight and dry weight were above the highest test concentration of 221 µg as/L.

The observed parameters (shoot length, fresh weight and dry weight) were not significantly different from the controls up to the highest test concentration and the NOEC was therefore determined to be ≥221 µg a.s./L (geometric mean measured concentration).

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen technical
	Batch no.:	AE F068300-01-10
	Purity:	99.2% w/w
	Appearance:	Yellow brown powder
	Date received:	30 September 2010
	Storage:	Store at 25 ± 5 °C. Keep in tightly closed containers in a dry, cool, well ventilated place.
	Expiry date:	27 February 2011
2.	**Test Organism:**	*Egeria densa*, Hydrocharitaceae, Alismatales, Monocotyledonous
	Source:	[redacted]
	Acclimatization:	The plants were held immersed for at least 10 days prior to the test start in water and sediment of the same quality as used in the test.
3.	**Test water:**	Smart & Barko medium (1985)
4.	**Sediment:**	Formulated sediment based on the artificial soil used in OECD Guideline 219 was used: • 4-5% peat (dry weight, according to 2 ± 0.5% organic carbon) as close to pH 5.5 to 6.0 as possible; it is important to use peat in powder form, finely ground (particle size < 1 mm) and only air dried. • 20% (dry weight) kaolin clay (kaolinite content preferably above 30%). • 75-76% (dry weight) quartz sand (fine sand should predominate with more than 50 per cent of the particles between 50 and 200 µm). • Deionised water was added to obtain moisture of the final mixture of about 30%. • If needed, calcium carbonate of chemically pure quality ($CaCO_3$) was added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5. For the batch of sediment containing nutrients, instead of deionised water, an aqueous nutrient medium (with 300 mg/L sediment of both ammonium chloride and sodium phosphate in

the appropriate amount of water) was added to obtain moisture of the final mixture of about 30%.

B. STUDY DESIGN AND METHODS

1. In-life phase: 09 – 23 December 2010

2. Exposure conditions

Test vessels:	2-L glass beakers (approximately 24 cm high and 11 cm diameter). Small plant pots (approx. 9 cm diameter and 8 cm high and around 350 mL volume, plastic, commercially available) were used as containers for potting the plants into the sediment. The sediment surface coverage was about 70% of the test vessel surface; the minimum overlaying water depth was 12 cm
Experimental design:	5 test concentrations (10, 31.6, 100, 316 and 1000 µg a.s./L) plus a control
Replicates:	6 replicates for the control and 3 replicates per treatment group. Each replicate contained 5 plants.
Temperature:	18.0 – 22.0 °C
pH:	7.89 – 8.92
Aeration:	None
Photoperiod:	16 hours light : 8 hours dark
Light intensity:	7378 - 7518 lux

3. Administration of the test item

The nominal concentrations were spaced by a factor of 3.16, due to the flat concentration-effect curve observed in the pre-test. A stock solution was prepared in acetone with a concentration of 101 mg test item/500 µL (stock solution 1). 50 µL of stock solution 1 were dispensed into a 1 L graduated glass flask and the acetone was left to evaporate. The flask was filled up to 1 L to achieve a concentration of 10.01 mg test item/L equivalent to 10.0 mg a.s./L (stock solution 2). The stock solution 2 was stirred at room temperature for 24 hours and then the 1 litre was added to 9 L growth medium to obtain the highest test concentration of 1000 µg a.s./L from which serial dilutions were made to give the remainder of the test solutions. The test media were stirred vigorously for 30 minutes at room temperature. The pH values of the test media were not adjusted.

4. Preparation of test vessels

For the test, the sediment was filled into standard planting pots. Since the standard planting pots have wholes at the bottom, first a filter paper was put on the bottom of the vessel. Afterwards an approximately 1 cm layer of the standard sediment was added. On top of this a 4 cm layer of sediment was added which had been supplemented with a nitrate and phosphate fertilizer (nutrient supplemented sediment). This was covered again with 1 cm of standard sediment without fertilizer (in order to provide sufficient nutrients to the plants via the sediment without enriching the water with further nutrients). A

fine/very thin layer (approximately 2 mm) of coarse quartz sand was added on the top of the sediment in order to reduce suspension of sediment into the water.

5. Test organism assignment and treatment

After the pre-culture, the plants were removed from the pre-culture and cleaned of sediment and surplus water; plants that were apparently not healthy were discarded at this stage. The plants were weighed. Shoots were then potted into the sediment and shoot length above sediment was measured. If required, the length of the plants above sediment was adjusted to 3 cm.

25 plants of the pre-culture were additionally harvested at this stage (only using the most homogenous individuals) and plant dry weight were determined to obtain the respective data for Day 0.

For the growth inhibition test, five plants were used per pot and test vessel and three replicates were prepared for each of the five treatments.

The pots with sediment and plants were placed into the glass beakers. Afterwards, the test vessels were carefully filled up with 2 L of the respective test media.

6. Measurements and observations

The correct application of the test item was confirmed by analytical measurements of aclonifen concentrations in the test media at test initiation and after the 14-Day exposure period.

During the 14-Day exposure period, shoot lengths were recorded at test start and on Days 4, 7, 11, and 14.

Total plant fresh weight was determined after absorbing remaining test medium attached to the plants by means of tissue paper. Dry weight was determined subsequently after weighing the fresh plants. The five plants per replicate were combined and the plants were dried in aluminium weighing boats at 105 °C for 24 hours.

Light intensities at the water surface were measured on Days 0 and 14. Oxygen contents and pH values of the test medium were recorded on Days 0, 7, and 14. Test media temperature was recorded using a data logger four times a day (Thermo Data Logger EL-USB-TC).

At the end of the growth test, all plants were harvested. Any symptoms (such as chlorosis or necrosis, roots) or other observations were recorded. Total plant wet weight (after careful absorption of attached test medium) was determined followed by the estimation of total plant dry weight.

7. Statistics/Data evaluation

Statistical calculations were made on the results obtained for individual vessels, not for individual plants. Data evaluation was done for shoot length increase, fresh and dry weight as well as for growth rate of the respective parameters at test termination. The relative values (in percent of test start) of the parameters were used for the evaluation.

EC_x values were calculated by probit analysis modified for continuous data using the computer program ToxRat Professional (ToxRat Solutions, Alsdorf, Germany). No Observed Effect Concentrations (NOEC) were calculated, using ANOVA, followed by [redacted] test, [redacted] or [redacted] t-test.

The evaluation was performed using mean measured concentrations. The replicates of each concentration plot were used for fitting concentration-response curves of the measured parameters, if meaningful concentration-effect relationships were observed.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Freshly prepared test solutions at test start and pooled samples of the test media at the end of the growth test were analysed for the test item using HPLC UV-VIS (LOQ 0.25 µg a.s./L). The measured concentrations in the test media were between 34.8 and 38.2% of nominal at test start and between 9.7 and 15.9% at test end. Due to deviations from the nominal concentrations >20%, the analysis of the results is based on the geometric mean measured test concentrations.

Table: Measured test concentrations of Aclonifen during the exposure to *Egeria densa*

Nominal concentration (µg a.s./L)	Measured concentration					
	Day 0		Day 14		Geometric mean measured	
	µg a.s./L	% nominal	µg a.s./L	% nominal	µg a.s./L	% nominal
Control	<LOD	-	<LOD	-	<LOD	-
10.0	3.64	36.4	0.97	9.7	1.88	18.8
31.6	12.08	38.2	3.61	11.4	6.60	20.9
100.0	38.08	38.1	13.86	13.9	22.97	23.0
316.0	107.18	33.9	50.13	15.9	73.30	23.2
1000	347.98	34.8	140.87	14.1	221.4	22.1

LOD: Limit of Detection = 0.025 µg a.s./L (lowest calibration point and 100 mL work up volume)

The validated method is summarised in Document M-CA4 (CA 4.1.2/74).

B. BIOLOGICAL DATA

There was a concentration dependent effect on the increase in shoot length of *Egeria densa* however there was no concentration dependent effect on the fresh and dry weight based on weight increase and weight growth rate.

Table: Percentage inhibition for plant shoot length, fresh weight and dry weight during the exposure of *Egeria densa* to Aclonifen

Geometric mean measured concentration (µg a.s./L)	% Inhibition after 14 days					
	Shoot length		Fresh weight		Dry weight	
	Increase	Growth rate	Increase	Growth rate	Increase	Growth rate
1.88	2.7	0.7	-4	-3.5	4.9	3.4
6.60	6.7	4.4	-42	-35.7	-53.4	-29.1
23.00	17.6	11.9	-54.8	-45.4	-65.4	-47.9
73.30	21.2	17.4	-32.2	-22.5	3.1	-0.3
221.0	14.2	9.6	-40.5	-33.9	-0.6	-6.5

- negative values indicate increase in the observed parameter compared to control

The test results were statistically analysed to determine the 14-Day EC_{50} values together with 95% confidence intervals for parameters showing concentration dependent effects as well as NOEC values.

C. VALIDITY CRITERIA

Specific criteria for macrophyte growth tests using *Egeria densa* have not been set yet. At the time of performing the study, validity criteria proposed by the AMRAP working group were used to assess the validity of the study.

Validity criterion	Required (AMRAP working group)	Achieved
Increase in biomass in controls (shoot length)	≥50%	123%
Continuous growth throughout the test duration	Required	Yes
Temperature	20 ± 2°C	18.0 – 21.0 °C

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint		Geometric mean measured concentration (µg a.s./L)	
		EC_{50}	NOEC
Shoot length	Relative increase	>221.0	≥221.0
	Growth rate	>221.0	≥221.0
Fresh weight	Relative increase	>221.0	≥221.0
	Growth rate	>221.0	≥221.0
Dry weight	Relative increase	>221.0	≥221.0
	Growth rate	>221.0	≥221.0

III. CONCLUSION

In a static growth inhibition test with the rooted macrophyte *Egeria densa* exposed to Aclonifen over 14 days the EC_{50} values for increase in shoot length, fresh weight and dry weight were above the highest test concentration of 221 µg as/L.

The observed parameters (shoot length, fresh weight and dry weight) were not significantly different from the controls up to the highest test concentration and the NOEC was therefore determined to be ≥221 µg a.s./L (geometric mean measured concentration).

[redacted] *(2011)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

In a static growth inhibition test with the rooted macrophyte *Egeria densa* exposed to Aclonifen over 14 days the EC_{50} values for increase in shoot length, fresh weight and dry weight were above the highest test concentration of 221 µg as/L.

The observed parameters (shoot length, fresh weight and dry weight) were not significantly different from the controls up to the highest test concentration and the NOEC was therefore determined to be ≥221 µg a.s./L (geometric mean measured concentration).

Due to the lack of concentration-dependent effects, EC_{10} and EC_{20} values could not be calculated, however as the E_rC_{50} value is the endpoint required for aquatic risk assessment this was not considered to affect the interpretation of the study results.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.7/09
Report Author:	[redacted]
Report Year:	2011
Report Title:	Toxicity of aclonifen technical to the aquatic macrophyte, Myriophyllum spicatum
Report No:	EBCLX019
Document No:	M-398530-01-1
Guideline(s) followed in study:	Higher Tier Study based on OECD 221
Deviations from current test guideline:	Current Guideline: OECD 239, 2014 Only 4 replicate control vessels containing 3 plants each were included in the study rather than 6 replicates containing 3 plants each. This deviation was not considered to have affected study integrity and validity.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to determine the dose-response effect of Aclonifen Technical to the rooted aquatic macrophyte, *Myriophyllum spicatum*, over an exposure period of 14 days. Growth in the study is defined as a change (yield) in total shoot length, total plant wet weights and total plant dry weights (shoots and roots). The EC_{50} was estimated for these growth parameters based on growth occurring between study Days 0 and 14.

The test system consisted of two to four replicate aquaria per treatment group. Each replicate contained four plants for a total of 8 to 16 plants per group. All plants within a replicate were planted into a single 125x65 mm crystallization dish containing 550 grams of artificial sediment. The rooted aquatic plants were submerged in the aquaria and following a 7-Day acclimation period, were exposed to nominal concentrations of 0.76, 2.4, 7.8, 25 and 80 µg a.s./L for 14 days. A control and solvent control group was included in the study. Following the 14-Day exposure period plants were sacrificed and measured.

Mean measured recoveries based on Day 0, Day 4, Day 7 and Day 14 sampling events and were within the range of 63 to 83% of the nominal concentrations. The initial (Day 0) measured recoveries ranged from 81 to 98% of the nominal test concentrations. The toxicity values were calculated based on the geometric mean measured concentrations of 0.48, 1.5, 5.4, 19.5 and 66.2 µg a.s./L.

The most sensitive endpoint in the 14-Day exposure of Aclonifen Technical to the rooted aquatic macrophyte *Myriophyllum spicatum* was Dry Weight Yield. The statistical NOEC, LOEC and E_bC_{50} for this endpoint were 1.5, 5.4 and 21.8 µg a.s./L, respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen Technical
   Batch no.: 18002
   Purity: 99.6%
   Appearance: Yellow-brown powder
   Date received: 09 December 2009
   Storage: Ambient (room temperature)
   Expiry date: 27 February 2011

2. **Test Organism:** *Myriophyllum spicatum*
   Source: [redacted]

3. **Test water:** Dilution water (processed hard water) used for culturing and testing consisted of spring water blended with reverse osmosis water to produce a hard water.

4. **Sediment:** A defined formulated sediment based on OECD 2178 was used with the following composition:
   - Clay 2017 g
   - Sand 7563 g
   - Peat moss 504g
   - 20XAAP Media 5042 mL

   Prior to mixing, peat moss was sieved to only include finely ground particles. The clay, sand and peat moss were autoclaved to sterilize. The 20XAAP media was prepared by adding sterile nutrient stock solutions to distilled water. The pH of the media was adjusted to 7.5. The peat moss and media were mixed using an electric mixer for approximately 24 hours to stabilize the pH.· Following mixing, calcium carbonate was added to adjust pH to 5.4.· The clay and sand were then combined with the peat moss suspension and mixed thoroughly. The pH was again

checked after all ingredients had been mixed together. The final sediment pH was 6.6.

B. STUDY DESIGN AND METHODS

1. In-life phase: 15 April – 06 May 2010

2. Exposure conditions

Test vessels:	4-L glass beakers filled with 3.5 L test solution
Experimental design:	5 test concentrations (0.76, 2.4, 7.8, 25 and 80 µg a.s./L) plus a control and solvent control (DMF, 100 µL/L)
Replicates:	4 (Control, Solvent Control, 0.76 µg a.s./L); 3 (2.4, 7.8, 25 µg a.s./L) and 2 (80 µg a.s./L). Each replicate contained 4 plants.
Temperature:	19.95 - 21.23 °C
pH:	8.2 – 10.2
Aeration:	None
Photoperiod:	24 hours light The planned light cycle, as outlined in the study protocol was 16 hours light and 8 hours dark. However, due to an oversight, the light banks were not plugged into the programmed timers during the study. Conducting the study under 24-Hour a day lighting did not appear to have any adverse effects on the plant growth or cause any undesirable conditions such as algae growth
Light intensity:	8210 to 10512 lux (mean 9185 lux)

3. Administration of the test item

A separate stock solution was prepared for each test concentration. Initially an 800 mg a.s./L stock solution was prepared for dosing the highest test concentration. This stock was serial diluted to other stock solutions at concentrations of 250, 78.1, 24.4 and 7.6 mg a.s./L, for dosing the test concentrations of 25, 7.8, 2.4 and 0.76 µg a.s./L, respectively. All stock solutions were prepared in 100-mL volumetric flasks with DMF as the diluent. Mixing was accomplished by inverting the flasks several times.

4. Test organism assignment and treatment

The definitive study consisted of a pre-exposure (establishment) and exposure phase. The pre-exposure phase lasted for seven days. The exposure phase lasted for 14 days.

At the start of the pre-exposure phase, shoots were cut from healthy cultures at a length of 7 cm. The leaves were removed from the bottom 2 cm of each shoot. Shoots were then planted 2 cm deep into 125x65 mm glass crystallization dishes containing 550 grams of artificial sediment. The surface of the sediment was then covered with a layer (approximately 100 mL) of autoclaved crushed coral. Four shoots were planted into each crystallization dish. The dishes containing the planted macrophytes were placed into 4-L beakers (25 cm tall, 15.5 cm diameter). The four plants in the crystallization dish held

in the 4-L beakers make up a single replicate. During the seven day pre-exposure phase each replicate vessel contained 3.5-L of dilution water.

The start of the exposure period was marked by the addition of stock solution to each exposure beaker, with the exception of the control beakers which received no stock solution and the solvent control vessels received DMF only. The stock was mixed into the test beakers using a disposable pipette for approximately one minute. Following 14 days of exposure, all plants were removed from the test system.

5. Measurements and observations

At the end of the 14-Day exposure period, length of the main shoot and all side shoots were measured, wet weights were measured, and following drying of plants for at least 72 hours, dry weight measurements were collected.

Temperature was recorded hourly and daily. pH and conductivity was measured on Days -7, 0, 7 and 14.

Samples for analytical verification of test concentrations were taken on Days 0, 4, 7 and 14.

6. Statistics/Data evaluation

Effects on yield for total shoot length, total plant wet weight and total plant dry weight were determined on a per plant basis, based on the growth of each plant during the 14 day growth intervals.

Raw or transformed data from treatment groups were compared to controls for normality and homogeneity of variance using the [redacted] test and [redacted] test of equal variance, respectively. If normality and homogeneity of variance were demonstrated for the raw or transformed values, then parametric analyses were conducted using analysis of variance (ANOVA) followed by [redacted] test. If normality and/or homogeneity of variance were not demonstrated on raw or transformed values, nonparametric procedures were used. The ranks of the raw values were determined, and then an analysis of variance and a one-tailed [redacted] test were performed on these ranks.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Table: Measured test concentrations of Aclonifen during the exposure to *Myriophyllum spicatum*

Nominal Concentration (µg a.s./L)	Measured concentration									
	Day 0		Day 4		Day 7		Day 14		Geometric mean	
	µg a.s./L	% nom	µg a.s./L	% nom	µg a.s./L	% nom	µg a.s./L	% nom	µg a.s./L	% nom
Control	<LOQ	-	<LOQ		<LOQ	-	<LOQ	-	<LOQ	-
Solv. control	<LOQ	-	<LOQ		<LOQ	-	<LOQ	-	<LOQ	-
0.76	0.62	81	0.62	68	0.43	57	0.38	50	0.48	0.1
2.4	2.0	85	1.8	77	1.3	54	1.2	49	1.5	0.4
7.8	7.1	91	6.1	78	4.7	61	4.1	53	5.4	1.6
25	24.2	97	21.0	84	18.9	76	14.9	60	19.5	4.7
80	78.2	98	68.0	85	65.5	82	55.0	69	66.2	11.6

% nom: percentage of nominal concentration
LOQ: Limit of Quantitation = 0.08 µg a.s./L

The validated method is summarised in Document M-CA4 (CA 4.1.2/75).

B. BIOLOGICAL DATA

Active growth of the control plants during the 14-Day exposure period was demonstrated by a total shoot length yield of 25.6 cm (from 14.2 cm on Day 0 to an average of 39.8 cm on Day 14).

Plants in the control vessels and the three lowest test concentrations appeared normal throughout the study. Plants in the two highest test concentrations (19.5 and 66.2 µg a.s./L) appeared semi-transparent to red in colour. At study termination roots appeared normal in the controls and all treatment groups.

Shoot length yield and wet weight yield was analyzed at test termination on study Day 14. There was no dose response trend for these endpoints and the statistical analysis showed that no levels were statistically different from the pooled control group.

Dry weight yield was analyzed at test termination on study day 14. Dunnett's test showed a statistically significant difference in the three highest treatment groups. Percent inhibitions as compared to the pooled control group was -9.4, 3.4, 39.4, 52.1 and 55.8% for the 0.48, 1.5, 5.4, 19.5 and 66.2 µg a.s./L test groups, respectively.

