Document Title

Summary of the ecotoxicological studies for spiroxamine

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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ERM
On behalf of Bayer AG
Crop Science Division

M-764402-01-2

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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

[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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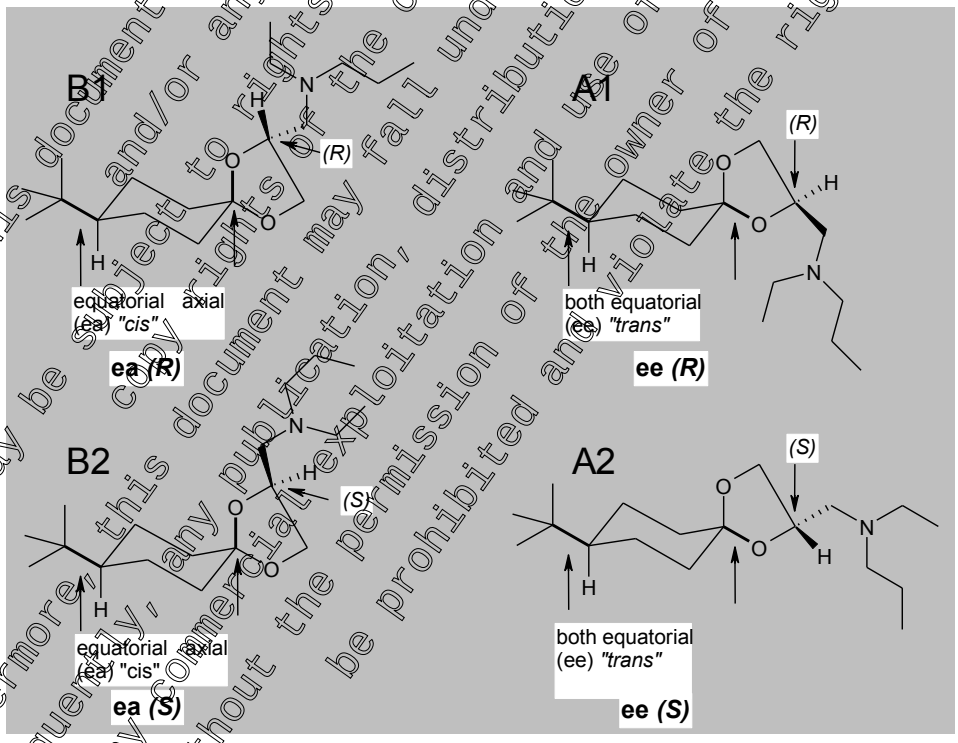
CA 8 ECOTOXICOLOGICAL STUDIES ON THE ACTIVE SUBSTANCE

Spiroxamine was included in Annex I to Council Directive 91/414/EEC in 1999 (Directive 1999/73/EC, Entry into Force on 1 September 1999). This Supplementary Dossier contains data which were not submitted at the time of the Annex I inclusion and first renewal of spiroxamine under Council Directive 91/414/EEC and which were therefore not evaluated during the first EU review and renewal. However, all studies submitted for the first approval and subsequent first renewal of spiroxamine have also been summarised according to current guidance and included in the dossier. Where studies meet relevant validity criteria, new robust study summaries have been provided in the appropriate dossier section. However, where studies do not meet relevant validity criteria and are not considered acceptable, less detailed summaries may have been provided alongside discussions of study deficiencies. All relied upon study reports are submitted in Document K for this second renewal of approval dossier or in Document K for the previous Annex I inclusion and first renewal submissions.

All data which were already submitted by Bayer AG (former Bayer CropScience) for the Annex I inclusion and first renewal under Council Directive 91/414/EEC are contained in the draft Re-Assessment Report (RAR) 2010 and its revised RAR 2017, and are included in the Baseline Dossier provided by Bayer AG.

Spiroxamine consists of four isomers (two diastereomers each with its corresponding two enantiomers which are in a 1:1 ratio) as shown in the schematic below. The isomer nomenclature presented in some historical documentation may differ with respect to the A/B and corresponding trans/cis notation as a result of a discrepancy in referencing, which is discussed in detail in position paper M-761468-01-1 (see CA 1.7/01). It is recommended that the stereo assignments depicted here, together with the A and B notation should be used exclusively going forward to ensure continuity of information throughout the dossier.