Table: Yield for plant shoots, wet weights and dry weights during the exposure of *Myriophyllum spicatum* to Aclonifen

Geometric mean measured concentration (µg a.s./L)	Length yield		Wet weight yield		Dry weight yield	
	cm	% Inhibition	g	% Inhibition	g	% Inhibition
Control	25.6	-	1.0024	-	0.1304	-
Solvent control	25.1	-	1.0805	-	0.1487	-
Pooled control	25.3	-	1.0414	-	0.1395	-
0.48	23.1	8.7	0.9252	11.4	0.1526	-9.4
1.5	25.4	-0.2	0.9941	4.5	0.1349	3.4
5.4	24.3	3.9	0.8073	22.5	0.0845	39.4[1]
19.5	23.9	5.7	0.7699	26.1	0.0668	52.1[1]
66.2	24.2	4.5	0.9102	12.6	0.0617	55.8[1]

[1]: Statistically significant difference from pooled control ([redacted] one-tailed test; $p \leq 0.05$)

The most sensitive endpoint was Dry Weight Yield. The statistical NOEC, LOEC and E_yC_{50} for this endpoint were 1.5, 5.4 and 21.8 µg a.s./L, respectively.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 239, 2014)	Achieved*
Increase in control mean total shoot length	Factor of 2	2.8
Increase in control mean total shoot fresh weight	Factor of 2	2.4
Control plants	No visual symptoms of chlorosis and should be visibly free from contamination by other organisms such as algae and/or bacterial films on the plants, at the surface	No abnormal symptoms or algal contamination

	of the sediment and in the test medium.	
Control mean coefficient of variation for yield (based on shoot fresh weight) between replicates	≤35%	29.8%

*Based on pooled controls

In the absence of a validated Test Guideline for assessing the effects of a chemical on the growth of *Myriophyllum spicatum,* the study was based on OECD Test Guideline 221: *Lemna* sp. Growth Inhibition Test (2006) and there were no specific validity criteria applicable to the study design.

In terms of the current version of OECD Test Guideline 239: Water-sediment *Myriophyllum spicatum* Toxicity Test (2014), all validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Geometric mean measured concentration (µg a.s./L)		
	E_yC_{50}	NOEC	LOEC
Total Shoot Length Yield	>66.2 [n.d.]	66.2	>66.2
Total Plant Wet Weight Yield	>66.2 [n.d.]	66.2	>66.2
Total Plant Dry Weight Yield	21.8 [10.4 – 45.8]	1.5	5.4

[95% confidence limits]
n.d.: not determined

III. CONCLUSION

The most sensitive endpoint in the 14-Day exposure of Aclonifen Technical to the rooted aquatic macrophyte *Myriophyllum spicatum* was Dry Weight Yield. The statistical NOEC, LOEC and E_yC_{50} for this endpoint were 1.5, 5.4 and 21.8 µg a.s./L, respectively.

[redacted] *(2011)*

Data Point:	KCA 8.2.7/10
Report Author:	[redacted]
Report Year:	2016
Report Title:	Aclonifen (tech.): Recalculation of growth inhibition study with myriophyllum spicatum
Report No:	M-543492-01-1
Document No:	M-543492-01-1
Guideline(s) followed in study:	not applicable
Deviations from current test guideline:	Not applicable. Report is a re-evaluation of previously generated study data
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A 14-Day static *Myriophyllum spicatum* growth inhibition study with the test item aclonifen (tech.) has been conducted (KCA 8.2.7/10, [redacted] 2011) considering the recommendations of the OECD test guideline 221, 2006 (*Lemna* sp. Growth Inhibition Test). In the report effects on yield for total shoot length, total plant wet weight and total plant dry weight were determined and the corresponding endpoints are consequently based on yield only.

However, processes in ecosystems are dominantly rate driven and therefore, the unit development per time (growth rate) is more suitable to measure effects in macrophytes. Also, growth rates and their inhibition can easily be compared between species, test durations and test conditions, which is not the case for yield or biomass based endpoints. Following current state of science, the recently published test guidelines for *Myriophyllum* sp. tests (OECD 238 and 239, both 2014) ask for determination of average specific growth rate as a response variable. Moreover, the EFSA Aquatic Guidance Document (AGD, 2013) lists growth rate as the preferred endpoint to be used in the risk assessment for macrophytes.

For the abovementioned reasons, assessment endpoints based on growth rate have been recalculated for the study at hand in agreement with current state of science and the relevant guidelines. This statement presents NOEC, LOEC and EC_{50} values for growth rate of total shoot length, total plant wet weight and total plant dry weight after 14 days of exposure as calculated by ToxRat Professional, version 2.10..

The static 14 days growth inhibition test with *Myriophyllum spicatum* provided the following effects on total shoot length, total plant wet weight and total plant dry weight on a per plant basis:

Table: Growth rate for plant shoots, wet weights and dry weights during the exposure of *Myriophyllum spicatum* to Aclonifen

Geometric mean measured concentration (µg a.s./L)	Total shoot length		Total plant wet weight		Total plant dry weight	
	Mean growth rate	% Inhibition	Mean growth rate	% Inhibition	Mean growth rate	% Inhibition
Control	0.072	-	0.060	-	0.042	-

Solvent control	0.072	-	0.063	-	0.045	-
Pooled control	0.072	-		-		-
0.48	0.068	5.56	0.056	8.61	0.046	0.00
1.5	0.072	0.00	0.059	3.83	0.042	3.84
5.4	0.070	2.32	0.051	17.3	0.028	34.8[1]
19.5	0.070	2.81	0.050	18.9	0.024	46.0[1]
66.2	0.070	2.14	0.056	8.14	0.022	48.6[1]

[1]: Statistically significant difference from pooled control ([redacted] Multiple sequential t-test Procedure)

The most sensitive measurement variable in this study was total plant dry weight resulting in a lowest E_rC_{50} of 42.01 µg a.s./L.

Table: Summary of endpoints

Endpoint – growth rate	Geometric mean measured concentration (µg a.s./L)		
	EC_{50}	NOEC	LOEC
Total Shoot Length	>66.2 [n.d.]	66.2	>66.2
Total Plant Wet Weight	>66.2 [n.d.]	66.2	>66.2
Total Plant Dry Weight	42.01 [n.d.]	1.5	5.4

[95% confidence limits]
n.d.: not determined

[redacted] *(2016)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The most sensitive endpoint in the 14-Day exposure of Aclonifen Technical to the rooted aquatic macrophyte *Myriophyllum spicatum* was dry weight growth rate. The statistical NOEC, LOEC and E_rC_{50} for this endpoint were 1.5, 5.4 and 42.01 µg a.s./L, respectively.

EC_{10} and EC_{20} values were not calculated, however as the E_rC_{50} value is the endpoint required for aquatic risk assessment this was not considered to affect the interpretation of the study results.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.7/11
Report Author:	[redacted]
Report Year:	2006
Report Title:	Lemna gibba G3 - Growth inhibition test with Aclonifen (tech.) in a water/sediment system using spiked medium (code: AE F068300 00 1D9Z0002)
Report No:	EBCLX009
Document No:	M-263844-01-1
Guideline(s) followed in study:	Higher tier study, conducted under principal consideration of OECD 221 "Lemna sp. Growth Inhibition Test" Revised Proposal for a New Guideline (October 2004)
Deviations from current test guideline:	Current Guideline: OECD 221, 2006 Test system was water/sediment, rather than water only. This deviation was not considered to have affected study integrity and validity.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

The effects of Aclonifen on the growth and reproduction of the aquatic monocotyledonous plant, *Lemna gibba,* were investigated in an exposure to nominal concentrations of 12, 24, 48, 96, 192 and 384 µg a.s./L plus a control and a solvent control. Fronds of *Lemna gibba* were exposed to Aclonifen for seven days in a static water-sediment system without test medium renewal.

Samples were analysed for the actual concentration of aclonifen present in the test medium at all freshly prepared and all aged treatment levels including controls (water-phase only). Measured test concentrations ranged from 96 to 103% of the nominal concentrations on day 0 and from less than the limit of determination (1.361 µg/L) to 7% of nominal after 7 days. The lower concentrations on Day 7correspond with the expected adsorptive to sediment properties of aclonifen. Therefore, the initial measured test concentration was used to calculate the study endpoints.

The most sensitive response variable was frond number resulting in an overall E_rC_{50} of 116 µg a.s./L.

The lowest NOEC (24.4 µg a.s./L) was based on visual effects and statistical data analysis for both frond number and dry weights of plants. Results reported based on initial nominal concentrations.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** Aclonifen technical
   Batch no.: 97013003
   Purity: 994 g/kg
   Expiry: April 2006 (retest)

2. **Test organism:** *Lemna gibba*
   Strain: G3
   Source: [redacted]

[redacted]

3.	**Treatment:**	Nominal test concentrations of 12.0, 24.0, 48.0, 96.0, 192 and 384 µg a.s./L
4.	**Test vessels:**	Glass dishes, diameter 10cm, total volume ca. 470 mL, covered with glass lids to permit gas exchange and illumination
	Test water:	20X-AAP medium, pH adjusted to 7.5 ± 0.1
	Test sediment:	Artificial sediment (using OECD 219) prepared 10 days before test start and comprising: 74% quartz, 5.0% sphagnum peat, 20% kaolin, Approx. 1% $CaCO_3$, Growth medium (450 mL/kg dry weight sediment) Moisture content of final mix 30-50% Organic carbon content 2 ± 0.5%

B. STUDY DESIGN AND METHODS

1.	**In-life phase:**	29 July to 28 October 2005
2.	**Exposure conditions:**	
	Temperature:	23.2 – 23.9°C (Days 0 – 7)
	pH:	8.0 – 8.5 (Days 0 – 7)
	Photoperiod:	Continuous illumination, mean 7680, range 6800 - 8830 lux

3. Dose preparation

Each test vessel comprised a sediment layer, approximately 0.7 cm covering the base of the vessel. Growth medium (200 mL) was slowly poured into the vessels, after covering the sediment with a sheet to prevent separation of sediment ingredients. The sheet was then removed, and the vessels were allowed to equilibrate in the dark and at room temperature for 10 days.

A primary stock solution was prepared by dissolving 41 mg test item in 10 mL dimethyl formamide (DMF), followed by 5 minutes stirring. Aliquots of the resulting stock solution were transferred to a dilution series to obtain test exposure concentrations by spiking the overlying water in the test vessel. A solvent control and control exposure treatment were run along with nominal test exposure concentrations of 12.0, 24.0, 48.0, 96.0, 192 and 384 µg a.s./L

Plants were transferred within 5 minutes of spiking the growth medium with test item.

4. Test organism assignment and treatment

Colonies used for test were from an inoculum culture 7-10 days old. Each test vessel contained a total of 12 fronds, with 3 replicates per treatment. The test vessels were placed in a random order and were repositioned each observation day (days 2, 5 and 7).

5. Measurements and observations

Frond counts were made on Days 0, 2, 5 and 7. On Day 14 (end of test) the colonies were collected from each of the replicate test vessels, rinsed with deionised or distilled water and blotted to remove excess water. After drying for at least one day at 60°C to a constant dry weight the colonies were

weighed. In the same way the starting biomass was measured (dry weights) of a triplicate of 12 fronds (taken from the same batch used as inoculum within this study).

Temperature was determined by continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature was recorded hourly by a data logger. The pH was measured in all freshly prepared and all aged test levels and the controls. The light was measured at least once during the test.

Water phase samples were analysed for the actual concentration of aclonifen present in all freshly prepared test levels on day 0, and in all aged test levels on Day 7 of the exposure period. Aliquots for freshly prepared test levels for Day 0 analyses were sampled from the prepared volume of each test treatment level. For sampling of aged test media, after removing of plant material from the test vessels on Day 7 the contents of all three replicate vessels were combined, and the pH was measured. Samples were analysed by HPLC-MS/MS.

6. Statistics

The LOEC determinations from the appropriate parameter (inhibition) were done, using the ANOVA procedure (α = 0.05, one sided) and properly selected multiple t-tests of a commercial program. Calculations were carried out using Microsoft Excel® spreadsheets. All further statistical evaluations were done using the commercial program ToxRat Professional, version 2.09 (2004).

H. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Samples were analysed for the actual concentration of aclonifen present in the test medium at all freshly prepared and all aged treatment levels including controls (water-phase only). Measured test concentrations ranged from 96 to 103% of the nominal concentrations on day 0 and from less than the limit of determination (1.361 µg/L) to 7% of nominal after 7 days. The lower concentrations on day 7correspond with the expected adsorptive to sediment properties of aclonifen. Therefore, the initial measured test concentration was used to calculate the study endpoints.

Table: Mean measured concentrations (µg/L) of aclonifen in the exposure solutions

Nominal concn (µg a.s./L)	Measured concn (µg a.s./L)			
	Day 0	% nominal	Day 7	% nominal
Control	<1.361	n.a	<1.361	n.a.
Solvent control	<1.361	n.a	<1.361	n.a.
12	11.9	99	<1.361	n.a.
24	24.7	103	<1.361	n.a.
48	47.5	99	2.72	6
96	91.8	96	5.42	6
192	186	97	11.9	6
384	369	96	25.8	7

n.a. = not applicable

The validated method is summarised in Document M-CA4 (CA 4.1.2/91).

B. BIOLOGICAL DATA

Frond numbers

Mean frond numbers are presented in the following table:

Table: Mean frond numbers over 7-day exposure to aclonifen technical

Nominal concn (µg a.s./L)	Day 0	Day 2	Day 5	Day 7	Growth rate µ	Doubling time (days)	% inhibition
	Mean (%CV)				(1/d)		
Control	12	25 (10.6)	85 (11.2)	160 (13.4)	0.369	1.9	-
Solvent control	12	24 (6.3)	80 (7.1)	155 (4.2)	0.366	1.9	-
Pooled control	12	25	83	158	0.367	1.9	-
12	12	25 (10.6)	89 (14.3)	182 (13.5)	0.388*	1.8	-5.5
24	12	25 (6.0)	93 (7.0)	170 (5.5)	0.379	1.8	-3.1
48	12	23 (4.3)	59 (8.5)	101 (7.5)	0.304*	2.3	19.3
96	12	20 (2.8)	37 (5.6)	53 (7.7)	0.211*	3.3	42.6
192	12	20 (7.8)	20 (17.3)	28 (16.3)	0.118*	6.0	67.9
384	12	20 (5.0)	13 (0)	13 (0)	0.011*	60.6	96.9

SD = Standard deviation

Negative % inhibition indicates growth relative to pooled control

* Statistically significant compared to pooled control (based on Student t-test for homogeneous variances with Bonferroni adjustment, α = 0.05)

Biomass

Plant biomass (dry weight) along with the corresponding confidence limits are presented below:

Table: Biomass (frond dry weight) after 7-day exposure to aclonifen technical

Nominal concn (µg a.s./L)	Final dry weight (mg)	%CV	Average growth rate µ (0→7 d) (1/day)	% inhibition
Control	21.8	12.2	0.431	-
Solvent control	20.6	8.4	0.423	-
Pooled control	21.2	-	0.427	-
12	24.3	6.3	0.445	-4.3
24	21.2	1.5	0.427	-0.1
48	11.5	2.1	0.339*	20.5
96	7.2	2.2	0.272*	36.2
192	5.9	5.0	0.245*	42.6
384	4.9	6.8	0.218*	48.8

Inoculum dry weight: 1.067 mg. This value subtracted from final dry weight

Negative % inhibition indicates growth relative to pooled control

* Statistically significant compared to pooled control (based on [redacted]t-test for inhomogeneous variances with Bonferroni adjustment, α = 0.05)

Shape of fronds

Visual observations on day 2 showed new fronds were smaller and slightly chlorotic at nominal concentrations of 48 µg a.s./L and above. By day 5 observation of chlorosis was more pronounced, with plants at 192 and 384 µg a.s./L most affected. By day 7

At test termination, slightly chlorotic to chlorotic, small fronds and fronds with less root formation were observed in the 0.011 and 0.020 mg a.i./L treatment solutions. Small fronds were observed in the 0.0049 mg a.i./L treatment level. Fronds exposed to the remaining treatment levels, the control and the solvent control were observed to be normal.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 221, 2006)	Achieved
Doubling time of frond number in the control <2.5 days (60 h), corresponding to approximately 7-fold increase in 7 days	<2.5d	1.9d

The frond number increased in the controls by a factor of 13.2 within 7 days corresponding to a doubling time (Td) of about 1.9 days, therefore the validity criterion was met and the study can be considered valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Parameter	Initial nominal concn (µg a.s./L)			
	Frond no.	95% confidence limit	Biomass (dry weight)	95% confidence limit
E_rC_{10}	38.8	25.2 – 50.4	25.0	0.91 – 57.2
E_rC_{20}	56.5	41.3 – 69.4	59.1	9.1 - 108
E_rC_{50}	116	98.3 - 136	305	170 - >384
NOEC	24	-	24	-
LOEC	48		48	-

III. CONCLUSION

The most sensitive response variable was frond number resulting in an overall E_rC_{50} of 116 µg a.s./L.

The lowest NOEC (24.4 µg a.s./L) was based on visual effects and statistical data analysis for both frond number and dry weights of plants. Results reported based on initial nominal concentrations.

[redacted] *(2006)*

Assessment and conclusion by applicant:

The validity criterion was met, therefore this study is considered to be acceptable.

The most sensitive response variable was frond number resulting in an overall E_rC_{50} of 116 µg a.s./L.

The lowest NOEC (24.4 µg a.s./L) was based on visual effects and statistical data analysis for both frond number and dry weights of plants. Results reported based on initial nominal concentrations.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.7/12
Report Author:	[redacted]
Report Year:	2018
Report Title:	Lemna gibba G3 – Growth inhibition test with aclonifen tech. (BCS-AG74518) under peak exposure conditions
Report No:	EBCL0005
Document No:	M-612847-01-1
Guideline(s) followed in study:	EU Directive 91/414/EEC Regulation (EC) Number 1107/2009 OECD Test Guideline 221 US EPA OCSPP 850.4400
Deviations from current test guideline:	Current Guideline: OECD 221, 2006 Test medium not stabilised as per the Guideline. This deviation was not considered to have affected study integrity and validity
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

The effects of Aclonifen, on the growth and reproduction of the aquatic monocotyledonous plant, *Lemna gibba,* were investigated in two different exposure designs.

In **design 1** the Lemna plants were exposed to three 24 hours lasting peaks at Day 0, Day 3 and Day 6. Between and after the peak exposure the plants were transferred to untreated growth medium.

In **design 2** the Lemna plants were exposed to two 24 hours lasting peaks on Day 0 and Day 7.

In both test designs the test duration was 14 days and after 7 days only 12 fronds per replicate were transferred to the second week to avoid nutrient and space constraints.

Frond numbers and total frond area of plants are recorded for both designs after 0, 3 (prior to exposure to the second peak in design 1), 5 and 7 days (before and after thinning each replicate to 12 fronds), 10, 12 days and at the test end. Growth and growth inhibition were calculated. The concentrations which inhibited the growth of this species by 10, 20, and 50 percent (EC_{10}, EC_{20}, EC_{50}) were determined.

In both designs the same peak concentrations were tested: 7.00, 21.3, 64.8, 197 and 600 µg a.s./L. Additionally, control and solvent control for each exposure design were performed in parallel and were handled in the same way as the respective test concentrations.

Samples were analysed for the actual concentration of aclonifen present in the test medium in freshly prepared and aged treatment levels including controls. Measured test concentrations ranged from 108 to 118% of nominal concentrations in freshly prepared solutions and from 104 to 119 in the aged solutions. Therefore, the study endpoints were calculated based on nominal test concentrations.

The exposure scenario with three 24 hours peaks on Day 0, Day 3 and Day 6 over the course of one week (design 1) resulted in E_rC_{50} values after 7 days of 447 and 127 µg a.s./L for frond number and frond area, respectively. After 14 days, the E_rC_{50} values were calculated to be 104 and 117 µg a.s./L for frond number and frond area, respectively.