Relevant information for classification as detailed in the "*Combined Draft (Renewal) Assessment Report prepared according to Regulation (EC) N° 1107/2009 and Proposal for Harmonised Classification and Labelling (CLH Report) according to Regulation (EC) N° 1272/2008 – Volume 1, Level 2*" is provided in Document N1, Section 9.2, and highlighted in light grey.

CA 8.1 Effects on birds and other terrestrial vertebrates

CA 8.1.1 Effects on Birds

The avian studies conducted with spiroxamine technical are summarised in the following table.

Table CA 8.1.1/01 Summary of avian toxicity studies with spiroxamine

Organism	Test item	Test type	Endpoints		Reference
Bobwhite quail (*Colinus virginianus*)	Spiroxamine	Acute oral toxicity	LD_{50} 565 mg a.s./kg bw	EU	M-008095-02-1
Canary (*Serinus canarius*)	Spiroxamine	Acute oral toxicity	LD_{50} 250 - 500 mg a.s./kg bw	EU	M-008100-01-1
Bobwhite quail (*Colinus virginianus*)	Spiroxamine	Short-term dietary toxicity	LC_{50} > 5000 mg a.s./kg diet (LDD_{50} > 357 mg a.s./kg bw/day)	EU	M-008081-02-1
Mallard duck (*Anas platyrhynchos*)	Spiroxamine	Short-term dietary toxicity	LC_{50} 5000 mg a.s./kg diet (LDD_{50} 871 mg a.s./kg bw/day)	EU	M-008047-02-1
Mallard duck (*Anas platyrhynchos*)	Spiroxamine	Short-term dietary toxicity	LC_{50} >312 mg a.s./kg diet (LDD_{50} >81.4 mg a.s./kg bw/day)	EU	M-008072-01-1
Bobwhite quail (*Colinus virginianus*)	Spiroxamine	Reproductive test	NOEC 29.3 mg a.s./kg diet NOEC 2.02 mg a.s./kg bw/day NOAEC 78.6 mg a.s./kg diet NOAEL 5.40 mg a.s./kg bw/day	EU	M-007470-03-1
Mallard duck (*Anas platyrhynchos*)	Spiroxamine	Reproductive test	NOEC 78.8 mg a.s./kg diet NOEL 10.6 mg a.s./kg bw/day	EU	M-008186-01-1

EU: previously evaluated as part of the original EU review and listed in EFSA conclusion and DAR
NEW: new study or data generated since the previous EU review or previously not submitted

CA 8.1.1.1 Acute oral toxicity to birds

Data Point:	KCA 8.1.1.1/01
Report Author:	[redacted]
Report Year:	1998
Report Title:	KWG 4168 (technical grade): Acute oral toxicity to bobwhite quail
Report No:	VB-028
Document No:	M-008095-02-1
Guideline(s) followed in study:	U.S. EPA E 71-1 (1982)
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2016), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Adult bobwhite quail (*Colinus virginianus*) were used to evaluate the acute oral toxicity of KWG 4168 over a period of 14 days.

Groups of ten birds, five per sex, were given a single oral dose of 125, 250, 500, 1000 or 2000 mg a.s./kg body weight (b.w.) or an untreated control. Following exposure, all groups were held for a 14-day observation period.

No mortalities were observed in the control and 125 mg a.s./kg b.w. groups. One mortality occurred in the dose level of 250 mg a.s./kg b.w. five hours after dosing and one bird showed apathy for two days. At the 500 mg a.s./kg b.w. dose rate, three birds died within two days and four others showed signs of intoxication however recovered within four days. Of the ten birds dosed with 1000 mg a.s./kg b.w., all exhibited signs of toxicity, with nine dying within two days. Half of the birds of the highest dose group, 2000 mg a.s./kg b.w. died within one hour of application of the test substance, with the remaining birds dying in the following two days.

Marked clinical signs of toxicity were apathy and loss of equilibrium. There were no visible sex-related differences in toxicity. Observations in post-mortem examination of the birds dosed with 500, 1000 or 2000 mg a.s./kg b.w. showed no compound-related lesions.