The exposure scenario of two 24 hours peaks on Day 0 and Day 7 over the course of two weeks (design 2) resulted in higher E_rC_{50} values of >600 and 469 µg a.s./L for frond number and frond area after 7 days, respectively. After 14 days, the E_rC_{50} values were calculated to be 154 and 127 µg a.s./L for frond number and frond area, respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** Aclonifen technical (BCS-AG74518)
   Batch no.: AE F068300-01-14
   Origin Batch ID: PEA1000295
   Purity: 99.5% w/w
   Expiry: 26 November 2016

2. **Test organism:** *Lemna gibba*
   Strain: G3
   Source: [redacted]

3. **Treatment:** In both test designs, nominal test concentrations were tested: control, solvent control, 7.00, 21.3, 64.8, 197 and 600 µg a.s./L

4. **Test vessels:** Glass dishes, diameter 10cm, total volume ca. 470 mL, covered with glass lids to permit gas exchange and illumination
   Test water: 20X-AAP medium, pH adjusted to 7.5 ± 0.1

B. STUDY DESIGN AND METHODS

1. **In-life phase:** 3 to 6 November 2016

2. **Exposure conditions:**
   Temperature: 24.2 – 24.7 (Days 0 – 14)
   pH: 7.5 – 9.1 (Days 0 – 14)
   Photoperiod: Continuous illumination, mean 6600, range 6530 - 6690 lux

3. **Dose preparation**

Prior to the start of each peak exposure the stock solution was prepared by solving 201 mg of the test substance ad 5 mL DMF intense stirring for 3 to 10 minutes. An adequate amount of the stock solution was transferred to a dilution series to obtain the concentration levels used in the study. A solvent control and control exposure treatment were run along with nominal test exposure concentrations of 7.00, 21.3, 64.8, 197 and 600 µg a.s./L.

The test item was applied into the freshly prepared test medium on Days 0, 3, 6 and 7. Plants were transferred within 5 minutes of spiking the growth medium with test item.

4. Test organism assignment and treatment

Two different peak exposure designs were performed:

- Design 1: 3 peaks (Day 0, 3 and 6), each lasting 24 hours
- Design 2: 2 peaks (Day 0 and 7), each lasting 24 hours

Between and after the peaks the plants were transferred to untreated growth medium.

Colonies used for test were from an inoculum culture 7-10 days old. Each test vessel contained a total of 12 fronds (3-4 fronds per plant), with 3 replicates per treatment. The test vessels were placed in a random order and were repositioned each observation day (Days 3, 5, 7, 10, 12 and 14).

To avoid nutrient depletion and space limitations in the test vessels, only 12 fronds of each replicate were transferred for both designs after Day 7.

5. Measurements and observations

Visual observations were made on Days 3, 5, 7, 10, 12 and 14, with frond counts and determination of total frond areas carried out using a Lemna Tec Scnalyzer machine, validated for such measurements.

Temperature was determined by continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature was recorded hourly by a data logger. The pH was measured in all freshly prepared and all aged test levels and the controls. The light was measured at least once during the test.

No remarkable observations of the test item in the test medium were recorded for the test concentrations 7.00 to 64.8 µg a.s./L. The medium of the test concentration of 197 µg a.s./L was slightly yellowish and the highest test concentration 600 µg a.s./L was yellowish

Samples were analysed for the actual concentration of aclonifen tech. present in all freshly prepared test levels on Day 0, 3, 6 and 7 and in all aged test levels on Day 1, 4, 7 and 8 of the exposure period. Aliquots of freshly prepared test levels for Day 0, 3, 6 and 7 analyses were sampled from the prepared volume of each treatment level. For sampling of aged test media, after transferring the plants in vessels with freshly prepared untreated media on Day 1, Day 4, Day 7 and on Day 8, the contents of all replicate vessels were combined, and the pH was measured. Aliquots from the combined test solutions were then submitted for analysis. Additionally, samples of the untreated medium were taken in between the peak exposure periods. However, these samples were not measured since the analytical results of the peak exposure samples and the biological results were clear and no further information could be gained by measuring these samples of the untreated media. Samples were analysed by HPLC-MS/MS.

6. Statistics

Calculations were carried out using Microsoft Excel® spreadsheets. All further statistical evaluations were done using the commercial program ToxRat Professional, version 3.2.1 (2015).

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Samples were analysed for the actual concentration of aclonifen present in the test medium at all freshly prepared and all aged treatment levels including controls.

The analytical measurements resulted in recoveries within 80 to 120% of nominal. In the controls no test substance was detected.

The results were based on nominal values since all measurements showed a correct dosing and proved the stability of the test item within the peak exposure.

Table: Measured concentrations (µg/L) of Aclonifen (aclonifen) in the exposure solutions – Test design 1 (three 24 hour peaks on day 0, 3 and 6)

Design 1, 1st peak (day 0 – 1)				
Nominal concn (µg a.s./L)	Measured concn (µg a.s./L)		% of nominal	
	Day 0	Day 1	Day 0	Day 1
Control	<0.625	<0.625	-	-
Solvent control	<0.625	<0.625	-	-
7.00	8.18	8.14	117	116
21.3	23.8	22.9	112	107
64.8	73.6	73.0	114	113
197	222	229	113	116
600	707	699	118	116
Design 1, 2nd peak (day 3 – 4)				
	Measured concn (µg a.s./L)		% nominal	
	Day 3	Day 4	Day 3	Day 4
Control	<0.625	<0.625	-	-
Solvent control	<0.625	<0.625	-	-
7.00	8.14	7.92	116	113
21.3	23.0	22.2	108	104
64.8	75.3	71.1	116	110
197	232	222	118	113
600	710	711	118	119
Design 1, 3rd peak (day 6 - 7)				
	Measured concn (µg a.s./L)		% nominal	
	Day 6	Day 7	Day 6	Day 7
Control	<0.625	<0.625	-	-
Solvent control	<0.625	<0.625	-	-
7.00	8.27	8.04	118	115
21.3	23.8	22.8	112	107
64.8	76.7	76.5	118	118
197	225	220	114	112
600	710	698	118	116

Limit of quantification (LoQ) = 0.625 µg a.s./L

Table: Measured concentrations (µg/L) of Aclonifen (aclonifen) in the exposure solutions – Test design 2 (two 24 hour peaks on day 0 and 7)

Design 2, 1st peak (day 0 – 1)				
Nominal concn (µg a.s./L)	Measured concn (µg a.s./L)		% of nominal	
	Day 0	Day 1	Day 0	Day 1
Control	<0.625	<0.625	-	-
Solvent control	<0.625	<0.625	-	-
7.00	8.18	8.14	117	116
21.3	23.8	22.9	112	107
64.8	73.6	73.0	114	113
197	222	229	113	116
600	707	699	118	116
Design 2, 2nd peak (day 7 - 8)				
	Measured concn (µg a.s./L)		% nominal	
	Day 7	Day 8	Day 7	Day 8
Control	<0.625	<0.625	-	-
Solvent control	<0.625	<0.625	-	-
7.00	8.06	7.52	115	107
21.3	23.3	22.2	110	104
64.8	73.1	72.7	113	112
197	215	213	109	108
600	669	670	112	112

Limit of quantification (LoQ) = 0.625 µg a.s./L

The validated method is summarised in Document M-CA4 (CA 4.1.2/92).

B. BIOLOGICAL DATA

Frond number

Mean frond numbers from test design 1 are presented in the following table:

Table: Frond counts, growth rate and % inhibition, Test design 1 (three 24 hour peaks on day 0, 3 and 6)

Nominal concn (µg a.s./L)	Day 0	Day 3	Day 5	Day 7	Growth rate µ	% inhibition
	Mean (%CV)				(1/d)	
Control	12	45.3 (2.5)	82.0 (12.4)	163 (17.7	0.371	-
Solvent control	12	39.3 (9.6)	86.3 (10.1)	168 (3.1)	0.377	-
7.00	12	45.7 (8.3)	92.3 (12.5)	186 (12.8)	0.391	-4.4-
21.3	12	36.3 (6.9)	72.0 (15.8)	115 (24.0)	0.320*	14.6
64.8	12	23.0 (7.5)	53.3 (22.0)	73.0 (14.5)	0.257*	31.3
197	12	21.3 (5.4)	38.3 (8.0)	44.7 (5.6)	0.188*	49.9
600	12	21.0 (20.8)	39.7 (3.9)	51.3 (13.0)	0.207*	44.7
Nominal concn (µg a.s./L)	**Day 7**	**Day 10**	**Day 12**	**Day 14**	**Growth rate µ**	**% inhibition**
	Mean (%CV)				**(1/d)**	

Control	12	39.3 (3.9)	76.3 (3.3)	157 (0.7)	0.367	-
Solvent control	12	43.0 (10.7)	86.7 (8.5)	151 (12.5)	0.361	-
7.00	12	37.0 (11.8)	79.7 (4.8)	156 (8.2)	0.366	-0.7
21.3	12	27.3 (15.2)	53.7 (17.7)	95.7 (11.5)	0.296*	18.7
64.8	12	13.0 (7.7)	29.0 (9.1)	47.0 (11.3)	0.194*	46.6
197	12	15.0 (6.7)	22.0 (23.6)	33.7 (12.0)	0.147*	59.7
600	12	13.3 (8.7)	15.7 (13.3)	18.7 (12.4)	0.062*	82.9

Negative % inhibition indicates growth relative to control
* Statistically significant compared to pooled control based on [redacted] Multiple sequential test procedure

Mean frond numbers from test design 2 are presented in the following table:

Table: Frond counts, growth rate and % inhibition, Test design 2 (two 24 hour peaks on days 0 and 7)

Nominal concn (µg a.s./L)	Day 0	Day 3	Day 5	Day 7	Growth rate µ	% inhibition
	Mean (%CV)				(1/d)	
Control	12	40.0 (7.5)	83.7 (12.0)	161 (7.2)	0.371	-
Solvent control	12	44.0 (13.6)	94.3 (13.0)	199 (15.5)	0.400	-
7.00	12	39.3 (8.9)	77.7 (9.8)	151 (6.0)	0.362*	6.1
21.3	12	35.7 (4.3)	78.7 (7.2)	149 (3.4)	0.360*	6.6
64.8	12	25.3 (4.6)	60.7 (6.2)	118 (10.0)	0.326*	15.5
197	12	21.0 (12.6)	48.7 (11.3)	96.3 (2.2)	0.298*	22.8
600	12	22.0 (7.9)	38.0 (11.5)	65.0 (6.7)	0.241*	37.4
Nominal concn (µg a.s./L)	**Day 7**	**Day 10**	**Day 12**	**Day 14**	**Growth rate µ**	**% inhibition**
	Mean (%CV)				(1/d)	
Control	12	40.7 (5.1)	88.7 (1.3)	164 (6.5)	0.374	-
Solvent control	12	44.7 (8.5)	96.7 (2.2)	179 (2.3)	0.386	-
7.00	12	37.7 (4.1)	84.7 (14.2)	161 (13.9)	0.370	2.6
21.3	12	26.0 (11.5)	50.7 (15.1)	105 (10.7)	0.309*	18.6
64.8	12	21.3 (7.2)	37.7 (23.2)	78.7 (13.9)	0.268*	29.6
197	12	16.7 (13.9)	23.0 (21.7)	42.3 (23.8)	0.177*	53.3

600	12	13.7 (4.2)	16.0 (12.5)	20.7 (7.4)	0.077*	79.6

Negative % inhibition indicates growth relative to control
* Statistically significant compared to pooled control (based on [redacted] Multiple sequential test procedure

Total frond area

Mean frond numbers from test design 1 are presented in the following table:

Table: Frond counts, growth rate and % inhibition, Test design 1 (three 24 hour peaks on day 0, 3 and 6)

Nominal concn (µg a.s./L)	Day 0	Day 3	Day 5	Day 7	Growth rate µ	% inhibition
	Mean (%CV)				(1/d)	
Control	12	45.3 (2.5)	82.0 (12.4)	163 (17.7	0.371	-
Solvent control	12	39.3 (9.6)	86.3 (10.1)	168 (3.1)	0.377	-
7.00	12	45.7 (8.3)	92.3 (12.5)	186 (12.8)	0.391	-4.4
21.3	12	36.3 (6.9)	72.0 (15.8)	115 (24.0)	0.320*	14.6
64.8	12	23.0 (7.5)	53.3 (22.0)	73.0 (14.5)	0.257*	31.3
197	12	21.3 (5.4)	38.3 (18.0)	44.7 (5.6)	0.188*	49.9
600	12	21.0 (20.8)	39.7 (3.9)	51.3 (13.0)	0.207*	44.7
Nominal concn (µg a.s./L)	**Day 7**	**Day 10**	**Day 12**	**Day 14**	**Growth rate µ**	**% inhibition**
	Mean (%CV)				**(1/d)**	
Control	12	39.3 (3.9)	76.3 (3.3)	157 (0.7)	0.367	-
Solvent control	12	43.0 (10.7)	86.7 (8.5)	151 (12.5)	0.361	-
7.00	12	37.0 (11.8)	79.7 (4.8)	156 (8.2)	0.366	-0.7
21.3	12	27.3 (13.2)	53.7 (17.7)	95.7 (11.5)	0.296*	18.7
64.8	12	13.0 (7.7)	29.0 (9.1)	47.0 (11.3)	0.194*	46.6
197	12	15.0 (6.7)	22.0 (23.6)	33.7 (12.0)	0.147*	59.7
600	12	13.3 (8.7)	15.7 (13.3)	18.7 (12.4)	0.062*	82.9

Negative % inhibition indicates growth relative to control
* Statistically significant compared to pooled control (based on [redacted] Multiple sequential test procedure

Total frond area from test design 1 are presented in the following table:

Table: Total frond area and % inhibition, Test design 1 (three 24 hour peaks on days 0, 3 and 6)

Nominal concn (µg a.s./L)	Day 0	Day 3	Day 5	Day 7	Growth rate µ	% inhibition

	Total frond area (mm²) (%CV)				(1/d)	
Control	111 (2.7	394 (4.5)	795 (13.4)	1449 (22.2)	0.364	-
Solvent control	100 (4.1)	349 (7.2)	744 (7.3)	1503 (5.5)	0.387	-
7.00	114 (14.5)	389 (19.5)	828 (17.1)	1699 (15.4)	0.386	-2.8
21.3	109 (2.8)	327 (3.4)	635 (7.2)	1008 (24.3)	0.314*	16.2
64.8	112 (10.8)	208 (9.6)	324 (16.7)	413 (15.4)	0.185*	50.6
197	115 (8.5)	193 (11.1)	252 (7.2)	300 (8.7)	0.138*	63.4
600	113 (8.0)	184 (13.1)	248 (12.0)	297 (9.2)	0.137*	63.4
Nominal concn (µg a.s./L)	**Day 7**	**Day 10**	**Day 12**	**Day 14**	**Growth rate µ (1/d)**	**% inhibition**
	Mean (%CV)					
Control	121 (4.6)	355 (6.2)	714 (3.9)	1318 (3.3)	0.341	-
Solvent control	124 (5.4)	382 (6.0)	773 (7.7)	1282 (10.0)	0.333	-
7.00	121 (8.5)	354 (9.9)	721 (10.0)	1296 (10.0)	0.339	-0.6
21.3	112 (11.0)	205 (23.9)	383 (26.0)	672 (23.3)	0.254*	24.4
64.8	82.0 (8.8)	120 (13.1)	131 (7.3)	234 (4.6)	0.215*	36.1
197	63.3 (43.0)	81.0 (19.9)	101 (15.8)	158 (15.2)	0.138*	59.2
600	65.3 (32.7)	80.3 (35.2)	88.0 (31.6)	98.0 (25.8)	0.060*	82.1

Negative % inhibition indicates growth relative to control
* Statistically significant compared to pooled control (based on [redacted] Multiple sequential test procedure

Total frond area from test design 2 are presented in the following table:

Table: Total frond area and % inhibition, Test design 2 (two 24 hour peaks on days 0 and 7)

Nominal concn (µg a.s./L)	Day 0	Day 3	Day 5	Day 7	Growth rate µ	% inhibition
	Total frond area (mm²) (%CV)				(1/d)	
Control	105 (2.2)	357 (7.2)	716 (9.9)	1383 (10.6)	0.368	-
Solvent control	114 (12.9)	393 (14.5)	818 (14.8)	1638 (15.4)	0.381	-
2.00	101 (10.9)	328 (10.9)	687 (13.6)	1306 (10.4)	0.366	2.2
21.3	107 (3.0)	328 (6.8)	676 (5.5)	1239 (3.4)	0.349*	6.7
64.8	115 (6.5)	214 (8.3)	420 (837)	758 (8.8)	0.270*	28.0

197	109 (9.6)	185 (12.7)	320 (6.7)	552 (4.8)	0.232*	38.0
600	115 (10.1)	193 (6.7)	287 (5.6)	408 (3.8)	0.182*	51.4
Nominal concn (µg a.s./L)	**Day 7**	**Day 10**	**Day 12**	**Day 14**	**Growth rate µ (1/d)**	**% inhibition**
	Mean (%CV)					
Control	127 (3.0)	39. (5.9)	795 (4.6)	1369 (8.2)	0.348	-
Solvent control	127 (7.4)	413 (4.2)	866 (0.9)	1481 (3.9)	0.251	
7.00	114 (3.9)	352 (9.4)	756 (10.7)	1442 (17.1)	0.361	-4.5
21.3	111 (6.5)	234 (8.5)	424 (11.8)	761 (9.9)	0.274*	20.5
64.8	82.3 (17.4)	124 (15.2)	207 (17.5)	382 (17.1)	0.219*	36.4
197	76.3 (5.5)	101 (5.8)	132 (13.5)	220 (19.0)	0.150*	56.6
600	77 (10.6)	95.0 (7.6)	104. (7.7)	121 (8.6)	0.064*	81.4

Negative % inhibition indicates growth relative to control

* Statistically significant compared to pooled control (based on [redacted] Multiple sequential test procedure

Growth effects

Within 7 days of exposure in design 1 sublethal effects in terms of small fronds were observed in each test concentration up to and including 21.3 µg a.s./L. Additionally, necrotic fronds were observed in 64.8, 197 and 600 µg a.s./L and white fronds in 600 µg a.s./L. The same results were observed at the end of week 2.

In design 2, within the first week, smaller fronds were observed in the concentrations from 21.3 to 600 µg a.s./L. Additionally necrotic fronds were observed in 197 and 600 µg a.s./L. In the second week smaller fronds were recorded in the test concentrations of 21.3 to 600 µg a.s./L. Necrotic fronds were observed at 64.8, 197 and 600 µg a.s./L. Additionally, white fronds were recorded in the highest test concentration.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 221, 2006)	Achieved
Doubling time of frond number in the control <2.5 days (60 h), corresponding to approximately 7-fold increase in 7 days	<2.5d	1.8 – 1.9d

The frond number increased in the controls by a factor corresponding to a doubling time (Td) of about 1.9 days after 7 days in test designs 1 and 2 and by a factor corresponding to a doubling time of 1.8d after 14 days in test designs 1 and 2, therefore the validity criterion was met and the study can be considered valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

		Nominal concn (µg a.s./L)			
		Test design 1		Test design 2	
		7 days	14 days	7 days	14 days
Mean frond number growth rate	E_rC_{50} (95% CI)	447 (333 – 653)	104 (93.7 – 115)	>600 (n.d.)	154 (134 – 178)
	E_rC_{20} (95% CI)	32.6 21.2 – 45.2)	22.5 (18.9 – 26.2)	125 (107 – 144)	32.8 (25.6 – 40.7)
	E_rC_{10} (95% CI)	8.31 (4.10 – 13.7)	10.1 (7.95 – 12.4)	31.3 (23.3 – 39.8)	14.6 (10.3 – 19.3)
	LOErC	21.3	21.3	≥7.00	21.3
	NOErC	7.00	7.00	≥7.00	7.00
Mean total frond area growth rate	E_rC_{50} (95% CI)	128 (111 – 149)	117 (101 – 136)	469 (418 – 532)	127 (110 – 148)
	E_rC_{20} (95% CI)	18.9 (14.5 – 23.5)	24.0 (18.5 – 29.8)	53.2 (46.3 – 60.4)	26.9 (21.0 – 33.1)
	E_rC_{10} (95% CI)	6.93 (4.76 – 9.40)	10.5 (7.27 – 14.1)	17.4 (13.7 – 20.7)	11.9 (8.42 – 15.8)
	LOErC	21.3	21.3	21.3	21.3
	NOErC	7.00	7.00	7.00	7.00

Test design 1: three 24 hour peaks on Day 0, 3 and 6

Test design 2: two 24 hour peaks on Day 0 and 7

III. CONCLUSION

The exposure scenario with three 24 hours peaks on Day 0, Day 3 and Day 6 over the course of one week (design 1) resulted in E_rC_{50} values after 7 days of 447 and 127 µg a.s./L for frond number and frond area, respectively. After 14 days, the E_rC_{50} values were calculated to be 104 and 117 µg a.s./L for frond number and frond area, respectively.

The exposure scenario of two 24 hours peaks on Day 0 and Day 7 over the course of two weeks (design 2) resulted in higher E_rC_{50} values of >600 and 469 µg a.s./L for frond number and frond area after 7 days, respectively. After 14 days, the E_rC_{50} values were calculated to be 154 and 127 µg a.s./L for frond number and frond area, respectively.

[redacted] *(2018)*

Assessment and conclusion by applicant:

The validity criterion was met therefore this study is considered to be acceptable.

The exposure scenario with three 24 hours peaks on Day 0, Day 3 and Day 6 over the course of one week (design 1) resulted in E_rC_{50} values after 7 days of 447 and 127 µg a.s./L for frond number and frond area, respectively. After 14 days, the E_rC_{50} values were calculated to be 104 and 117 µg a.s./L for frond number and frond area, respectively.

The exposure scenario of two 24 hours peaks on Day 0 and Day 7 over the course of two weeks (design 2) resulted in higher E_rC_{50} values of >600 and 469 µg a.s./L for frond number and frond area after 7 days, respectively. After 14 days, the E_rC_{50} values were calculated to be 154 and 127 µg a.s./L for frond number and frond area, respectively.

Assessment and conclusion by RMS:

Data Point:	KCA 8.2.7/13
Report Author:	[redacted]
Report Year:	2018
Report Title:	Lemna gibba G3 - Growth inhibition test with aclonifen tech. (BCS-AG74518) under peak exposure conditions (peaks on day 0 and 3)
Report No:	EBCL0022
Document No:	M-612732-01-1
Guideline(s) followed in study:	EU Directive 91/414/EEC Regulation1107/2009 (Europe) OECD Test Guideline 221 US EPA OCSPP 850.4400
Deviations from current test guideline:	Current Guideline: OECD 221, 2006 pH slightly above the recommended pH range of 7.5 ± 0.1. This deviation was not considered to have affected study integrity and validity.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

The effects of Aclonifen, on the growth and reproduction of the aquatic monocotyledonous plant, *Lemna gibba,* were investigated under pulsed exposure conditions. The plants were exposed to the test item under defined conditions with two 24-hour lasting peaks on Day 0 and Day 3. Between and after the exposure peaks the plants were transferred to untreated nutrient medium. The nominal concentrations of 7.00, 21.3, 64.8, 197 and 600 µg a.s./L in comparison to a pooled control (control and solvent control) were tested. At start of the second week (Day 7) only 12 fronds out of each replicate were transferred to the freshly prepared exposure media to avoid space limitations in the test vessels and nutrient depletion. The complete test duration was 14 days.

Frond numbers and total frond area of plants were recorded on Day 0, 3 (prior to exposure to the second peak), 5 and 7 (before and after thinning each replicate to 12 fronds), 10, 12 and 14. Growth and growth inhibition were determined. The concentrations which inhibited the growth of this species by 10, 20, and 50 percent (EC_{10}, EC_{20}, EC_{50}) were determined.

Samples were analysed for the actual concentration of aclonifen present in the test medium in freshly prepared and aged treatment levels including controls. Measured test concentrations ranged from 104 to 118% of nominal concentrations in freshly prepared solutions and from 104 to 117 in the aged solutions. Therefore, the study endpoints were calculated based on nominal test concentrations.

At the end of the first week after the two peak exposures, the E_rC_{50} was calculated to be 421 µg a.s./L for frond number and 90.1 µg a.s./L for frond area. At the end of the second week the effects were reduced resulting in E_rC_{50} values of greater than 600 µg a.s./L for both frond number and frond area.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:** Aclonifen technical (BCS-AG74518)
   Batch no.: AE F068300-01-15
   Origin Batch ID: PEA1000563
   Purity: 99.5% w/w
   Expiry: 15 August 2018

2. **Test organism:** *Lemna gibba*
   Strain: G3
   Source: [redacted]

3. **Treatment:** In both test designs nominal test concentrations were tested: control, solvent control, 7.00, 21.3, 64.8, 197 and 600 µg a.s./L

4. **Test vessels:** Glass dishes, diameter 10cm, total volume ca. 470 mL, covered with glass lids to permit gas exchange and illumination
   Test water: 20X-AAP medium, pH adjusted to 7.5 ± 0.1

B. STUDY DESIGN AND METHODS

1. **In-life phase:** 20 April to 19 May 2017
2. **Exposure conditions:**
   Temperature: 23.7 – 24.2°C (Days 0 – 14)
   pH: 7.9 – 9.1 (Days 0 – 14)
   Photoperiod: Continuous illumination, mean 6810, range 6600 - 6970 lux
3. **Dose preparation**

Prior to the start of each peak exposure the stock solution was prepared by solving 30.2 mg of the test substance in 5 mL dimethylformamide (DMF). An adequate amount of the stock solution was transferred to a dilution series to obtain the concentration levels used in the study. A solvent control and control exposure treatment were run along with nominal test exposure concentrations of 7.00, 21.3, 64.8, 197 and 600 µg a.s./L.

The test item was applied into the freshly prepared test medium on Day 0 and 3.

4. **Test organism assignment and treatment**

Colonies used for test were from an inoculum culture 7-10 days old. Each test vessel contained a total of 12 fronds (3-4 fronds per plant), with 3 replicates per treatment. The test vessels were placed in a random order and were repositioned each observation day (Days 3, 5, 7, 10, 12 and 14).

To avoid nutrient depletion and space limitations in the test vessels, only 12 fronds of each replicate were transferred for both designs after Day 7.

5. Measurements and observations

Visual observations were made on Days 3, 5, 7, 10, 12 and 14, with frond counts and determination of total frond areas carried out using a Lemna Tec Scnalyzer machine, validated for such measurements.

Temperature was determined by continuous measurement in one additional incubated glass vessel filled with the same amount of de-ionised water as in the test vessels. Temperature was recorded hourly by a data logger. The pH was measured in all freshly prepared and all aged test levels and the controls. The light was measured at least once during the test.

For the analysis of the test item concentrations, duplicate samples of the freshly prepared test media on Day 0 and 3 (start of the peak exposures) were taken from all test levels and the controls. Duplicates samples of aged test media were taken from pooled replicates of each test level and the controls on Days 1 and 4 (end of the peak exposures). All samples were stored deep-frozen (at about ≤ -18°C) immediately after sampling and were kept stored under these conditions until analysis. One of each duplicate sample were analysed for the actual concentration of aclonifen in all freshly prepared test levels of Days 0 and 3 and in all aged test levels of Days 1 and 4 of the exposure period. The B-samples, stored as retain samples were not measured. Samples were analysed by HPLC-MS/MS.

6. Statistics

Calculations were carried out using Microsoft Excel® spreadsheets. All further statistical evaluations were done using the commercial program ToxRat Professional, version 3.2.1 (2015).

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The analytical measurements resulted in recoveries within 80 to 120% of nominal. In the controls no test substance was detected.

The results were based on nominal values since all measurements showed a correct dosing and proved the stability of the test item within the peak exposure.

No remarkable observations of the test item in the test medium were recorded for the test concentrations 7.00 to 197 µg a.s./L. The medium of the highest test concentration of 600 µg a.s./L was slightly yellowish.

Table: Measured concentrations (µg/L) of Aclonifen (aclonifen) in the exposure solutions

Exposure Peak 1 (day 0 – 1)				
Nominal concn (µg a.s./L)	Measured concn (µg a.s./L)		% of nominal	
	Day 0	Day 1	Day 0	Day 1
Control	<0.625	<0.625	-	-
Solvent control	<0.625	<0.625	-	-
7.00	8.18	8.20	117	117
21.3	23.0	23.0	108	108
64.8	71.0	68.7	109	106
197	208	204	106	104
600	624	658	104	109
Exposure Peak 2 (day 3 – 4)				
	Measured concn (µg a.s./L)		% nominal	
	Day 3	Day 4	Day 3	Day 4
Control	<0.625	<0.625	-	-
Solvent control	<0.625	<0.625	-	-
7.00	8.29	7.90	118	113
21.3	22.8	22.4	107	105
64.8	70.6	69.7	109	108
197	220	218	112	111
600	644	626	107	104

Limit of quantification (LoQ) = 0.625 µg a.s./L

The validated method is summarised in Document M-CA4 (CA 4.1.2/93).

B. BIOLOGICAL DATA

Frond number

Mean frond numbers from test design 1 are presented in the following table:

Table: Frond counts, doubling time and % inhibition of average growth rates

Nominal concn (µg a.s./L)	Day 0	Day 3	Day 5	Day 7	Growth rate µ	% inhibition
	Mean (%CV)				(1/d)	
Control	12	33.0 (5.2)	65.0 (10.1)	129.7 (15.2)	0.339	-
Solvent control	12	37.0 (9.7)	76.7 (10.5)	149.7 (8.7)	0.360	-
7.00	12	32.3 (4.7)	65.7 (2.3)	131.3 (6.3)	0.342	2.2
21.3	12	33.0 (13.2)	66.7 (8.7)	105.0 (10.0)	0.309*	11.5
64.8	12	22.7 (11.1)	40.7 (13.5)	59.3 (8.6)	0.228*	34.8
197	12	19.3 (7.9)	30.7 (7.5)	44.3 (3.4)	0.187*	46.6
600	12	21.7 (25.4)	31.0 (16.8)	43.7 (10.6)	0.184*	47.3
Nominal concn (µg a.s./L)	**Day 7**	**Day 10**	**Day 12**	**Day 14**	**Growth rate µ**	**% inhibition**
	Mean (%CV)				**(1/d)**	
Control	12	40.0 (2.5)	87.0 (4.1)	170.0 (9.7)	0.378	-

Solvent control	12	39.3 (6.4)	77.3 (9.5)	145.0 (13.1)	0.355	-
7.00	12	37.7 (9.3)	77.0 (8.1)	152.3 (10.0)	0.363	1.1
21.3	12	37.7 (8.1)	70.3 (1.6)	131.7 (7.1)	0.342*	6.7
64.8	12	35.3 (7.1)	70.0 (13.6)	121.3 (18.1)	0.329*	10.3
197	12	31.7 (7.9)	60.0 (11.5)	100.3 (10.0)	0.303*	17.4
600	12	22.7 (15.5)	39.7 (8.1)	65.3 (7.2)	0.242*	34.0

Negative % inhibition indicates growth relative to control

* Statistically significant compared to pooled control (based on [redacted] Multiple sequential test procedure

Total frond area

Total frond area from test design 1 are presented in the following table:

Table: Total frond area and % inhibition of their average growth rates

Nominal concn (µg a.s./L)	Day 0	Day 3	Day 5	Day 7	Growth rate µ	% inhibition
	Total frond area (mm^2) (%CV)				(1/d)	
Control	102.7 (5.7)	248.3 (6.5)	514.3 (9.9)	1098.7 (13.0)	0.338*	-
Solvent control	110.3 (5.9)	288.0 (11.5)	613.0 (11.2)	1231.0 (8.0)	0.344	-
7.00	103.0 (5.0)	260.0 (4.4)	556.7 (4.5)	1098.0 (8.0)	0.338	1.0
21.3	111.7 (10.3)	262.3 (17.1)	496.0 (15.4)	769.0 (14.5)	0.275*	19.4
64.8	105 (8.1)	170.3 (8.4)	221.7 (13.4)	285.0 (12.6)	0.142*	58.3
197	109.3 (4.6)	169.7 (3.2)	190.3 (3.5)	239.0 (1.8)	0.112*	67.2
600	108 (10.5)	157.3 (11.9)	185.3 (13.4)	224.0 (12.0)	0.104*	69.5
Nominal concn (µg a.s./L)	**Day 7**	**Day 10**	**Day 12**	**Day 14**	**Growth rate µ**	**% inhibition**
	Mean (%CV)				**(1/d)**	
Control	111.3 (8.5)	374.3 (9.5)	783.0 (2.8)	1465.0 (9.6)	0.368	-
Solvent control	109 (6.9)	346.3 (8.6)	687.0 (11.9)	1216.7 (13.6)	0.344	-
7.00	105.3 (8.5)	331.7 (13.9)	682.7 (13.4)	1259.7 (11.1)	0.354	0.5
21.3	107.7 (9.5)	300.7 (7.9)	578.7 (8.1)	1042.0 (11.5)	0.324*	9.0
64.8	68.0 (4.4)	205.7 (9.2)	405.7 (11.2)	762.7 (16.1)	0.344*	3.3
197	68.3 (5.9)	182.7 (8.2)	345.7 (6.4)	618.3 (6.8)	0.315*	11.6
600	66.7 (7.1)	140.7 (11.1)	260.7 (8.9)	450 (7.8)	0.273*	23.4

Negative % inhibition indicates growth relative to control

* Statistically significant compared to pooled control (based on [redacted] Multiple sequential test procedure

Growth effects

By Day 3 sublethal effects (smaller fronds and detached fronds) were observed at 64.8 µg a.s./L and above. Within 7 days of exposure sublethal effects in terms of small, necrotic and detached fronds were observed in each test concentration up to and including 64.8 µg a.s./L.

Observations from Day 10 to the end of the study (day 14) found smaller fronds in the nominal exposure concentrations of 64.8 and 197 µg a.s./L with plants in the 600 µg a.s./L exposure concentration additionally being observed to have necrotic fronds.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 221, 2006)	Achieved
Doubling time of frond number in the control <2.5 days (60 h), corresponding to approximately 7-fold increase in 7 days	2.5d	1.9 – 2.0d

The frond number increased in the controls by a factor corresponding to a doubling time (Td) of about 2.0 days after 7 days and by a factor corresponding to a doubling time of 1.9d after 14 days, therefore the validity criterion was met and the study can be considered valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

		Nominal concn (µg a.s./L)	
		7 days	**14 days**
Mean frond number growth rate	E_rC_{50} (95% CI)	421 (357 – 508)	>600
	E_rC_{20} (95% CI)	32.8 (26.4 – 39.6)	204 (152 – 264)
	E_rC_{10} (95% CI)	8.7 (6.1 – 11.6)	62.2 (34.3 – 91.2)
	LOErC	21.3	21.3
	NOErC	7.00	7.00
Mean total frond area growth rate	E_rC_{50} (95% CI)	90.1 (84.4 – 96.1)	>600
	E_rC_{20} (95% CI)	15.2 (13.6 – 16.9)	472 (348 – 709)
	E_rC_{10} (95% CI)	6.0 (5.1 – 6.9)	117 (69.0 – 165)
	LOErC	21.3	21.3
	NOErC	7.00	7.00

24 hour peak on Day 0 and 3

III. CONCLUSION

At the end of the first week after the two peak exposures, the E_rC_{50} was calculated to be 421 µg a.s./L for frond number and 90.1 µg a.s./L for frond area. At the end of the second week the effects were reduced resulting in E_rC_{50} values of greater than 600 µg a.s./L for both frond number and frond area.

[redacted] *(2018)*

Assessment and conclusion by applicant:

The validity criterion was met, therefore this study is considered to be acceptable.

At the end of the first week after the two peak exposures, the E_rC_{50} was calculated to be 421 µg a.s./L for frond number and 90.1 µg a.s./L for frond area. At the end of the second week the effects were reduced resulting in E_rC_{50} values of greater than 600 µg a.s./L for both frond number and frond area.

Assessment and conclusion by RMS:

CA 8.2.8 Further testing on aquatic organisms

No further testing on aquatic organisms was required.

CA 8.3 Effect on arthropods

Table 8.3-1: Summary of the effects of Aclonifen on arthropods

Test Species	Test Item	Time-scale Test type / substrate	Endpoint	Reference
Bees				
Honey bee *Apis mellifera* L.	Aclonifen	48 h Acute oral	**LD_{50} > 106.8 µg a.s./bee**	KCA 8.3.1.1.1/01 KCA 8.3.1.1.2/01 M-174936-01-1 [redacted], 1999
		48 h Acute contact	**LD_{50} > 100 µg a.s./bee**	
Bumble bee *Bombus terrestris* L.	Aclonifen	48 h Acute oral	**LD_{50} > 130.36 µg a.s./bee**	KCA 8.3.1.1.1/02 KCA 8.3.1.1.2/02 M-567133-01-1 [redacted], 2016
		48 h Acute contact	**LD_{50} > 150 µg a.s./bee**	
Honey bee *Apis mellifera* L	Aclonifen	8 d repeated exposure Larval toxicity	NOED = 25.0 µg a.s./larval stage	KCA 8.3.1.3/01 M-600773-01-1 [redacted], 2017
Honey bee *Apis mellifera* L	Aclonifen	22 d repeated exposure Larval toxicity	**NOED = 40.0 µg a.s./larva**	KCA 8.3.1.3/02 M-578600-01-1 [redacted], 2017

Endpoints in **bold** were used in the risk assessment

CA 8.3.1 Effects on bees

CA 8.3.1.1 Acute toxicity to bees

CA 8.3.1.1.1 Acute oral toxicity

Data Point:	KCA 8.3.1.1.1/01
Report Author:	[redacted]
Report Year:	1999
Report Title:	Final report - Laboratory Testing for Toxicity (Acute Contact and Oral LD50) of ACLONIFEN on Honey Bees (Apis mellifera L.) (Hymenoptera, Apidae)
Report No:	R007442
Document No:	M-174936-01-1
Guideline(s) followed in study:	EPPO: Bulletin 22, 203-215 No. 170 (1992)
Deviations from current test guideline:	Current Guideline: OECD 213/214, 1998 Starvation time extended from up to 120 minutes to up to 135 minutes to ensure bees were hungry. Complete uptake of contaminated food lasted up to 9 h (instead of up to 3 h) as bees avoided contaminated food. 5 µL droplet volume used rather than 1 µL recommended in the guideline. Environmental conditions slightly outside of recommended range. These deviations are not considered to have affected the integrity or outcome of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

An acute test was conducted to determine the acute oral and contact effect of aclonifen on mortality and behaviour of the honey bee, *Apis mellifera*. The test included a solvent control, a CO_2 treated negative control (contact test only) and test groups (100, 50, 25, 12.5 and 6.3 µg a.s./bee, nominal for both contact and oral testing, plus a toxic standard (dimethoate, 0.2 µg a.s./bee). Additionally, bees were assessed for any behavioural effects.

The contact test was 48 hours duration. There was no mortality in any aclonifen test treatments and the 48-hour LD_{50} was >100 µg a.s./bee. No behavioural effects were observed in any test treatment. There was 3.3% mortality in the CO_2 control treatment but no mortality in the solvent control.

The oral test was 48 hours duration. There was no mortality in any aclonifen test treatments and the 48-hour LD_{50} was >106.8 µg a.s./bee. No behavioural effects were observed in any test treatment. There was 3.3% mortality in the solvent control.

The toxicity of aclonifen was tested in both an acute contact and an oral toxicity test on honey bees. The LD_{50} (48 h) was >100 µg a.s./bee in the contact toxicity test and the LD_{50} (48 h) was >106.8 µg a.s./bee in the oral toxicity test.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen
	Batch no.:	97013/03
	Active Ingredient / Purity:	995 g/kg
	Appearance:	Yellow powder
	Storage:	Room temperature in the dark
	Expiry date:	18 December 2000 (re-analysis date)
2.	**Reference item:**	Perfekthion EC
	Batch no.:	98-1
	Active Ingredient / Purity:	396 g/L dimethoate
3.	**Test Organism:**	Worker honey bee, *Apis mellifera* L.
	Age:	Four to six weeks old female
	Source:	[redacted]
	Feeding:	Commercial ready-to-use syrup for honey bees (30% saccharose, 31% glucose, 39% fructose). Supplied by Apiinvest, Co. Südzucker AG, D-97199 Ochsenfurt

A. STUDY DESIGN AND METHODS

1.	**In-life phase:**	25 to 28 May 1999
2.	**Exposure conditions**	
	Test vessels:	Stainless steel cages 10 cm x 8.5 cm x 5.5 cm (length x width x height) with removable glass sheet
	Experimental design:	*Contact:* CO_2 control, CO_2/solvent control, test item 100, 50, 25, 12.5 and 6.3 µg a.s./bee; Dimethoate (toxic standard) 0.20 µg a.s./bee *Oral:* CO_2 control, test item 100, 50, 25, 12.5 and 6.3 µg a.s./bee; Dimethoate (toxic standard) 0.20 µg a.s./bee
	Replicates:	3 replicates per test item dose level, controls and toxic standard, consisting of 10 bees in one cage per test concentration
	Temperature:	27 - 28°C
	Relative humidity:	40 - 57%
	Photoperiod:	Darkness (except during observation)
3.	**Administration of the test item**	

Contact toxicity test

Bees were collected from edge positioned honeycombs without anaesthetic. Bees were anaesthetised with CO_2 until completely immobilised immediately before application of test treatments. A single 5 µL droplet of aclonifen in appropriate carrier (acetone) was placed on the ventral bee thorax using a Burkhard Applicator. For the control 1 x 5 µL droplet tap water containing acetone was used. The toxic standard was dimethoate (0.2 µg a.s./bee).