Based on the results of this study, the LD_{50} was determined to be 565 mg a.s./kg b.w. with 95% confidence intervals of 413 to 773 mg a.s./kg b.w. The NOED and LOED were 125 and 250 mg a.s./kg b.w., respectively.

I. Materials and Methods

A. Materials

Test Material KWG 4168

Lot/Batch #: 898114002

Purity: 97.8%

Description: Clear liquid

Stability of test compound: Not reported

Reanalysis/Expiry date: 27 January 1994

Density:	Not reported
Treatments	
Test rates:	125, 250, 500, 1000 or 2000 mg a.s./kg body weight
Solvent/vehicle:	None
Analysis of test concentrations:	No (dose enclosed within capsule)
Test organisms	
Species:	Bobwhite quail (*Colinus virginianus*) aged 19 weeks
Source:	[redacted]
Acclimatisation period:	14-day acclimation period
Feeding:	Laying hen ration available *ad libitum*, except for an 18-hour fasting period prior to dosing
Test design	
Test vessel:	Stainless steel wire cages (18 x 23 x 13 cm)
Replication:	Five female and five male birds per treatment
No. of animals/vessel:	Individually housed
Duration of test:	14 days
Environmental test conditions	
Temperature:	20 ± 2°C
Relative humidity:	30 - 90%
Photoperiod:	Natural day length

B. Study Design

This study was conducted to assess the acute oral toxicity of KWG 4168 on adult bobwhite quail (*Colinus virginianus*). Dose levels for this study were selected based upon the results of a previous range-finding test.

Five male and five female birds were randomly assigned a treatment or control group, and housed individually in 18 x 23 x 13 cm stainless steel wire cages. Cages followed a natural photoperiod and were maintained at 20 ± 2°C with a relative humidity of 30 to 90%.

After a 14-day acclimation period, food was withheld from all groups for approximately 18 hours prior to dose administration. The test substance was administered by oral application of one gelatine capsule per bird. Dose levels used in the study were 125, 250, 500, 1000 and 2000 mg a.s./kg body weight (b.w.) and an empty capsule-only control.

Observations for mortality and toxicity were made continuously for the first hour, *ca.* hourly for the remainder of the first day and then daily on workdays for 14 days. Body weights were measured prior to test initiation (day -1), on study day 7 and at test termination. Feed consumption for each group was recorded on days 3, 7 and 14. At test termination, all surviving birds were sacrificed, and those in the 500, 1000 and 2000 mg a.s./kg b.w. dose groups were necropsied as well as all birds that died during the in-life phase of the study.

II. Results and Discussion

Validity criteria were not assessed as part of the study.

No mortalities were observed in the control and 125 mg a.s./kg b.w. groups.

Half of the birds of the highest dose (2000 mg a.s./kg b.w.) group died within one hour of application of the test substance, with the remaining birds dying in the following two days. Of the ten birds dosed with 1000 mg a.s./kg b.w., all exhibited signs of toxicity, with nine dying within two days.

At the 500 mg a.s./kg b.w. dose rate, three birds died within two days and four others showed signs of intoxication however recovered within four days. One mortality occurred in the dose level of 250 mg a.s./kg b.w. five hours after dosing and one bird showed apathy for two days.

Marked clinical signs of toxicity were apathy and loss of equilibrium. There were no visible sex-related differences in toxicity. Observations in post-mortem examination of the birds dosed with 500, 1000 or 2000 mg a.s./kg b.w. showed no compound-related lesions. Pale livers and pancreas were observed in some birds that had died during the study, however such findings were frequently observed in dead birds and not deemed to be treatment-related.