Oral toxicity test

Bees were collected from edge positioned honeycombs without anaesthetic. Bees were starved for 135 minutes in all treatment groups prior to application of test item. Approximately 30 mg aclonifen contaminated food (1 part solvent, 19 parts ready to use syrup).

Treated food was offered in syringes, which were weighed before and after introduction to cages. Duration of uptake did not exceed 3 hours, except in highest treatment group where uptake lasted 9 hours. After treatment, the syringes containing treated food were removed, weighed and replaced with fresh untreated food.

4. Measurements and observations

Observation of the bees was undertaken at the following times:

- 1, 2 and 4 hours (first day)
- 24 hours, 48 hours following days

Any cases of mortality and/or poisoning or behavioural abnormalities of the bees (e.g. food refusal, apathy, moving coordination problems) were recorded.

5. Statistics/Data evaluation

No mortality or behavioural changes were noted during the study, therefore, statistical analysis was not required.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Dose levels of test item in oral test were 106.8, 46.3, 30.6, 14.4 and 7.0 µg a.s./ bee.

No analytical verification of the dosing solutions for the contact test was performed.

No analytical verification of dose levels of dimethoate (toxic standard) were performed.

B. BIOLOGICAL DATA

Contact toxicity test

No behavioural abnormalities were observed in the test treatments at any time.

Table: Mean mortality and behavioural abnormalities of the bees in the contact toxicity test

Dose (µg/bee)	1h		4h		24h		48h	
	Mortality	Behav. abnorm.	Mortality	Behav. abnorm.	Mortality	Behav. abnorm.	Mortality	Behav. abnorm.
100	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0
12.5	0	0	0	0	0	0	0	0
6.25	0	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	0
Solvent control	0	0	0	0	0	0	0	0
Toxic Standard								
0.20	0	0	0	23.3	96.7	3.3	96.7	3.3

Results are averages from three replicates (ten bees each) per dosage/control

Oral toxicity test

No behavioural abnormalities were observed in the test treatments at any time.

Table: Mean mortality and behavioural abnormalities of the bees in the oral toxicity test

Dose (µg/bee)	1h		4h		24h		48h	
	Mortality	Behav. abnorm.	Mortality	Behav. abnorm.	Mortality	Behav. abnorm.	Mortality	Behav. abnorm.
100	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0
12.5	0	0	0	0	0	0	0	0
6.25	0	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	0
Solvent control	0	0	3.3	0	3.3	0	3.3	0
Toxic Standard								
0.20	0	0	16.7	20.0	86.7	6.7	90.0	3.3

Results are averages from three replicates (ten bees each) per dosage/control

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 213/214, 1998)	Achieved
Mortality in controls	≤10%	3.3% (contact test) 3.3% (oral test)
Oral LD_{50} of the toxic standard (dimethoate)	0.10 – 0.35 µg a.i./bee	90% mortality after 48h at 0.2 µg a.i./bee
Contact LD_{50} of the toxic standard (dimethoate)	0.10 – 0.30 µg a.i./bee	96.7% mortality after 48h at 0.2 µg a.i./bee

The study was conducted according to EPPO test guideline (1992). The OECD 213/214 validity criteria regarding control mortality were met. The toxic standard showed 90 and 96.7% mortality for oral and contact test. Therefore it is considered that this study is valid for risk assessment purposes.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoints (µg a.s./bee)	1h	4h	24h	48h
Contact LD_{50} [95% confidence limits]	>100	>100	>100	>100
Oral LD_{50} [95% confidence limits]	>106.8	>106.8	>106.8	>106.8

III. CONCLUSION

The toxicity of aclonifen was tested in both an acute contact and an oral toxicity test on honey bees. The LD_{50} (48 h) was >100 µg a.s./bee in the contact toxicity test. The LD_{50} (48 h) was >106.8 µg a.s./bee in the oral toxicity test.

[redacted] *(1999)*

Assessment and conclusion by applicant:

The OECD 213/214 validity criteria regarding control mortality were met. The toxic standard showed 90 and 96.7% mortality for oral and contact test. Therefore, it is considered that this study is valid for risk assessment purposes.

The toxicity of aclonifen was tested in both an acute contact and an oral toxicity test on honey bees. The LD_{50} (48 h) was >100 µg a.s./bee in the contact toxicity test. The LD_{50} (48 h) was >106.8 µg a.s./bee in the oral toxicity test.

Assessment and conclusion by RMS:

Data Point:	KCA 8.3.1.1.2/02
Report Author:	[redacted]
Report Year:	2016
Report Title:	Aclonifen tech.: Acute oral and contact toxicity to the bumble bee, Bombus terrestris L. under laboratory conditions
Report No:	S15-00341
Document No:	M-567133-01-1
Guideline(s) followed in study:	OECD Guidelines No. 213 and No. 214 (1998), OEPP/EPPO 170 (4) (2010), VAN DER STEEN (2001) and recommendations of the ICPPR bumble bee ring test group (2015)
Deviations from current test guideline:	Current Guideline: OECD 246/247, 2017 Behavioural abnormalities in reference item treatment were not recorded. This deviation was considered not to have affected the integrity or outcome of the study
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

An acute test was conducted to determine the acute oral and contact effect of aclonifen on mortality and behaviour of the bumble bee, *Bombus terrestris L.*. The test included a solvent control and test groups 46.79, 57.33, 82.40, 95.75 and 130.36 µg a.s./bee, actual uptake for oral testing, plus a toxic standard (dimethoate, 1.43 µg a.s./bee). The contact test treatment concentrations were 52.2, 68, 89, 115 and 150 µg a.s./bee (nominal).

In the control and solvent control groups of the oral toxicity test 0% and 3.3% mortality was observed during the 48-hour test period, respectively. In the test treatment groups of the oral toxicity test at the second highest dose of 95.75 µg a.s./bumble bee (based on actual uptake) a mortality of 3.3% (corrected mortality: 0%) was observed after 48 hours. No mortality was observed at the end of the 48-hour observation period in any other test treatment.

In the control and solvent control groups of the contact toxicity test 10% and 6.7% mortality was observed during the 48 h test period, respectively. In the test treatment groups of the contact toxicity test at the highest dose of 150 µg a.s./bumble bee a mortality of 3.3% (corrected mortality: -3.6%) was observed after 48 hours. The maximum mortality of 6.7% (corrected mortality: 0.0%) in the contact toxicity test was observed at the dose of 89 µg a.s./bumble bee.

In both the oral and contact toxicity tests no remarkable sublethal effects were observed during the test period of 48 hours.

In the reference item groups of the oral and contact toxicity tests mortalities of 90% and 100%, respectively were within the required range. The validity criteria were met, thus the test is considered to be valid.

The LD_{50} (48 h) was >150 µg a.s./bee in the contact toxicity test and the LD_{50} (48 h) was >130.36 µg a.s./bee in the oral toxicity test.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen technical (AE F068300)
   Batch no.: PEA 1000235
   Active Ingredient / Purity: 99.5% w/w (analysed)
   Appearance: Yellow solid
   Storage: Room temperature in the dark
   Expiry date: 26 November 2016 (re-analysis date)

2. **Reference item:** Perfekthion EC
   Batch no.: FRE-001226
   Active Ingredient / Purity: 400 g/L dimethoate

3. **Test Organism:** Adult worker bumble bee *Bombus terrestris* L.
   Age: Not specified
   Source: [redacted]
   Feeding: 50% (w/v) aqueous sucrose solution. Fed ad-libitum during acclimatisation and test period, except during starvation and feeding of test treatment solutions (oral toxicity test only)

B. STUDY DESIGN AND METHODS

1. **In-life phase:** 29 September to 01 October 2015

2. **Exposure conditions**
   Test vessels: Bees were housed individually in Nicot cages (queen bee schooling cages; slightly conical perforated plastic cylinder, base approx. 1 cm radius, height 7 cm
   Experimental design: *Contact:* Solvent control, test item 52.2, 68, 89, 115 and 150 µg a.s./bee (nominal);
   Dimethoate (toxic standard) 13 µg a.s./bee
   Oral: Solvent control; test item 52.2, 68, 89, 115 and 150 a.s./bee (nominal);

	Mean actual uptake calculated as 46.79, 57.33, 82.40, 95.75 and 130.36 µg a.s./bee; Dimethoate (toxic standard) 1.5 µg a.s./bee; mean actual calculated uptake 1.43 µg a.s./bee
Replicates:	30 replicates (1 bee) per test item dose level, controls and toxic standard
Temperature:	24.3 – 25.0°C
Relative humidity:	55.9 – 63.8%
Photoperiod:	Darkness (except during application and observation)

3. Administration of the test item

Stock solutions of test/reference item, using acetone as a solvent. In the oral toxicity test for the preparation of the highest dose level of 150 µg a.s./bumble bee, aqueous sucrose solution (w/v) containing 1% tween and 1% xanthan was used. Further dilutions of the stock solution were prepared using 50% (w/v) aqueous sucrose solution containing 5% acetone, 1% tween and 1% xanthan in order to get the required dose levels of application solution(s). For the reference item deionised water was used as solvent. In the oral toxicity test one further dilution of the stock solution was prepared using 50% (w/v) aqueous sucrose solution in order to get the required dose level.

Contact toxicity test

Bees were randomly collected from hive and introduced to test units, under test conditions, 1 day before test start. Bees were anaesthetised with CO_2 until completely immobilised immediately before application of test treatments. A single 2 µL droplet of aclonifen in appropriate carrier (acetone) was placed on the dorsal bee thorax using a Burkhard applicator. For the control 1 x 2 µL droplet tap water containing acetone was used.

Oral toxicity test

Bees were randomly collected from hive and introduced to test units, under test conditions, 1 day before test start. Bees were starved for approximately 2 hours in all treatment groups prior to application of test item.

Treated food was offered in syringes, which were weighed before and after introduction to cages using calibrated equipment. Duration of uptake did not exceed 4 hours. After treatment, the syringes containing treated food were removed, weighed and replaced with fresh untreated food.

4. Measurements and observations

Observation of the bees was undertaken at the following times:

- 4 hours (first day)
- 24 hours, 48 hours following days

Any cases of mortality and/or poisoning or behavioural abnormalities of the bees (e.g. food refusal, apathy, moving coordination problems) were recorded.

5. Statistics/Data evaluation

Fisher's Exact Binomial Test with Bonferroni Correction (one-sided, α = 0.05) was used to evaluate significant difference between solvent control and test treatment mortality at the end of the test. Statistical analyses were conducted using ToxRat Professional 3.1.0.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

No analytical verification of the dosing solutions for the contact test was performed.

No analytical verification of dose levels of dimethoate (toxic standard) were performed.

B. BIOLOGICAL DATA

Contact toxicity test

No remarkable sublethal effects were observed in the test treatments at any time.

Table: Mortality of the bees in the contact toxicity test

Dose (µg a.s./bee)	Mortality (%)		Corrected mortality (%)	
	24h	48h	24h	48h
52.2	0	0	-3.4	-7.2
68	0	0	-3.4	-7.2
89	3.3	6.7	0	0
115	0	3.3	-3.4	-3.6
150	3.3	3.3	0	-3.6
Control	0	10	-	-
Solvent control	3.3	6.7	-	-
Reference item (Perfekthion)				
13	93.3	100	93.3	100

Oral toxicity test

No remarkable sublethal effects were observed in the test treatments at any time.

Table: Mortality of the bees in the oral toxicity test

Target dose (µg/bee)	Mean actual uptake (µg a.s./bee)	Mortality (%)		Corrected mortality (%)	
		24h	48h	24h	48h
52.2	46.79	0	0	0	-3.4
68	57.33	0	0	0	-3.4
89	82.40	0	0	0	-3.4
115	95.75	3.3	3.3	3.3	0
150	130.36	0	0	0	-3.4
Control		0	0	-	-
Solvent control	-	0	3.3	-	-
Reference item (Perfekthion)					
1.5	1.43	90	90	-	-

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 246/247, 2017)	Achieved
Mortality in controls – oral test	≤10%	3.3%
Mortality in controls – contact test	≤10%	10%
Mortality in reference item group – oral test	≥50%	90%
Mortality in reference item group – contact test	≥50%	100%

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoints (µg a.s./bee)	24h	48h	NOED
Contact LD_{50} [95% confidence limits]	>150	>150	≥150
Oral LD_{50} [95% confidence limits]	>130.36	>130.36	≥130.36

III. CONCLUSION

The toxicity of aclonifen technical was tested in both an acute contact and an oral toxicity test on honey bees. The LD_{50} (48 h) was >150 µg a.s./bee in the contact toxicity test. The LD_{50} (48 h) was >130.36 µg a.s./bee in the oral toxicity test.

[redacted] *(2016)*

Assessment and conclusion by applicant:

The OECD 213/214 validity criteria regarding control mortality were met. The toxic standard showed 90 and 100% mortality for oral and contact test, respectively. Therefore, it is considered that this study is valid for risk assessment purposes.

The toxicity of aclonifen technical was tested in both an acute contact and an oral toxicity test on honey bees. The LD_{50} (48 h) was >150 µg a.s./bee in the contact toxicity test. The LD_{50} (48 h) was >130.36 µg a.s./bee in the oral toxicity test.

Assessment and conclusion by RMS:

CA 8.3.1.1.2 Acute contact toxicity

Data Point:	KCA 8.3.1.1.1/01
Report Author:	[redacted]
Report Year:	1999
Report Title:	Final report - Laboratory Testing for Toxicity (Acute Contact and Oral LD50) of ACLONIFEN on Honey Bees (Apis mellifera L.) (Hymenoptera, Apidae)
Report No:	R007442
Document No:	M-174936-01-1
Guideline(s) followed in study:	EPPO: Bulletin 22, 203-215 No. 170 (1992)
Deviations from current test guideline:	Current Guideline: OECD 213/214, 1998 Starvation time extended from up to 120 minutes to up to 135 minutes to ensure bees were hungry. Complete uptake of contaminated food lasted up to 9 h (instead of up to 3 h) as bees avoided contaminated food. 5 µL droplet volume used rather than 1 µL recommended in the guideline. Environmental conditions slightly ourtside of recommended range. These deviations are not considered to have affected the integrity or outcome of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Please refer to Section 8.3.1.1.1/01 for a full summary of this study.

Data Point:	KCA 8.3.1.1.2/02
Report Author:	[redacted]
Report Year:	2016
Report Title:	Aclonifen tech.: Acute oral and contact toxicity to the bumble bee, Bombus terrestris L. under laboratory conditions
Report No:	315-00341
Document No:	M-567133-01-1
Guideline(s) followed in study:	OECD Guidelines No. 213 and No. 214 (1998), OEPP/EPPO 170 (4) (2010), VAN DER STEEN (2005) and recommendations of the ICPPR bumble bee ring test group (2015)
Deviations from current test guideline:	Current Guideline: OECD 246/247, 2017 Behavioural abnormalities in reference item treatment were not recorded. This deviation was considered not to have affected the integrity or outcome of the study
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Please refer to Section 8.3.1.1.1/02 for a full summary of this study.

CA 8.3.1.2 Chronic toxicity to bees

No chronic toxicity studies on the active ingredient, aclonifen, have been perfomed on bees. Studies on the representative formulation containing aclonifen are presented in the product dossier.

CA 8.3.1.3 Effects on honeybee development and other honeybee life stages

Data Point:	KCA 8.3.1.3/01
Report Author:	[redacted]
Report Year:	2017
Report Title:	Aclonifen technical - Honey bee (Apis mellifera L.) larval toxicity test (Repeated exposure)
Report No:	S15-04235
Document No:	M-600773-01-1
Guideline(s) followed in study:	Regulation (EC) No. 1107/2009 Directive 2003-01 (Canada/PMRA) US EPA OCSPP 850.3UPP OECD Draft Guidance Document on Honey bee (Apis mellifera) Larval Toxicity Test, Repeated Exposure (Version dated April 2015) and OECD Guideline for the Testing of Chemicals 237, Honey bee (Apis mellifera) Larval Toxicity Test, Single Exposure (2013)
Deviations from current test guideline:	Current Guideline: OECD Guidance Document No. 239, 2016 The test was performed over an 8-day period rather than 22 days. This deviation was not considered to have affected study integrity and validity.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The study was conducted to determine possible effects of aclonifen technical on the honey bee larvae, *Apis mellifera* L. from repeated feeding exposure in an 8-day *in vitro* test.

The test included a control, a solvent control and five test item groups (40.3, 81.2, 162.3, 324.7 and 649.3 mg a.s./kg diet, equivalent to cumulative doses of 6.3, 12.5, 25, 50 and 100 µg a.s/larva/ development period). In addition, a reference item (dimethoate, 48 mg a.s./kg diet, equivalent to a cumulative dose of 7.4 µg a.s/larva/ development period). Additionally, bees were assessed for any behavioural effects.

Analysis of the treated larval diet for each test treatment including control and solvent control were analysed. Measured concentrations ranged from 82 to 101% of nominal. Measured concentrations remained within ±20%, therefore, results were based on nominal test concentrations.

After an 8-day repeated honey bee larval exposure with aclonifen technical study the NOEC was determined to be 162.3 mg aclonifen/kg diet. The equivalent NOED was 25.0 µg aclonifen/larva/development period. The LC_{10} was calculated to be 202 mg aclonifen/kg diet, equivalent

to an LD_{10} of 31.1 µg aclonifen/larva/development period. The LC_{20} was calculated to be 235.0 mg aclonifen/kg diet, equivalent to an LD_{20} of 36.2 µg aclonifen/larva/development period. The LC_{50} was calculated to be 313.8 mg aclonifen/kg diet, equivalent to an LD_{50} of 48.3 µg aclonifen/larva/development period.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen SC 600 PEA F068300
	Batch no.:	PEA 1000325
	Active Ingredient / Purity:	99.5% w/w, analysed
	Appearance:	Yellow solid
	Storage:	Room temperature in the dark
	Expiry date:	26 November 2016
2.	**Reference item:**	BAS 152 I (Dimethoate technical)
	Batch no.:	35016A161
	Active Ingredient / Purity:	98.8% w/w
3.	**Test Organism:**	First instar larvae (L1) honey bees (*Apis mellifera* L.)
	Age:	1st instar
	Source:	[redacted]
	Feeding:	None prior to test

B. STUDY DESIGN AND METHODS

1. In-life phase: 26 – 29 July 2017

2. Exposure conditions

Test vessels:	Crystal polystyrene grafting cells, diameter 9mm, in 48-well cellular culture plate. Culture plates were wetted with 15% (w/v) glycerol solution and placed in hermetically sealed Plexiglas desiccator, containing dishes filled with saturated K_2SO_4 solution in order to maintain water saturated atmosphere. All desiccators placed in same incubator
Experimental design:	Control, solvent control and five test item groups (40.3, 81.2, 162.3, 324.7 and 649.3 mg a.s./kg diet, equivalent to cumulative doses of 6.3, 12.5, 25, 50 and 100 µg a.s/larva/ development period Reference item (dimethoate, 48 mg a.s./kg diet, equivalent to a cumulative dose of 7.4 µg a.s/larva/ development period
Replicates:	45 larvae from three different hives
Temperature:	28.5 – 35.0°C
Relative humidity:	38.4 – 100%

Photoperiod: Darkness (except during application and observation)

3. Administration of the test item

Dose preparation

Test item stock solutions were prepared freshly at each application day. Test item and solvent control solutions were prepared using acetone as a solvent.

The larval diet was prepared freshly in advance, divided into aliquots and subsequently stored deep-frozen (≤ - 18 °C) until use. On each feeding day the required amount of diet was thawed and warmed in the incubator before use. The diet was prepared with deionized, autoclaved water using the following ingredients:

- Diet A: 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: 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: 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

Oral treatment

Each larva was fed once a day (except on day 2) with a standardized amount of artificial diet. On Day 1 each larva was fed with 20 µL of untreated diet A, on Day 3 each larva was fed with 20 µL of treated or untreated diet B, on Day 4 each larva was fed with 30 µL of treated or untreated diet C, on Day 5 each larva was fed with 40 µL of treated or untreated diet C, on Day 6 each larva was fed with 50 µL of treated or untreated diet C.

4. Measurements and observations

Mortality was assessed before feeding on Days 4 to 6 as well as on Days 7 and 8. Larvae were recorded as dead if no respiration (movement of spiracles) was observed. Any dead larvae were systematically removed. Other observations (larval appearance and size) were assessed qualitatively in comparison to the solvent control. On Day 8 (last day) the presence of uneaten food was recorded qualitatively.

Analytical samples were taken directly from the prepared diets prior to feeding. Two sub-samples (1 for analysis, 1 retained) of 2.5 mL were taken and the weight of each sample recorded.

No samples of reference feeding solutions were taken. Samples were stored frozen (-18°C) with 1 hour of sampling until required for analysis.

Analytical determination was conducted by Bayer, Crop Science Division, Monheim am Rhein, Germany.

5. Statistics/Data evaluation

The percent cumulative mortality was calculated for each treatment group and was corrected for control mortality according to the formula of [redacted] (1925) and modified by [redacted] (1947).

A multiple sequentially-rejective Fisher Test after [redacted] (one-sided greater, $\alpha = 0.05$) was used to evaluate whether there are significant differences between the mortality data of the solvent control and the test item treatment group and to determine the NOEC and LOEC based on mortality.

The corresponding NOED (No Observed Effect Dose) and LOED were calculated by taking into account the density of the larval diet (1.1 g/cm^3) and cumulative feeding volume per larva (140 µL diet).

Fisher's Exact Binomial Test (one-sided greater, $\alpha = 0.05$) was used to evaluate where was a significant difference between mortality in the reference treatment group compared to the solvent control.

Probit analysis using linear maximum likelihood regression was used to calculate the LC_{10}, LC_{20} and LC_{50}. The corresponding LD_{10}, LD_{20} and LD_{50} were calculated by taking into account the density of the larval diet (1.1 g/cm^3) and cumulative feeding volume per larva (140 µL diet).

Statistical calculations were made by using the statistical program TOXRAT Professional 3.1.0.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

The mean measured concentrations determined in the feeding solutions ranged from 93 to 98% of nominal.

Table: Analytical verification of feeding solutions

Nominal concentration (mg a.s./kg diet)	Lowest and highest concn of aclonifen from day 3 to 6 (mg aclonifen/kg diet)	Lowest and highest recovery from day 3 to 6 (%)
Control	<LoD	-
Solvent control	<LoD	-
40.6	33.3	82
	40.8	100
81.2	68.3	84
	81.8	101
162.3	145.8	90
	162.3	100
324.7	290.4	89
	326.1	100
649.3	531.6	82
	578.6	89

LoD (limit of detection) = 0.001 mg aclonifen/kg diet

The validated method is summarised in Document M-CA4 (CA 4.1.2/95).

B. BIOLOGICAL DATA

Table: Effects of aclonifen on honey bee larvae from repeated exposure

Concn (mg aclonifen/kg diet)	Cumulative mortality (%)					Adjusted mortality (%)			
	Days								
	4	5	6	7	8	5	6	7	8
Control	0	2.2	2.2	2.2	2.2	-	-	-	-
Solvent control	0	0	0	2.2	2.2	-	-	-	-
40.6	0	0	0	0	0	0	0	-2.2	-2.2
81.2	0	0	0	0	0	0	0	-2.2	-2.2

162.3	0	0	2.2	2.2	2.2	0	2.2	0	0
324.7	0	0	4.4	13.3	55.6*	0	4.4	11.3	54.6
649.3	2.2	4.4	37.8*	82.2*	97.8*	4.4	37.8	81.8	97.8
Reference item (48 mg/kg)	13.3**	42.2**	66.7**	95.6**	100**	40.9	66.0	95.5	100

* Significant increase compared to solvent control (Fisher's Exact Test with Bonferroni Correction, one-side greater, $\alpha = 0.05$)

** Significant increase compared to solvent control (Fisher's Exact Test, one-side greater, $\alpha = 0.05$)

Uneaten food was observed in all treatment groups on day 8. Larvae in the three highest test treatments

C. VALIDITY CRITERIA

Validity criterion	Required (OECD Guidance Document No. 239, 2016)	Achieved
Average mortality in control treatment (Days 3 – 8)	≤15%	2.2% in control and solvent control
Adult emergence rate (Day 22)	≥70%	Not applicable as study performed over 8 days only
Average mortality in reference item treatment (Day 8)	≥50%	100%

All relevant validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint		mg aclonifen/kg diet (95% confidence interval)
Day 8	LOEC	324.7
	NOEC	162.3
	LC_{10}	202.0 (163.0 – 231.7)
	LC_{20}	235.0 (198.6 – 264.2)
	LC_{50}	313.8 (280.9 – 350.6)
		µg aclonifen/larva/development period (95% confidence interval)
	LOED	50.0
	NOED	25.0
	LD_{10}	31.1 (25.1 – 35.7)
	LD_{20}	36.2 (30.6 – 40.7)
	LD_{50}	48.3 (43.3 – 54.0)

III. CONCLUSION

Measured concentrations remained within ±20%, therefore, results were based on nominal test concentrations.

After an 8-day repeated honey bee larval exposure with aclonifen technical study the NOEC was determined to be 162.3 mg aclonifen/kg diet. The equivalent NOED was 25.0 µg aclonifen/larva/development period.

The LC_{10} was calculated to be 202 mg aclonifen/kg diet, equivalent to an LD_{10} of 31.1 µg aclonifen/larva/development period.

The LC_{20} was calculated to be 235.0 mg aclonifen/kg diet, equivalent to an LD_{20} of 36.2 µg aclonifen/larva/development period.

The LC_{50} was calculated to be 313.8 mg aclonifen/kg diet, equivalent to an LD_{50} of 48.3 µg aclonifen/larva/development period.

[redacted] (2017)

Assessment and conclusion by applicant:

All relevant validity criteria were satisfied and therefore this study can be considered to be valid.

After an 8-Day repeated honey bee larval exposure with aclonifen technical study the NOEC was determined to be 162.3 mg aclonifen/kg diet. The equivalent NOED was 25.0 µg aclonifen/larva/development period

The LC_{50} after 8 days of repeated exposure was determined to be 313.8 mg aclonifen/kg diet, equivalent to an LD_{50} of 48.3 µg aclonifen/larva/development period.

Assessment and conclusion by RMS:

Data Point:	KCA 8.3.1.3/02
Report Author:	[redacted]
Report Year:	2017
Report Title:	Repeated exposure of aclonifen to honey bee (Apis mellifera) larvae under laboratory conditions (in vitro)
Report No:	16 10 48 139 B
Document No:	M-578600-01-1
Guideline(s) followed in study:	EU Directive 91/414/EEC, Regulation (EC) No 1107/2009 (2009); US EPA OCSPP Not Applicable; Directive 2003 01 (CANADA/PMRA)
Deviations from current test guideline:	Current Guideline: OECD Guidance Document No. 239, 2016 None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The study was conducted to determine possible effects of aclonifen on the honey bee larvae, *Apis mellifera* from repeated exposure under laboratory conditions (*in vitro*).

The test included a control, a solvent control and five test item groups (40.3, 81.2, 162.3, 324.7 and 649.3 mg a.s./kg diet, equivalent to cumulative doses of 6.3, 12.5, 25, 50 and 100 µg a.s/larva/ development period). A reference item (dimethoate, 48 mg a.s./kg diet, equivalent to a cumulative dose of 7.4 µg a.s/larva/ development period) was included in the study design. In addition to mortality, bees were assessed for any behavioural effects.

Analysis of the treated larval diet for each test treatment including control and solvent control were analysed. Measured concentrations ranged from 84 to 115% of nominal. Measured concentrations remained within ±20%, therefore, results were based on nominal test concentrations.

The ED_{50} (successful adult emergence up to Day 22) were determined to be 80.0 µg a.s./larva, respectively. The respective LOED was 80.0 µg a.s./larva, the NOED was 40.0 µg a.s./larva.

The EC_{50} (successful adult emergence up to Day 22) were determined to be 520 mg a.s./kg food, respectively, while the respective LOEC was 519 mg a.s./kg food and the corresponding NOEC was 260 mg a.s./kg food.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Item: Aclonifen technical
Batch no.: PEA 1000325
Active Ingredient / Purity: 99.5% w/w analysed
Appearance: Yellow powder
Storage: Room temperature in the dark
Expiry date: 26 November 2016

2. Reference item: BAS 152 I (Dimethoate technical)
Batch no.: 35015A161
Active Ingredient / Purity: 98.8% w/w

3. Test Organism: First instar larvae (L1) honey bees (*Apis mellifera* L.)
Age: 1st instar, 1 day old
Source: [redacted]
Feeding: None prior to test

B. STUDY DESIGN AND METHODS

1. In-life phase: 5 to 26 September 2016

2. Exposure conditions

Test vessels: Crystal polystyrene grafting cells, diameter 9mm, in 48-well cellular culture plate. Culture plates were placed on adjustable warming plate set to 34.5ºC. Test was conducted in a Binder KBF 720 climatic chamber
After day 8 relative humidity was decreased and honey bee pupae were transferred into emergence boxes on day 15. Each

	culture plate was covered with a perforated lid and equipped with a syringe containing 50% w/v sucrose solution
Experimental design:	Control, solvent control and five test item groups (32, 65, 130, 260 and 519 mg a.s/kg food, equivalent to doses of 5.0, 10, 20, 40 and 80 µg total a.s./larva Reference item (dimethoate, 48 mg a.s./kg diet, equivalent to a cumulative dose of 7.4 µg a.s/larva/ development period
Replicates:	Three replicates of 12 larvae were used. Therefore, a total number of 36 bees for each control, test item concentration treatment and for the reference treatment were set up
Temperature:	Target: 34.5 ± 0.5 °C; Achieved: 34.0 – 35.0°C
Relative humidity:	Target: day 1 to 8, 95 ± 5%; Achieved: 94 - 98% Target: day 8 to 15, 80 ± 5%; Achieved: 76 - 79% Target: day 15 to 22, around 50%; Achieved: 48 - 52%
Photoperiod:	Darkness (except during application and observation)

3. Administration of the test item

Dose preparation

Test item stock solutions were prepared freshly at each application day. Test item and solvent control solutions were prepared using acetone as a solvent. Test solutions were placed in an ultrasonic bath for several minutes. Final feeding solutions were prepared by mixing previously compounded stock solution with untreated final diet at a fixed volumetric ratio. Final diets were placed on a multiple vortexer for 5 minutes to ensure even distribution.

Application of control, test and reference item took place from day 3. Final diets were warmed to 34.5°C in a climate chamber and vortexed again before feeding.

4. Measurements and observations

Mortality: Number of dead larvae (immobile or which does not react to contact is noted as dead) were assessed daily on Days 4 to 8 (larvae) and day 15 (pupae). Larval mortality included all individuals, which had died between Days 3 and 8, while dead individuals between Days 8 and 22 were termed 'pupal mortality'. Together they were termed 'total mortality'.

Adult emergence: At the end of the test (Day 22), bees which emerged successfully were counted. Lifeless pupae and bees or those unable to leave the breading cups on their own accord, were marked as dead. In order to correct the effects observed in the treatment group by the control (i.e. background mortality) any calculations were performed using 'mortality' rather than 'adult emergence'.

Other observations included amounts of unconsumed food and/or substantially undersized larvae.

All final diets were samples in duplicate directly after preparation (Days 3, 4, 5 and 6). Analytical samples were stored frozen (-18°C) until required for analysis. Analysis was conducted by reversed phase high performance liquid chromatography (RP-HPLC) with MS-MS detection.

5. Statistics/Data evaluation

The percent cumulative mortality was calculated for each treatment group and was corrected for control mortality according to the formula of [redacted] (1925) and modified by [redacted] (1947).

The Step-down Chochran-Armitage test was used (one-sided greater, $\alpha = 0.05$) to evaluate significant differences between mortality data in the control and the test treatments and to determine the NOEC/NOED. A Trimmed Spearman-Karber procedure was used for calculation of ED/EC_{50} values. A Weibull regression was used to determine the EC/ED_{10} and EC/ED_{20} values.

Statistical calculations were made by using the statistical program TOXRAT Professional (Ratte, 2015).

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Measured concentrations of aclonifen in test samples ranged from 84 to 115%, with no aclonifen detected in either the control or the solvent control. The concentrations remained within ±20%, therefore, results were determined based on nominal test concentrations.

Table: Analytical verification of feeding solutions

Nominal concentration (mg a.s./kg)	Sampling time (days)	Measured concn (mg/kg)	% of nominal	Mean % of nominal
Control	3 - 6	n.d.	-	-
Solvent control	3 - 6	n.d.	-	-
519.67	3	557.3	107	104
	4	528.0	102	
	5	526.0	101	
	6	552.6	106	
259.83	3	267.9	103	106
	4	258.5	99	
	5	299.4	115	
	6	277.6	107	
129.92	3	128.8	99	92
	4	122.0	94	
	5	118.5	91	
	6	109.4	84	
64.96	3	63.85	98	100
	4	64.82	100	
	5	67.56	104	
	6	62.53	96	
32.48	3	33.84	104	99
	4	33.39	103	
	5	30.97	95	
	6	30.51	94	

n.d. not detected

The validated method is summarised in Document M-CA4 (CA 4.1.2/76).

B. BIOLOGICAL DATA

After 120 hours of repeated oral exposure (Day 8) larval mortalities ranged from 5.6 - 8.3% in the controls. Pupal mortality (between Days 8 and 22) was 14.7% in the control and 9.1% in the solvent control. The control group showed a total mortality of 16.7 - 19.4% by Day 22. At the end of the test (Day 22), adult emergence rates between 80.6 and 83.3% were determined.

In the test item group larval mortalities at Day 8 ranged between 0 and 38.9%. Pupal mortalities ranged between 2.9 and 31.8% in the test item treatment groups. Total mortalities by Day 22 ranged between 8.3 and 58.3%. The adult honey bees emerged at rates ranging between 41.7 and 91.7% following an application of 80.0, 40.0, 20.0, 10.0 and 5.0 µg a.s./larva, respectively, during the larval stages. Only the larvae treated with 80.0 µg a.s./larva showed a statistically significantly increased mortality when compared to the solvent control. The statistical evaluation of the adult emergence rate was done using all absolute mortality data, in order to correct the adult emergence rate with control mortality.

Table: Effects of aclonifen on honey bee larvae from repeated exposure

Dose (µg a.s./larva)	Concn (mg a.s./kg food)	Day 8			Day 22		
		Larval mortality (%)		Mean other obs.[a] (%)	Overall mortality (%)		Emergence rate (%)
		abs.	corr.		abs.	corr.	abs.
Control	-	5.6	0	0	19.4	0	80.6
Solvent control	-	8.3	0	0	16.7	0	83.3
80	519	38.9	36.3	56.5	58.3*	50	41.7
40	260	2.8	-6.1**	0	16.7	0	83.3
20	130	5.6	-3.0**	0	8.3	-10**	91.7
10	65	0	-9.1**	0	11.1	-6.7	88.9
5.0	32	5.6	-3.0**	0	16.7	0	83.3
Reference item	48	83.3	82.4	10	94.4	93.1	5.6

Results based on mean of 3 replicates (12 larvae each replicate)
a Other observations (e.g. remaining food)
* Statistically significant compared to control
** Negative values indicate higher mortality in control group than in treatment group

C. VALIDITY CRITERIA

Validity criterion	Required (OECD Guidance Document No. 239, 2016)	Achieved
Average mortality in control treatment (Days 3 – 8)	≤15%	5.6 - 8.3% in control and solvent control
Adult emergence rate (Day 22)	≥70%	80.6 – 83.3% in control and solvent control
Average mortality in reference item treatment (Day 8)	≥50%	83.3%

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint (up to day 22)		µg aclonifen/larva (95% confidence interval)
Test item doses	LOED	80.0
	NOED	40.0
	ED_{10}	80.0 (71.3 – 89.8)
	ED_{20}	47.3 (25.2 – 88.9)
	ED_{50}	18.0 (9.6 – 33.8)
		mg aclonifen/kg food (95% confidence interval)
Test item concentrations	LOEC	519
	NOEC	260
	EC_{10}	520 (463 – 583)
	EC_{20}	308 (163 – 581)
	EC_{50}	116 (62 – 219)

III. CONCLUSION

Measured concentrations remained within ±20%, therefore, results were based on nominal test concentrations.

The $ED_{50/20/10}$ (successful adult emergence up to Day 22) were determined to be 80.0/47.3/18.0 µg a.s./larva, respectively. The respective LOED was 80.0 µg a.s./larva, the NOED was 40.0 µg a.s./larva.

The $EC_{50/20/10}$ (successful adult emergence up to Day 22) were determined to be 520/308/116 mg a.s./kg food, respectively, while the respective LOEC was 519 mg a.s./kg food and the corresponding NOEC was 260 mg a.s./kg food.

[redacted] *(2017)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

After a 22-Day repeated honey bee larval exposure with aclonifen technical study the NOEC was determined to be 260 mg aclonifen/kg food. The equivalent NOED was 40.0 µg aclonifen/larva.

The EC_{50} after 22 days of repeated exposure was determined to be 520 mg aclonifen/kg food, equivalent to an ED_{50} of 80.0 µg aclonifen/larva.

Assessment and conclusion by RMS:

CA 8.3.1.4 Sub-lethal effects

No studies to assess the sub-lethal effects of the active ingredient, aclonifen, have been perfomed on bees.

CA 8.3.2 Effects on non-target arthropods other than bees

No studies on the active ingredient, aclonifen, have been perfomed on non-target arthropods other than bees. Studies on the representative formulation containing aclonifen are presented in the product dossier.

Data Point:	KCA 8.3.2/01
Report Author:	[redacted]; [redacted]
Report Year:	1992
Report Title:	A study of the acute toxicity for aleochara bilineata (staphylinidae) of SAG 127 01 H
Report No:	R007268
Document No:	M-174575-01-1
Guideline(s) followed in study:	IOBC/WPRS (Samsoe-Petersen)
Deviations from current test guideline:	Current Guideline: Grimm et al., 2000 The test was performed over a 5-Day exposure period rather than the current requirement of 28 days
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

In the previous submission (DAR, 2006), this study was presented in the active ingredient section. This study was performed on the representative formulation and hence is sumarized in full in Section 10.3.2.1 of the product dossier.

Data Point:	KCA 8.3.2/02
Report Author:	[redacted]; [redacted]
Report Year:	1992
Report Title:	A study of the acute toxicity for Poecilus cupreus (Carabidae) of SAG 127 01
Report No:	R007267
Document No:	M-174573-01-1
Guideline(s) followed in study:	BBA: VI 23-2.1.8
Deviations from current test guideline:	Current Guideline: BBA VI 23-2.1.8 None
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

In the previous submission (DAR, 2006), this study was presented in the active ingredient section. This study was performed on the representative formulation and hence is sumarized in full in Section 10.3.2.1 of the product dossier.

Data Point:	KCA 8.3.2/03
Report Author:	[redacted]
Report Year:	1992
Report Title:	A study of the acute toxicity for pardosa sp. (spiders) of SAG 127 01
Report No:	R007269
Document No:	M-174577-01-1
Guideline(s) followed in study:	BBA (July 28, 1987)
Deviations from current test guideline:	Current Guideline: BBA VI, 23-2.1.8, 1991 None
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

In the previous submission (DAR, 2006), this study was presented in the active ingredient section. This study was performed on the representative formulation and hence is sumarized in full in Section 10.3.2.1 of the product dossier.