Table CA 8.1.1.1/01-1 Mortality and signs of toxicity of birds after acute oral exposure to KWG 4168

Dose (mg a.s./kg b.w.)	Mortality (%)			Signs of toxicity (%)[1]		
	Males	Females	Total	Males	Females	Total
Control	0	0	0	0	0	0
125	0	0	0	0	0	0
250	0	20	10	0	40	20
500	40	20	30	60	80	70
1000	80	100	90	100	100	100
2000	100	100	100	100	100	100

[1] Apathy

Table CA 8.1.1.1/01-2 Body weights and body weight change of birds after acute oral exposure to KWG 4168

Dose (mg a.s./kg b.w.)		Males			Females		
		Day -1	Day 7	Day 14	Day -1	Day 7	Day 14
Control	Mean *SD*	182.0 *15.0*	184.8 *14.7*	186.0 *15.3*	173.0 *8.6*	174.4 *12.3*	175.2 *14.0*
125	Mean *SD*	180.6 *16.2*	184.2 *18.0*	179.6 *14.5*	182.8 *19.5*	186.0 *23.7*	191.0 *26.2*
250	Mean *SD*	175.0 *14.9*	173.2 *10.0*	173.0 *10.7*	168.2 *11.1*	164.0 *10.9*	165.5 *9.3*
500	Mean *SD*	171.6 *12.8*	168.7 *9.6*	174.0 *13.1*	162.0 *18.7*	160.0 *11.5*	166.0 *13.4*
1000	Mean *SD*	177.6 *5.9*	195.0 -	206.0 -	165.2 *10.3*	- -	- -
2000	Mean *SD*	173.6 *10.1*	- -	- -	174.6 *8.5*	- -	- -

Table CA 8.1.1.1/01-3 Mean and total feed consumption during exposure and observation periods

Dose (mg a.s./kg b.w.)	Mean daily feed consumption (g feed/bird/day)			Total daily feed consumption (g feed/day)		
	Days 0-3	Days 3-7	Days 7-14	Days 0-3	Days 3-7	Days 7-14
Control	12.2	15.5	14.4	366	621	1010
125	16.8	19.1	16.4	503	765	1148
250	8.9	16.5	15.6	239	595	985
500	3.8	16.9	17.3	88	472	849
1000	4.8	20.0	22.0	43	80	154
2000	0.8	-	-	5	-	-

III. Conclusion

Adult bobwhite quail (*Colinus virginianus*) were used to evaluate the acute oral toxicity of KWG 4168 over a period of 14 days.

Groups of ten birds, five per sex, were given a single oral dose of 125, 250, 500, 1000 or 2000 mg a.s./kg body weight (b.w.) or an untreated control. Following exposure, all groups were held for a 14-day observation period.

Based on the results of this study, the LD_{50} was determined to be 565 mg a.s./kg b.w. with 95% confidence intervals of 413 to 773 mg a.s./kg b.w. The NOED and LOED were 125 and 250 mg a.s./kg b.w., respectively.

Assessment and conclusion by applicant:

The study was conducted to the U.S. EPA E 71-1 (1982) test guideline but the test methodology used is considered to be consistent with the requirements of the current OECD 223 test guideline and is therefore considered acceptable.

The study was assessed against the current OECD test guideline "OECD 223: Avian acute oral toxicity test", adopted 29 July 2016.

Validity criteria according to OECD 223 were met:

- Control mortality to not exceed 10% at the end of the test (actual: 0.0%)

The study is therefore considered acceptable.

Based on the results of this study, the LD_{50} was determined to be 565 mg a.s./kg b.w.

Data Point:	KCA 8.1.1.1/02
Report Author:	[redacted]
Report Year:	1989
Report Title:	KWG 4168: Vogeltoxizitaet oral am Kanarienvogel (Serinus canarius), weiblich, orientierend
Report No:	VK-335
Document No:	M-008100-01-1
Guideline(s) followed in study:	Not reported
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated, not accepted RAR (2010), RAR (2017) Due to poor test design and lacking reliable endpoints the study cannot be used for risk assessment purposes. The acute oral LD50 of spiroxamine in the canary (Serinus canarius) was estimated to be between 250 - 500 mg as/kg bw.
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

A non-GLP screening study (acute oral toxicity test) on a non-standard bird species was conducted with female *Serinus canarius*.

Groups of five birds were exposed for seven days to gavage dosed dietary concentrations of 25, 50, 100, 250, 500 and 1000 mg a.s./kg bw.

The LD_{50} was determined to be 250 – 500 mg/kg bw.

I. Materials and Methods

A. Materials

Test Material KWG 4168 (F)

Lot/Batch #: 17001/89

Purity: 94.2 %

Description: Not reported

Stability of test compound: Not reported

Reanalysis/Expiry date: Not reported

Density: Not reported

Treatments

Test rates: 25, 50, 100, 250, 500 and 1000 mg a.s./kg bw

Solvent/vehicle: Deionised water

Analysis of test concentrations: Not reported

Test organisms

Species: Canary (*Serinus canarius*) – female

Source: Not reported

Acclimatisation period:	Not reported
Feeding:	Not reported
Test design	
Test vessel:	Not reported
Replication:	Not reported
No. of animals/vessel:	Not reported
Duration of test:	Not reported
Environmental test conditions	
Mean temperature:	Not reported
Relative humidity:	Not reported
Photoperiod:	Not reported

B. Study Design

A non-GLP screening study (acute oral toxicity test) on a non-standard bird species was conducted with female *Serinus canaries.*

Groups of five birds were exposed for seven days to gavage dosed dietary concentrations of 25, 50, 100, 250, 500 and 1000 mg a.s./kg bw. Birds were starved for an hour prior to dosing, dosing was conducted *via* a gavage.

II. Results and Discussion

Table CA 8.1.1.1/02-1 Results summary

Applied amount of active substance (mg a.s./kg bw)	Finding (dead / inserted)	Remarks
1000	5/5	-
500	5/5	-
250	1/5	Vomit, cramps, lateral position
100	0/5	Low cramps, apathy
50	0/5	Certain cramps, apathy
25	0/5	-

III. Conclusion

The LD_{50} was determined to be 250 – 500 mg a.s./kg bw.

Assessment and conclusion by applicant:

This non-GLP screening study on a non standard bird species was originally considered as non relevant. However, it supports the toxicity profile reported for Bobwhite quail and is therefore presented as supporting information only.

A control group does not appear to have been tested in this study therefore it is not possible to assess this against the OECD 223 test guideline criterion.

The LD_{50} was determined to be 250 – 500 mg a.s./kg bw.

CA 8.1.1.2 Short-term dietary toxicity to birds

Data Point:	KCA 8.1.1.2/01
Report Author:	[redacted]
Report Year:	1998
Report Title:	KWG 4168 (technical grade): 5-day dietary LC50 to bobwhite quail
Report No:	VB-025
Document No:	M-008081-02-1
Guideline(s) followed in study:	OECD 205 (1984) U.S. EPA E 71-2 (1982)
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2016), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Young bobwhite quail (*Colinus virginianus*) were used to evaluate the dietary toxicity of KWG 4168 over a period of five days.

Groups of ten birds were exposed for five days to dietary concentrations of 313, 625, 1250, 2500 or 5000 mg a.s./kg feed or an untreated control. Following exposure, all groups were held for a three-day observation period on untreated feed.

Based on the results of this study the subacute dietary LC_{50} of technical graded KWG 4168 to bobwhite quail was determined to be >5000 mg a.s./kg feed (equivalent to >357 mg a.s./kg bw/day). The LOAEC and NOAEC were 2500 and 1250 mg a.s./kg feed, respectively.

A decrease in body weight during the exposure period was noted in the group treated with 5000 mg a.s./kg diet, corresponding with a reduction in feed intake of the birds during the treatment phase, indicating the unpalatability of KWG 4168.

I. Materials and Methods

A. Materials

Test Material KWG 4168

Lot/Batch #:	89811/4002
Purity:	96.6%
Description:	Clear liquid
Stability of test compound:	Stable under the conditions of this study (recoveries of 105 – 107% after 24 hours)
Reanalysis/Expiry date:	11 August 1994
Density:	Not reported

Treatments

Test rates:	313, 625, 1250, 2500 and 5000 mg a.s./kg
Solvent/vehicle:	40.0 g peanut oil
Analysis of test concentrations:	Yes, mean measured values of a.s. in diet 90.9 – 116% of nominal

Test organisms	
Species:	Bobwhite quail (*Colinus virginianus*) aged 14 days
Source:	[redacted]
Acclimatisation period:	Acclimated in cages similar to the study cages in size, temperature and lighting
Feeding:	Hatchling starter ration available *ad libitum*
Test design	
Test vessel:	Stainless steel wire cages (70 x 45 x 20 cm).
Replication:	No replicates
No. of animals/vessel:	Ten unsexed birds
Duration of test:	Eight days
Environmental test conditions	
Temperature:	28°C
Relative humidity:	Not reported
Photoperiod:	16 h light : 8 h dark

B. Study Design

This study was conducted to assess the dietary toxicity of KWG 4168 on bobwhite quail (*Colinus virginianus*). Dietary test concentrations were selected based on the results of a preliminary range-finding study.