Data Point:	KCA 8.3.2/04
Report Author:	[redacted]
Report Year:	1999
Report Title:	Final Report – Effects of EXP04209E on the Lacewing Chrysoperla carhea Steph. (Neuroptera, Chrysopidae) in the Laboratory
Report No:	R008586
Document No:	M-177360-01-1
Guideline(s) followed in study:	IOBC/WPRS 1988; ring-test group (Vogt 1995, Vogt et al. in prep.)
Deviations from current test guideline:	Current Guideline: IOBC-WPRS 1988 None
Previous evaluation:	yes, evaluated and accepted Source: DAR, Vol. 3 B9 (9.5 table 9.5-7), August 2006 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

In the previous submission (DAR, 2006), this study was presented in the active ingredient section. This study was performed on the representative formulation and hence is sumarized in full in Section 10.3.2.1 of the product dossier.

Data Point:	KCA 8.3.2/05
Report Author:	[redacted]
Report Year:	2000
Report Title:	Effects of EXP04209E on the wolf spider Pardosa sp (Araneae, Lycosidae) in the Laboratory - Extended Laboratory Study
Report No:	B002997
Document No:	M-238654-01-1
Guideline(s) followed in study:	BBA: VI, 23-2.1.9 (1994) Draft
Deviations from current test guideline:	Current Guideline: BBA VI, 23-2.1.9 (1994) Draft A natural soil (LUFA 2.1) was used instead of quartz sand as the substrate. The time interval of checks for mortality, sublethal effects and food consumption were slightly changed. Deionized water rather than tap water was used as the test vehicle. Acceptable control mortality was reduced from 10% to 8.8%. The acclimatisation period was 3 days before the start of the experiment rather than 7 days. The above deviations were considered not to have had any adverse scientific effect on the outcome of the study.
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

In the previous submission (DAR, 2006), this study was presented in the active ingredient section. This study was performed on the representative formulation and hence is sumarized in full in Section 10.3.2.2 of the product dossier.

CA 8.3.2.1 Effects on Aphidius rhopalosiphi

Data Point:	KCA 8.3.2.1/01
Report Author:	[redacted]
Report Year:	1999
Report Title:	Effects of EXP 04209E on the aphid parasitoid Aphidius rhopalosiphi (Hymenoptera, Aphidiidae) in the laboratory
Report No:	R006177
Document No:	M-172247-01-1
Guideline(s) followed in study:	IOBC/WPRS 1988
Deviations from current test guideline:	Current Guideline: IOBC/WPRS 1988 None
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

In the previous submission (DAR, 2006), this study was presented in the active ingredient section. This study was performed on the representative formulation and hence is sumarized in full in Section 10.3.2.1 of the product dossier.

CA 8.3.2.2 Effects on Typhlodromus pyri

Data Point:	KCA 8.3.2.2/01
Report Author:	[redacted]
Report Year:	1999
Report Title:	Effects of EXP04209E on the predatory mite typhlodromus pyri Scheuten (Acari, Phytoseiidae) in the Laboratory
Report No:	R006155
Document No:	M-172210-01-1
Guideline(s) followed in study:	IOBC/WPRS 1988; [redacted] improvements 1995
Deviations from current test guideline:	Current Guideline: IOBC/WPRS 1988 None
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

In the previous submission (DAR, 2006), this study was presented in the active ingredient section. This study was performed on the representative formulation and hence is sumarized in full in Section 10.3.2.1 of the product dossier.

Data Point:	KCA 8.3.2.2/02
Report Author:	[redacted]
Report Year:	2003
Report Title:	Toxicity to the predatory mite Typhlodromus pyri Scheuten (Acari, Phytoseidae) in the laboratory Aclonifen water miscible suspension concentrate 600 g/L code: AE F068300 00 SC50 A203
Report No:	C033823
Document No:	M-232137-01-1
Guideline(s) followed in study:	ESCORT [redacted] 2001; IOBC: [redacted] 2000
Deviations from current test guideline:	Current Guideline: IOBC ([redacted] 2000) None
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

In the previous submission (DAR, 2006), this study was presented in the active ingredient section. This study was performed on the representative formulation and hence is sumarized in full in Section 10.3.2.1 of the product dossier.

Data Point:	KCA 8.3.2.2/03
Report Author:	[redacted]
Report Year:	2000
Report Title:	EXP04209E: An Extended Laboratory Study to Evaluate the Effects on the predaceous Mite Typhlodromus pyri Scheuten (Acari: Phytoseiidae)
Report No:	B002976
Document No:	M-238634-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: [redacted] 1992 Age of protonymphs is not exactly known, but is expected to be less than 24 hours
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2001 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

In the previous submission (DAR, 2006), this study was presented in the active ingredient section. This study was performed on the representative formulation and hence is sumarized in full in Section 10.3.2.2 of the product dossier.

CA 8.4 Effects on non-target soil meso and macrofauna

Table 8.4-1: Summary of the effects of Aclonifen on non-target soil meso and macrofauna

Test Species	Test Item	Duration	Endpoint	Reference
Earthworms				
Eisenia andrei	Aclonifen	Acute 14 days	NOEC = 100 mg a.s./kg d.w. EC_{50} = 300 mg a.s./kg d.w.	KCA 8.4/01 M-174306-01-1 [redacted] 1990

d.w.: Dry weight

Data Point:	KCA 8.4/01
Report Author:	[redacted]; [redacted]
Report Year:	1990
Report Title:	The acute toxicity of aclonifen, CME127 to earthworms (Eisenia foetida)
Report No:	R007146
Document No:	M-174306-01-1
Guideline(s) followed in study:	EU (=EEC): 87/302/EEC; OECD: 207
Deviations from current test guideline:	Current Guideline: OECD 207 (1984) None
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

In the previous submission (DAR, 2006), this study was evaluated and accepted as valid for risk assessment purposes. This study design and endpoint is no longer required for the registration of active ingredients in the EU and hence a summary of this study is not presented in this dossier.

CA 8.4.1 Earthworm, sub-lethal effects

No earthworm sub-lethal studies on the active ingredient, aclonifen, have been perfomed. Studies on the representative formulation containing aclonifen are presented in the product dossier.

Data Point:	KCA 8.4.1/01
Report Author:	[redacted]
Report Year:	1995
Report Title:	Assessment of Sublethal Effects of EXP4209 - (Official German Regristration Name: Bandur) - on Eisenia foetida in artificial soil - (Determination of Effects on Reproduction)
Report No:	R007431
Document No:	M-174912-01-1
Guideline(s) followed in study:	BBA; VI, 2-2; ISO; 11268-2
Deviations from current test guideline:	Current Guideline: ISO Guideline 11268-2 and BBA Guideline VI, 2-2 None
Previous evaluation:	yes, evaluated, not accepted Source: DAR, Vol 3 B9 (9.6.2), August 2006 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Now is no longer acceptable

~~In the previous submission (DAR, 2006), this study was presented in the active ingredient section despite it being performed on the representative formulation. The study was evaluated and not accepted as valid for risk assessment purposes. Therefore a summary of this study is not presented in this dossier.~~

In the previous submission (DAR, 2006), this study was presented in the active ingredient section. This study was performed on the representative formulation and hence is sumarized in full in Section 10.4.1.1 of the product dossier.

CA 8.4.2 Effects on non-target soil meso and macrofauna (other than earthworms)

CA 8.4.2.1 Species level testing

No studies on non-target soil meso and macrofauna (other than earthworms) on the active ingredient, aclonifen, have been perfomed. Studies on the representative formulation containing aclonifen are presented in the product dossier.

Data Point:	KCA 8.4.2.1/01
Report Author:	[redacted]
Report Year:	2002
Report Title:	AE F068300 00 SC50 A203 = EXP04209E (Bandur): Laboratory dose-repons test to evaluate effect on survival and reproduction of the predaceous mite Hypoaspis aculeifer Canestrini (Acari, Laelapidae) in standard soil (LUFA 2.1)
Report No:	C029557
Document No:	M-217404-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 226, 2008 The test was performed to the outdated Bakker test design with 14-day mortality, 7-day mating and 7-day reproduction phases rather than a single 14-day mortality and reproduction phase
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

In the previous submission (DAR, 2006), this study was presented in the active ingredient section. This study was performed on the representative formulation and hence is discussed in Section 10.4.2.1 of the product dossier.

CA 8.5 Effects on nitrogen transformation

Table 8.5-1: Summary of the effects of Aclonifen on soil nitrogen transformation processes

Test item	Time scale	Endpoint	Reference
Aclonifen	28 days	**No adverse effect after 28 days at a maximum tested concentration of 15 kg a.s./ha (20 mg a.s./kg)**	KCA 8.5/01 M-218214-01-2 [redacted], 1984

Test item	Time scale	Endpoint	Reference
Aclonifen	5 days	No adverse effect after 5 days at a maximum tested concentration of 13.5 kg a.s./ha (18 mg a.s./kg)	KCA 8.5/02 M-174171-01-1 [redacted], 1994

Endpoint in **bold** was used in the risk assessment

Data Point:	KCA 8.5/01
Report Author:	[redacted]
Report Year:	1984
Report Title:	Study to determine the effect of Aclonifen (CME 127) on soil microflora - Definitive Report
Report No:	R007411
Document No:	M-218214-01-2
Guideline(s) followed in study:	--
Deviations from current test guideline:	Current Guideline: OECD 216, 2010 None
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Laboratory experiments were carried out to determine the effect of Aclonifen (CME 127) on the nitrogen cycle. The study was carried out in accordance with the 1981 BBA-Richtlinienentwurf (draft guideline of the Federal Institute of Biology).

The test item was applied at 2 rates, 4 mg/kg and 20 mg/kg equivalent to 3 kg a.s./ha and 15 kg a.s./ha respectively, to two different soils (sand and loam). 0.5% of powdered lucerne meal (containing approx. 2.3% of nitrogen) was added as organic substrate.

The nitrogen cycle was unaffected by aclonifen. After 2-4 weeks, nitrification of added ammonium sulphate was complete. There was no loss in the ammonium- and nitrate-nitrogen balance.

Ammonification and nitrification of Lucerne meal was very slow which was not caused by aclonifen but by the slight N-mineralization of Lucerne. A subsequent test using sand with horn meal (instead of Lucerne) showed that mineralization progressed rapidly.

The results of this study indicate that soil nitrification and respiration were unaffected by aclonifen at levels up to 15 kg as/ha. The deviation between treated and control soil was lower than 25% at the end of the evaluation. Therefore, the impact of aclonifen on soil was considered negligible.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen (CME 127)
	Batch no.:	Not provided
	Active Ingredient / Purity:	99.7%
	Expiry date:	Not provided
2.	**Test Soils:**	Two types of soil were selected, the one being used for fruit (Ingelheim sand) and the other for vegetable cultivation (Schwabenheim loam). No agrochemicals or organic manure had been applied to either soil for several years. Mixed samples were taken from the top soil (0-10 cm) directly before the start of the study.
	Source:	[redacted]
	Pre-treatment:	The soil was sampled, air dried (if required) and sieved (2 mm) prior to use.

B. STUDY DESIGN AND METHODS

1. In-life phase: 11 August 1983 – 06 February 1984

2. Exposure conditions

Test vessels: 500-mL wide-mouthed bottles which were firmly closed

Experimental design:

i. Untreated soil containing 20 mg N as $(NH_4)_2SO_4$/100 g of dried soil.
ii. Soil containing 20 mg N as $(NH_4)_2SO_4$ and 0.4 mg CME 127/100 g of dried soil.
iii. Soil containing 20 mg N as $(NH_4)_2SO_4$ and 2.0 mg CME 127/100 g of dried soil.
iv. Untreated soil containing 11.5 mg N as lucerne/100 g of dried soil.
v. Soil containing 11.5 mg N as lucerne and 0.4 mg CME 127/100 g of dried soil.
vi. Soil containing 11.5 mg N as lucerne and 2.0 mg CME 127/100 g of dried soil.
vii. Untreated soil containing 20 mg N as horn meal/100 g of dried soil (Ingelheim sand).
viii. Soil containing 20 mg N as horn meal and 0.4 mg CME 127/100 g of dried soil (Ingelheim sand).
ix. Soil containing 20 mg N as horn meal and 2.0 mg CME 127/100 g of dried soil (Ingelheim sand).

Temperature: 21 ± 1°C

Moisture content: 40% of water holding capacity (WHC)

Photoperiod: In darkness

3. Administration of the test item

Dose preparation and dosing

Individual soil samples equivalent to 100 g of dried soil were spread out over an area of about 100 cm^2 for treatment. Each quantity of active ingredient was dissolved in 1 mL of acetone and evenly applied to the soil using a 1-mL sprayer. After evaporation of the acetone, the samples were mixed thoroughly using a Krupps mixing apparatus.

In order to examine the nitrification process, nitrogen was added to the soils (test sections i - iii) at the rate of 20 mg N/100 g of dried soil by mixing an aqueous solution of $(NH_4)_2SO_4$ containing 4.71 g/100 mL.

0.5% of lucerne meal (about 11.5 mg N/100 g of soil) was added to other soil samples (test sections iv to vi) for the purpose of investigating nitrogen mineralization. Owing to the incomplete mineralization of lucerne discernible in the interim period, the BBA-Richtlinienentwurf (BBA draft guideline) was amended, and a further series of tests carried out with Ingelheim sand. Horn meal was used instead of lucerne meal (test sections vii - ix) at the rate of 165 mg per 100 g of dried soil (equivalent to 20 mg N).

The water content of the soils was adjusted to 40% of their maximum water-holding capacity. Provision was made for each section of the test to be repeated twice at 40 g.

4. Measurements and observations

Samples were taken on days 0, 7, 14, 28 and 56.

Nitrogen cycle/nitrification: On each sampling day, the samples (i - iii) were extracted by shaking for one hour in 200 mL of potassium aluminium sulphate solution and the amounts of ammonium-nitrogen (NH_4-N) and nitrate-nitrogen (NO_3-N) were determined using a colorimetric method or ion-selective electrode.

Nitrogen cycle/mineralisation: The soil samples in sections iv - ix were extracted by shaking for one hour in 50 mL of aluminium sulphate solution. The supernatant liquid phase was decanted and the NO_3-N and NH_4-N content determined using an ion-selective electrode.

5. Statistics/Data evaluation

No statistical analysis of the generated data was performed.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

No analytical verification was required.

B. BIOLOGICAL DATA

The nitrogen cycle was unaffected by aclonifen. After 2-4 weeks, nitrification of added ammonium sulphate was complete. There was no loss in the ammonium- and nitrate-nitrogen balance.

Ammonification and nitrification of Lucerne meal was very slow which was not caused by aclonifen but by the slight N-mineralization of Lucerne. A subsequent test using sand with horn meal (instead of Lucerne) showed that mineralization progressed rapidly.

Table: Nitrification of ammonium sulphate: values expressed in mg N/100 g dried soil and in % of control ( )

Day	Sand			Loam		
	Control	0.4 mg aclonifen	2 mg aclonifen	Control	0.4 mg aclonifen	2 mg aclonifen
0	19.3	19.3 (100)	19.3 (100)	19.7	19.0 (96.4)	19.1 (97)
7	20.3	20.3 (100)	20.3 (100)	18.1	17.5 (96.7)	21.3 (117.7)
14	18.3	19.9 (108.7)	19.1 (104.4)	19.7	20.1 (102)	20.5 (104)
28	19.5	18.2 (93.3)	18.2 (93.3)	20.3	20.0 (98.5)	20.3 (100)
56	21	20.3 (96.6)	20.3 (96.6)	20.0	20.0 (100)	20.0 (100)

Table: Ammonification and nitrification of Lucerne meal and horn meal: values expressed in mg N/100 g dried soil and in % of control ( )

Day	Sand/Lucerne meal			Sand/horn meal			Loam/Lucerne meal		
	Control	0.4 mg aclonifen	2 mg aclonifen	Control	0.4 mg aclonifen	2 mg aclonifen	Control	0.4 mg aclonifen	2 mg aclonifen
0	0.3	0.3 (100)	0.2 (66.6)	1.2	1.2 (100)	1.2 (100)	0.2	0.4 (200)	0.4 (200)
7	0.1	0.1 (100)	<0.1 (<100)	6.2	7.7 (124.2)	6.1 (98.4	0.6	0.6 (100)	0.7 (116.6)
14	<0.1	0.1 (<100)	0.1 (<100)	11.1	9.2 (82.9)	9.4 (84.7)	1.0	0.9 (90)	1.1 (110)
28	0.9	0.9 (100)	1.1 (122.2)	10.8	8.6 (79.6)	10.8 (100)	1.9	1.9 (100)	2.1 (110.5)
56	2.8	2.8 (100)	3.3 (117.8)	14.6	14.6 (100)	13.8 (94.5)	1.7	1.9 (111.7)	2.6 (152.9)

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 216, 2010)	Achieved
Variation between controls	≤15%	0%[1]

[1]: on Day 56

The validity criterion was satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Effect
Nitrogen transformation	No adverse effect after 56 days at a maximum tested concentration of 20 mg a.s/kg soil dry weight (equivalent to field application rate of 15 kg a.s./ha)

III. CONCLUSION

The results of this study indicate that soil nitrification and respiration were unaffected by aclonifen at levels up to 15 kg a.s./ha. The deviation between treated and control soil was lower than 25% at the end of the evaluation. Therefore the impact of aclonifen on soil was considered negligible

[redacted] *(1984)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

Aclonifen had no adverse impact on soil nitrate transformation at levels up to 15 kg a.s./ha. It can be concluded that aclonifen will not have any adverse long-term influence on soil microflora.

Assuming a soil incorporation depth of 5 cm and a bulk soil density of 1.5, the the test concentration of 15 kg a.s./ha was estimated to be equivalent to 20 mg a.s./kg.

Assessment and conclusion by RMS:

Data Point:	KCA 8.5.02
Report Author:	[redacted]
Report Year:	1994
Report Title:	A laboratory assessment of the effects of Aclonifen on asymbiotic nitrogen fixation in soils
Report No:	R007082
Document No:	M-174171-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Not applicable, no specific guideline cited in report
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The effect of aclonifen on soil microbial activity in terms of asymbiotic nitrogen fixation was determined.

The test item was applied at 2 rates to 2 soils to give 3.6 mg a.s./kg soil or 18 mg a.s./kg soil which is equivalent to 2.7 kg a.s./ha or 5 times that concentration distributed to a depth of 5 cm. The effect of aclonifen on asymbiotic nitrogen fixation was investigated by measuring the rate of ethylene production from glucose amended soil samples spiked with acetylene at daily intervals for up to 5 days.

The clay loam soil had ethylene production rates of between 50 and 100 times greater than the sandy loam soil which was considered to have a poor asymbiotic nitrogen fixing capacity. The poor nitrogen fixation rates in the sandy loam soil resulted in variations between replicates of the treatments. These variations gave rise to significant differences in the treatments which were not dose related and were

not sequential in time. By Day 5 there were no significant differences between the control and the treatments.

Aclonifen, when added to clay loam and sandy loam soils up to 13.5 kg a.s./ha, did not adversely affect asymbiotic nitrogen fixation.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test Item:**	Aclonifen Technique (RPA 099795)
	Batch no.:	9229932
	Active Ingredient / Purity:	995 g/kg
	Appearance:	Yellow powder
	Expiry date:	22 November 1995
	Storage:	Ambient
2.	**Reference item:**	Dinoterb
	Purity:	99.9%
3.	**Test Soils:**	Clay loam and sandy loam
	Source:	[redacted]
	Pre-treatment:	The Soils used in the study were obtained from Rhone-Poulenc Agriculture Limited. They had been sieved to pass a 2mm screen prior to despatch. On receipt, the soils were conditioned at 22 ± 2°C for 8 and 21 days at a moisture content of 22.62% (clay loam) and 13.98% (sandy loam) respectively.

B. STUDY DESIGN AND METHODS

1. In-life phase: 31 May – 18 June 1994

2. Exposure conditions

Experimental design:	Two test concentrations (3.6 and 18.0 mg test item/kg dry soil weight) plus one control; five replicates of each
Temperature:	21 ± 1°C
Moisture content:	Clay loam: 22.62% Sandy loam: 13.98%

3. Administration of the test item

The test item was distributed in acid washed silicon sand (3.6 mg or 18.0 mg test substance/10 g sand). 10 g of the sand/test item mixture was mixed with 1kg soil (dry weight) to give the required target dose levels.