All birds were apparently healthy after arrival to the test facility, and were phenotypically similar to wild birds. At 14 days of age, ten birds of unknown sex weighing 26 to 41 g were randomly allocated to each of the treatment and control diets. Test vessels were stainless steel wire 70 x 45 x 20 cm cages which were maintained at a mean cage temperature of 28°C.

Test diets were prepared by mixing the bird feed, KWG 4168 and 40.0 g peanut oil in a glass beaker, then grinding the pre-mix to homogeneity, and finally adding the required amount of feed to obtain the desired nominal concentrations. Nominal test concentrations were 313, 625, 1250, 2500 and 5000 mg a.s./kg diet. Measured concentrations in the test diet ranged from 90.9 to 116% of nominal.

Birds were given relevant treated or control feed for five days, after which they received an untreated diet during a three-day observation period. Feed consumption was recorded daily during the exposure period and at the end of the observation period. Food and fresh tap water were available *ad libitum* throughout the study.

Birds were weighed at test initiation, on day 5, and at test termination. Observations for mortality and clinical signs of intoxication were recorded daily throughout the study. Necropsy examinations were conducted on all birds. Control birds and those receiving the 313, 625 or 1250 mg a.s./kg diet were not necropsied due to no abnormalities being observed.

Analytical method

Samples of feed were analysed using the validated analytical method VE-006, report reference M-008047-02-1 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria were not assessed in the study report.

The measured concentrations of spiroxamine in the test diets are summarised below.

Table CA 8.1.1.2/01-1 Measured concentration of KWG 4168 in the animal feed

Nominal dietary concentration (mg a.s./kg diet)	Measured concentration of nominal (%)
Control	-
313	116
625	114
1250	105
2500	98.4
5000	90.9

Mortality of 10 and 20% occurred in the 2500 and 5000 mg a.s./kg diet test groups, respectively. It was unable to be judged whether these mortalities were due to toxicity or to starvation because of the unpalatability of test diet, however clinical signs of toxicity were not observed.

Post-mortem examinations of birds that survived or died during the study showed no gross lesions or macroscopic organ alterations.

Table CA 8.1.1.2/01-2 Cumulative mortality of birds after dietary exposure to KWG 4168

Nominal dietary concentration (mg a.s./kg diet)	Cumulative mortality by day									Total (%)
	0	1	2	3	4	5	6	7	8	
Control	0	0	0	0	0	0	0	0	0	0.0
313	0	0	0	0	0	0	0	0	0	0.0
625	0	0	0	0	0	0	0	0	0	0.0
1250	0	0	0	0	0	0	0	0	0	0.0
2500	0	0	0	0	0	1	1	1	1	10.0
5000	0	0	0	1	1	2	2	2	2	20.0

A decrease in body weight during the exposure period was observed in the 5000 mg a.s./kg diet group, which coincided with a reduction in the feed intake of the birds. This secondary effect on growth is therefore not assumed to be due to toxicity or a treatment-related adverse effect.

Table CA 8.1.1.2/01-3 Body weights and body weight change of birds after dietary exposure to KWG 4168

Nominal dietary concentration (mg a.s./kg diet)	Body weight (g)			Body weight increase (g)		
	Day 0	Day 5	Day 8	Days 0-5	Days 5-8	Days 0-8
Control	33.1	44.0	50.8	10.9	6.8	17.6
313	35.0	43.4	51.3	8.3	8.0	16.3
625	33.4	39.4	46.5	6.1	7.0	13.1
1250	33.8	38.7	44.2	4.9	5.4	10.3
2500	34.9	36.7	41.0	1.8	4.4	6.2
5000	34.4	32.6	38.0	-1.8	5.5	3.6

Table CA 8.1.1.2/01-4 Feed consumption and a.s. intake of KWG 4168 during exposure and observation periods

Nominal dietary concentration (mg a.s./kg diet)	Mean daily feed consumption (g feed/bird/day)			Mean a.s. intake	
	Days 1-5	Days 5-8	Days 0-8	mg a.s./bird/day	mg a.s./kg bw/day
Control	6.1	6.2	6.1	0.0	0.0
313	6.0	6.9	6.3	1.9	48.4
625	4.8	5.5	5.1	3.0	82.4
1250	4.4	5.0	4.6	5.5	152.2
2500	3.8	5.7	4.4	9.4	262.7
5000	2.4	5.9	3.6	12.0	357.4

III. Conclusion

Young bobwhite quail (*Colinus virginianus*) were used to evaluate the dietary toxicity of KWG 4168 over a period of five days.

A decrease in body weight during the exposure period was noted in the group treated with 5000 mg a.s./kg diet, corresponding with a reduction in feed intake of the birds during the treatment phase, indicating the unpalatability of KWG 4168.

Based on the results of this study the subacute dietary LC_{50} of technical graded KWG 4168 to bobwhite quail was determined to be >5000 mg a.s./kg feed (equivalent to >357 mg a.s./kg bw/day). The LOAEC and NOAEC were 2500 and 1250 mg a.s./kg feed, respectively.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline "OECD 205: Avian dietary toxicity test", adopted 4 April 1984 which is still the current version.

Validity criteria according to the OECD 205 (1984) guideline were met:

- Control mortality to not exceed 10% at the end of the test (actual: 0.0%)
- Test substance to remain at least 80% of nominal in the diet throughout the first five days of the test period (actual: 90.9 to 116%)
- The lowest treatment level should not result in any compound-related mortality or toxicity (actual: no toxicity at 313 mg a.s./kg diet)

The study is therefore considered acceptable.

Based on the results of this study the subacute dietary LC_{50} of technical graded KWG 4168 to bobwhite quail was determined to be >5000 mg a.s./kg feed (equivalent to >357 mg a.s./kg bw/day).

Data Point:	KCA 8.1.1.2/02
Report Author:	[redacted]
Report Year:	1998
Report Title:	KWG 4168 (technical grade): 5-day dietary LC50 to mallard duck
Report No:	VE-006
Document No:	M-008047-02-1
Guideline(s) followed in study:	OECD 205 (1984) U.S. EPA E 71-2 (1982) (now OCSPP 850.2200 (2012))
Deviations from current test guideline:	Yes Methods: SANCO/3029/99 rev. 4 No linearity data, no chromatograms, method not described
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Young mallard ducks (*Anas platyrhynchos*) were used to evaluate the dietary toxicity of KWG 4168 over a period of five days.

Groups of ten birds were exposed for five days to dietary concentrations of 313, 625, 1250, 2500 or 5000 mg a.s./kg feed or an untreated control. Following exposure, all groups were held for a three-day observation period on untreated feed.

The LOAEC was 5000 mg a.s./kg feed based on signs of intoxication and mortality. The NOAEC was determined to be 2500 mg a.s./kg feed.

Based on the results of this study the subacute dietary LC_{50} of technical graded KWG 4168 to mallard ducks was determined to be 5000 mg a.s./kg feed (equivalent to 871 mg a.s./kg bw/day).

I. Materials and Methods

A. Materials

Test Material KWG 4168

Lot/Batch #: 898114002

Purity: 96.6%

Description: Clear liquid

Stability of test compound: Stable under the conditions of this study (recoveries of 105 – 107% after 24 hours)

Reanalysis/Expiry date: 11 August 1994

Density: Not reported

Treatments

Test rates: 313, 625, 1250, 2500 and 5000 mg a.s./kg feed

Solvent/vehicle: 60.0 g peanut oil

Analysis of test concentrations: Yes, mean measured values of a.s. in diet 83.4 – 99.9% of nominal

Test organisms

Species: Mallard ducks (*Anas platyrhynchos*) aged 7 days

Source:	[redacted]
Acclimatisation period:	Acclimated in cages similar to the study cages in size, temperature and lighting
Feeding:	Hatchling starter ration available *ad libitum*
Test design	
Test vessel:	PE-foil coated stainless wire cages of 100 x 70 x 70 cm
Replication:	No replicates
No. of animals/vessel:	Ten unsexed birds
Duration of test:	Eight days
Environmental test conditions	
Temperature:	27 – 33°C
Relative humidity:	40 – 60%
Photoperiod:	16 h light : 8 h dark

B. Study Design

This study was conducted to assess the dietary toxicity of KWG 4168 on mallard duck (*Anas platyrhynchos*). Dietary test concentrations were selected based on the results of a preliminary range-finding study.