4. Measurements and observations

The activity of the nitrogen fixation enzyme complex - nitrogenase - was measured by determining the rate of ethylene production from the enzymatic reduction of acetylene in glucose amended soils.

For the glucose amendment, 10 mg glucose/g soil (dry weight) was added as a solution in water. For the clay loam soil, 3.8 mL of water containing 800 mg glucose was added to each 80 g dry weight soil aliquot. For the sandy loam soil, 1.1 mL water containing 800 mg glucose was added to each 80g dry weight soil aliquot.

On 0, 1, 2, 3 and 4 days (5 days for clay loam), approximately 10% acetylene based on the void volume of bottle after soil addition was added using 2 syringe device by withdrawing 6.5 mL air through septum and replacing with 6.5 mL acetylene. The vessels were incubated in the presence of acetylene for approximately 24 hours.

5. Statistics/Data evaluation

No statistical analysis of the generated data was performed.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

No analytical verification was required.

B. BIOLOGICAL DATA

The clay loam soil had ethylene production rates of between 50 and 100 times greater than the sandy loam soil which was considered to have a poor asymbiotic nitrogen fixing capability. The pattern of nitrogen fixation activity in both soils was typical for a limited energy source addition, reaching a peak between 2 and 3 days after glucose amendment and reducing to effectively zero after 4 days when the glucose was exhausted.

No significant differences were observed between treatments for the clay loam soil at the greatest rates of nitrogen fixation as measured by acetylene reduction. The poor nitrogen fixation rates in the sandy loam soil resulted in variations between replicates of the treatments. These variations gave rise to significant differences in the treatments which were not dose related (see Day 2). These differences are not considered to be due to the treatments and thus not agronomically important.

Table: The effect of aclonifen in a clay loam soil on the rate of acetylene reduction to ethylene (µmole/h) (average of 5 replicate determinations):

Treatment	Time (days)				
	0	1	2	3	5
Control	n.d.	0.034	1.783	1.677	0.944
2.7 kg a.s./ha	n.d.	0.036	1.989	1.787*	0.899
13.5 kg a.s./ha	n.d.	0.046*	1.934	1.724	1.025

Time (days) = time after glucose amendment when soil spiked with acetylene
*significant difference (ANOVA; p=0.05)
n.d.= none detected

Table: The effect of aclonifen in a sandy loam soil on the rate of acetylene reduction to ethylene (µmole/h) (average of 5 replicate determinations):

Treatment	Time (days)				
	0	1	2	3	5
Control	n.d.	0.003	0.045	0.021	0.0004
2.7 kg a.s./ha	n.d.	0.004	0.091*	0.055	0.0003
13.5 kg a.s./ha	n.d.	0.004	0.066	0.057	0.001

Time (days) = time after glucose amendment when soil spiked with acetylene
*significant difference (ANOVA; p=0.05)
n.d.= none detected

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 216, 2010)	Achieved
Variation between controls	15%	n.d.

n.d.: not determined as only mean values reported

The test was not performed in accordance with any standardised test guideline and hence confirmation of validity is not possible. The study is acceptable for use as additional information.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint	Effect
Nitrogen transformation	No adverse effect after 5 days at a maximum tested concentration of 13.5 kg a.s./ha

III. CONCLUSION

Aclonifen, when added to clay loam and sandy loam soils up to 13.5 kg a.s./ha, did not adversely affect asymbiotic nitrogen fixation.

[redacted] *(1994)*

Assessment and conclusion by applicant:

The test was not performed in accordance with any standardised test guideline and hence confirmation of validity is not possible. The study is acceptable for use as additional information.

Aclonifen, when added to clay loam and sandy loam soils up to 13.5 kg a.s./ha (18 mg a.s./kg), did not adversely affect asymbiotic nitrogen fixation.

Assessment and conclusion by RMS:

CA 8.6 Effects on terrestrial non-target higher plants

No studies on the active ingredient, aclonifen, have been perfomed on terrestrial non-target plants. Studies on the representative formulation containing aclonifen are presented in the product dossier.

CA 8.6.1 Summary of screening data

No screening studies have been performed.

CA 8.6.2 Testing on non-target plants

No studies on the active ingredient, aclonifen, have been perfomed on terrestrial non-target plants. Studies on the representative formulation containing aclonifen are presented in the product dossier.

CA 8.7 Effects on other terrestrial organisms (flora and fauna)

No additional studies on the active ingredient, aclonifen, have been performed.

CA 8.8 Effects on biological methods for sewage treatment

Table 8.8-1: Summary of data on the effects of aclonifen on biological methods for sewage treatment

Test item	Test species	Time scale	Endpoint	Reference
Aclonifen	Activated sewage sludge micro-organisms	3 hours	EC_{50} > 1000 mg a.s./L[1]	KCA 8.8/02 M-177356-01-1 [redacted], 1999
Aclonifen	Activated sewage sludge micro-organisms	3 hours	**EC_{50} > 100 mg a.s./L**	KCA 8.8/03 M-664091-01-1 [redacted], 2019

[1]: Study does not meet the validity criteria of OECD 209 (2010)

Endpoint in **bold** was used in the risk assessment

Data Point:	KCA 8.8/01
Report Author:	[redacted]
Report Year:	1994
Report Title:	Bandur EXP04209 - Acute toxicity in bacteria (Pseudomonas putida).
Report No:	R007904
Document No:	M-175842-02-1
Guideline(s) followed in study:	DIN: 38/412
Deviations from current test guideline:	Current Guideline: DIN 38412-16; 1985 None
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

In the previous submission (DAR, 2006), this study was presented in the active ingredient section. This study was performed on the representative formulation and hence is sumarized in full in Section 10.8 of the product dossier.

Data Point:	KCA 8.8/02
Report Author:	[redacted]
Report Year:	1999
Report Title:	ACLONIFEN: Assessment of the inhibitory effect on respiration of activated sewage sludge
Report No:	R008584
Document No:	M-177356-01-1
Guideline(s) followed in study:	EU (=EEC): 87/302/EEC; OECD: 209; USEPA (=EPA): OPPTS 850.6800
Deviations from current test guideline:	Current Guideline: OECD 209, 2010 The control oxygen uptake rate of 9.84 mg O2/g/h was lower than the current guideline requirement of 20 mg O2/g/h
Previous evaluation:	yes, evaluated and accepted Source: Study list relied upon, December 2011 (RMS: DE)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

A study was performed to assess the effect of the test material on the respiration of activated sewage sludge. The method followed that described in the OECD Guidelines for Testing of Chemicals (1984) No 209 "Activated Sludge, Respiration Inhibition Test", EEC Commission Directive 87/302/EEC and US EPA Draft Ecological Effects Test Guidelines OPPTS 850.6800 (1996).

Following preliminary range-finding studies, activated sewage sludge was exposed to an aqueous dispersion of the test material item at a concentration of 1000 mg/l (three replicate flasks) for a period of 3 hours at 21°C with the addition of a synthetic sewage as a respiratory substrate.

The rate of respiration was determined after 30 minutes and 3 hours contact time and compared to data for the control and a reference material, 3,5-dichlorophenol.

The effect of the test item on the respiration of activated sewage sludge gave a 3-Hour EC_{50} of greater than 1000 mg/L. The No Observed Effect Concentration (NOEC) after 3 hours exposure was 1000 mg/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen
   Batch no.: OP9750062
   Active Ingredient / Purity: 995 g/kg
   Appearance: Yellow powder
   Date received: 01 October 1999
   Storage: Room temperature in the dark

2. **Reference item:** 3,5-dichlorophenol
   Batch no.: 90204 PS02611ES

3. **Test Species:** Activated sewage sludge micro-organisms from a domestic waste water treatment plant
   Source: [redacted]
   Pre-treatment: The activated sewage sludge sample was maintained on continuous aeration in the laboratory at a temperature of 21°C and was used on the day of collection. The pH of the sample was 7.9 and the suspended solids equal to 3.9 g/L prior to use

4. **Test water:** Laboratory tap water dechlorinated by passage through an activated carbon filter
   Total hardness: Approximately 100 mg/L as $CaCO_3$

B. STUDY DESIGN AND METHODS

1. In-life phase: 04 October – 13 October 1999

2. Exposure conditions

Test vessels: 500 mL conical flask
Experimental design: Single test concentration (1000 mg/L, three replicates) plus one control (two replicates) and reference item (3.2, 10 and 32 mg/L, single replicate)
Loading: 1.56 g suspended solids/L

Temperature: 21°C
Aeration: Compressed air (approximately 0.5 – 1 litre per minute)

3. Administration of the test item

Dose preparation and dosing

An amount of test item (500 mg) was dispersed in approximately 250 mL of water and subjected to ultrasonication (approximately 30 minutes). Synthetic sewage (16 mL), activated sewage sludge (200 mL) and water were added to a final volume of 500 mL to give the required concentration of 1000 mg/L.

Preparation of test system

At time "0" 16 mL of synthetic sewage was diluted to 300 mL with diluent and 200 mL of activated sewage sludge added in a 500 mL conical flask (first control) and the mixture aerated with compressed air. Thereafter at 15-minute intervals the procedure was repeated with appropriate amounts of the test or reference item being added. Finally a second control was prepared.

4. Measurements and observations

As each vessel reached 30 minutes contact time an aliquot was removed from the conical flask and poured into the measuring vessel (250 ml darkened glass Biological Oxygen Demand (BOD) bottle) and the rate of respiration measured using a Yellow Springs dissolved oxygen meter fitted with a BOD probe. The contents of the measuring vessel were stirred constantly by magnetic stirrer. The rate of respiration for each flask was measured over an approximate 10 minute period (between approximately 7.9 mg O_2/L and 1.3 mg O_2/L). This procedure was repeated after 3 hours contact time.

5. Statistics/Data evaluation

Percentage inhibition of respiration rate for the reference item was plotted against concentration and the EC_{50} values derived by inspection of the fitted line.

The EC_{50} value for the test item was determined by examination of the respiration inhibition data.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Analytical verification was not required.

B. BIOLOGICAL DATA

The results obtained are summarised in the following table:

Table: Oxygen consumption rates and percentage inhibition from the exposure of activated sewage sludge micro-organisms to Aclonifen

Concentration (mg/L)		30 minutes contact time		3 hours contact time	
		O_2 consumption rate (mg O_2/L/min)	% inhibition	O_2 consumption rate (mg O_2/L/min)	% inhibition
Control	R_1	0.53	-	0.51	-
	R_2	0.53	-	0.51	-
Aclonifen 1000	R_1	0.68	[28]	0.52	[2]

	R_2	0.73	[38]	0.54	[6]
	R_3	0.85	[60]	0.54	[6]
Reference Item	3.2	0.47	11	0.41	20
	10	0.37	30	0.22	57
	32	0.13	75	0.09	82

$R_1 – R_3$ = Replicates 1 – 3
[increase in respiration rate as compared to controls]

No significant inhibition of respiration rate occurred at the single test concentration of 1000 mg/L and hence the EC_{50} for aclonifen was estimated to be greater than 1000 mg/L.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 209, 2010)	Achieved
Oxygen uptake rate in controls	≥20 mg O_2/g/h	9.81 mg O_2/g/h
Coefficient of variation between controls	≤30%	0%
EC_{50} for 3,5-dichlorophenol	2–25 mg/L	8.5 mg/L

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint (mg/L)	EC	95% Confidence limits
EC_{50}	>1000	ND

ND = Not determined

III. CONCLUSION

The EC_{50} for respiration inhibition was greater than 1000 mg Aclonifen/L, the highest concentration tested.

[redacted] *(1999)*

Assessment and conclusion by applicant:

In the previous submission (DAR, 2006), this study was evaluated and accepted as valid for risk assessment purposes. The study was performed according to OECD Test Guideline 209 (1984) which was in force at the time of performing the test and all relevant validity criteria were satisfied.

In terms of the current version of OECD 209 (2010), the validity criteria relating to the coefficient of variation between controls and the 3-hour EC_{50} of the reference item 3,5-dichlorophenol were satisfied, however the control oxygen uptake rate was lower than the current guideline requirement of 20 mg O_2/g/h.

Due to the failure to satisfy one of the current guideline validity criteria, the test is not valid according to current requirements.

Therefore as this study does not meet current OECD guideline validity criteria, it should be considered as supportive only.

Assessment and conclusion by RMS:

Data Point:	KCA 8.8/03
Report Author:	[redacted]
Report Year:	2019
Report Title:	Activated sludge, respiration inhibition test with aclonifen
Report No:	EBCL0208
Document No:	M-664091-01-1
Guideline(s) followed in study:	OECD Guideline 209 'Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation)' (adopted: 22 July 2010) and considered the Question-and-Answer Document by the German Federal Environment Agency (Version 2012-03-02). This test method is in most essential parts equal to Council Regulation (EC) No 440/2008, Method C.11 "Biodegradation Activated Sludge Respiration Inhibition Test" (2008).
Deviations from current test guideline:	Current Guideline: OECD 209, 2010 The sludge concentration was 800 mg/L instead of 1500 mg/L. Only 3 concentrations of the test item were used. These deviations were not considered to have affected study integrity and validity.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A study was performed to assess the effect of the test material on the respiration of activated sewage sludge. The study was conducted in accordance with OECD Guideline 209 'Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation)' (adopted: 22 July 2010) and considered the Question-and-Answer Document by the German Federal Environment Agency (Version 2012-03-02). This test method is in most essential parts equal to Council Regulation (EC) No 440/2008, Method C.11 "Biodegradation Activated Sludge Respiration Inhibition Test" (2008).

The activated sludge was exposed to Aclonifen at a limit test item concentration of 100 mg/L. The respiration rate of each mixture was determined after aeration periods of 3 hours.

Aclonifen showed no statistical significant difference of respiration inhibition of activated sludge between control and a limit test item concentration of 100 mg/L. The effect of the test item on the respiration of activated sewage sludge gave a 3-Hour EC_{50} of greater than 100 mg/L. The No Observed Effect Concentration (NOEC) after 3 hours exposure was 100 mg/L.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test Item:** Aclonifen
   Batch no.: AE F068300-01-28
   Active Ingredient / Purity: 99.9% w/w

Appearance:	Yellow powder
Expiry date:	13 November 2020
Storage:	Not reported

2. Reference item: 3,5-dichlorophenol
Batch no.: A0357150

3. Test Species: Activated sewage sludge micro-organisms from a domestic waste water treatment plant

Source: [redacted]

Pre-treatment: The sludge was settled and the supernatant was decanted. After centrifuging the sludge (15 min at 3500 rpm and 20°C) the supernatant was decanted again. Approximately 1 g of the wet sludge was dried in order to calculate the amount of wet sludge to achieve a concentration of activated sludge of 3 g/L (dry weight) suspended solids. The calculated amount of sludge was dissolved in synthetic medium and then filled up to a defined end volume with deionised water.
The activated sewage sludge sample was maintained on continuous aeration in the laboratory at a temperature of 20 ± 2 °C and was fed daily with synthetic medium. The pH of the sample was 7.5

4. Test water: Deionised water

B. STUDY DESIGN AND METHODS

1. In-life phase: 28 January - 06 February 2019

2. Exposure conditions

Test vessels:	300 mL glass Erlenmeyer flasks
Experimental design:	100 mg test item/L, 3 replicates
	100 mg test item/L with (Allylthiourea) ATU, 2 replicates
	Control, 6 replicates
	Control with ATU, 4 replicates
	Reference item; 2.5, 5.0, 10, 20 and 40 mg/L
Loading:	800 mg suspended solids/L
Temperature:	20 ± 2 °C
Aeration:	Continuous aeration

3. Administration of the test item

Dose preparation and dosing

Direct weighings were prepared to give the different test item concentrations. The test item was added into Erlenmeyer flasks (incubation vessels) to about 130 mL deionised water and was stirred before testing (equilibration phase) overnight for 17 hours.

Preparation of test system

8 mL of the synthetic medium and 100 mL of activated sludge were added to the dissolved test item. The mixture was filled up with deionised water to 250 mL and aerated at 20 ± 2 °C.

The exposure medium with the reference substance was prepared by adding 8 mL of the synthetic medium, 100 mL of activated sludge and a defined amount of the stock solution to achieve the test concentrations, and was filled up with deionised water to 250 mL and aerated at 20 ± 3°C.

Control vessels (inoculated sample without test item) were prepared the same way.

Additional vessels to determine the physico-chemical oxygen consumption were prepared containing the test item, and the synthetic medium but no activated sludge.

To determine the heterotrophic oxidation four additional controls and two replicates with the test item concentration 100 mg/L, all containing 1.25 mL of ATU-solution (N-allylthiourea) which equals to a final concentration of 11.6 mg ATU/L, were prepared.

4. Measurements and observations

Oxygen consumption and temperature were measured and recorded after an aeration time of 3 hours in all these vessels starting with control 1-3. Thereafter the pH as well and then the other test vessels were measured. Control 4-6 terminated the measurements.

5. Statistics/Data evaluation

Statistical analysis of the mean respiration rate was performed using a Student-t test.

II. RESULTS AND DISCUSSION

A. ANALYTICAL VERIFICATION

Analytical verification was not required.

B. BIOLOGICAL DATA

The results obtained are summarised in the following tables:

Table: Respiration rates after 3 hours incubation period, percentage inhibition, temperature and pH values in the test performed without ATU (total respiration)

Treatment (mg/L)		Respiration rate (mg/L/h)	Mean Temp. (°C)	pH-	Inhibition (%)
Control 1	--	23.984	20.4	8.4	--
Control 2	--	23.247	20.2	8.4	--
Control 3	--	21.715	19.5	8.5	--
Control 4	--	22.422	19.1	8.5	--
Control 5	--	24.024	19.4	8.4	--

Control 6	--	18.898	19.6	8.4	--
Control, mean (CV)		22.381 (8.616)	--	--	--
Test item	100	22.941	19.3	8.4	0.000
Test item	100	22.142	19.5	8.4	1.070
Test item	100	22.152	19.5	8.5	1.025
Test item, mean (CV)	100	22.412 (2.046)	--	--	0.698
Physico-chemical oxygen consumption control	100	0.276	19.5	7.5	--
Reference compound	2.5	20.233	19.9	8.5	9.597
Reference compound	5	16.167	19.1	8.4	27.768
Reference compound	10	11.563	19.9	8.5	48.335
Reference compound	20	6.464	19.2	8.5	71.117
Reference compound	40	4.639	19.4	8.5	79.272

CV = Coefficient of variance

Table: Respiration rates after 3 hours incubation period, percentage inhibition, temperature and pH values in the test performed with ATU (heterotrophic respiration)

Treatment (mg/L)		Respiration rate (mg/L/h)	Mean Temp. (°C)	pH-	Inhibition (%)
Control 1	--	20.975	19.6	8.4	--
Control 2	--	20.088	19.3	8.4	--
Control 3	--	23.902	19.3	8.4	--
Control 4	--	17.672	19.6	8.4	--
Control, mean		20.659	--	--	--
Test item	100	22.736	19.6	8.4	0.000
Test item	100	22.844	19.6	8.4	0.000
Test item, mean	100	22.790	--	--	0.000

Aclonifen showed no statistical significant difference of respiration inhibition of activated sludge between the control and a limit test item concentration of 100 mg/L and hence the EC_{50} for aclonifen was estimated to be greater than 100 mg/L. Correspondingly the NOEC was ≥100 mg/L.

C. VALIDITY CRITERIA

Validity criterion	Required (OECD 209, 2010)	Achieved
Oxygen uptake rate in controls	≥20 mg O_2/g/h	27.977 mg O_2/g/h
Coefficient of variation between controls	≤30%	8.6%

EC_{50} for 3,5-dichlorophenol	2 – 25 mg/L	10.967 mg/L

All validity criteria were satisfied and therefore this study can be considered to be valid.

D. TOXICITY ENDPOINTS

Table: Summary of endpoints

Endpoint (mg/L)	EC_x	95% Confidence limits
EC_{50}	>100	ND
NOEC	≥100	ND

ND = Not determined

III. CONCLUSION

The EC_{50} for respiration inhibition was greater than 100 mg Aclonifen/L, the highest concentration tested.

[redacted] *(2019)*

Assessment and conclusion by applicant:

All validity criteria were satisfied and therefore this study can be considered to be valid.

The EC_{50} for respiration inhibition is greater than 100 mg Aclonifen/L.

Assessment and conclusion by RMS:

CA 8.9 Monitoring data

No data is required.