All birds were apparently healthy after arrival to the test facility, and were phenotypically similar to wild birds. At six days of age, ten birds of unknown sex weighing 68.2 to 123.5 g were randomly allocated to each of the treatment and control diets. Test vessels were PE-foil coated stainless steel wire 100 x 70 x 70 cm cages which were maintained at a mean cage temperature of 27 to 33°C.

Test diets were prepared by mixing the bird feed, KWG 4168 and 60.0 g peanut oil in a glass beaker, then grinding the pre-mix to homogeneity, and finally adding the required amount of feed to obtain the desired nominal concentrations. Nominal test concentrations were 313, 625, 1250, 2500 and 5000 mg a.s./kg. Measured concentrations in the test diet ranged from 83.4 to 99.9% of nominal.

Birds were given relevant treated or control feed for five days, after which they received an untreated diet during a three-day observation period. Feed consumption was recorded daily during the exposure period and at the end of the observation period. Food and fresh tap water were available *ad libitum* throughout the study.

Birds were weighed at test initiation, on day 5 and at test termination. Observations for mortality and clinical signs of intoxication were recorded daily throughout the study. Necropsy examinations were conducted on all birds of the 2500 and 5000 mg a.s./kg diet groups. Control birds and those receiving the 313, 625 or 1250 mg a.s./kg diet were not necropsied due to no abnormalities being observed.

Analytical method

Samples of feed were analysed using the validated analytical method VE-006, report reference M-008047-02-1 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria were not assessed in the study report.

The measured concentrations of spiroxamine in the test diets are summarised below.

Table CA 8.1.1.2/02-1 Mean measured concentration of KWG 4168 in the animal feed

Nominal dietary concentration (mg a.s./kg diet)	Mean measured concentration of nominal (%)
Control	-
313	86.4
625	99.9
1250	93.8
2500	86.0
5000	83.4

Mortalities and clinical signs of toxicity (slight apathy, tumbling) were observed for the birds exposed to 5000 mg a.s./kg feed from the fourth day of exposure onwards. Total mortality at the end of the test was 0.0, 0.0, 0.0, 0.0, 0.0 and 50% in the control, 313, 625, 1250, 2500 and 5000 mg a.s./kg diet test groups, respectively. Thus, the LC_{50} was considered to be 5000 mg a.s./kg diet.

Post-mortem examinations of surviving birds from the 2500 and 5000 mg a.s./kg diet groups showed no gross lesions or macroscopic organ alternations, while the examination of those birds that died in the 5000 mg a.s./kg diet group showed pale discolouration of the pancreas, liver and kidneys as well as enlarged gall bladders.

Table CA 8.1.1.2/02-2 Cumulative mortality of birds after dietary exposure to KWG 4168

Nominal dietary concentration (mg a.s./kg diet)	Cumulative mortality by day									Total (%)
	0	1	2	3	4	5	6	7	8	
Control	0	0	0	0	0	0	0	0	0	0.0
313	0	0	0	0	0	0	0	0	0	0.0
625	0	0	0	0	0	0	0	0	0	0.0
1250	0	0	0	0	0	0	0	0	0	0.0
2500	0	0	0	0	0	0	0	0	0	0.0
5000	0	0	0	0	2	5	5	5	5	50

During the five-day exposure period, a dose-related reduction in feed intake was evident in groups receiving 625, 1250, 2500 and 5000 mg a.s./kg diet. The reduced feed intake led to diminished body weight gain in all treated groups over the exposure period, a growth depression that could not be compensated in any of the treated groups during the following three-day observation period on untreated feed.

Table CA 8.1.1.2/02-3 Feed consumption and a.s. intake of KWG 4168 during exposure and observation periods

Nominal dietary concentration (mg a.s./kg diet)	Mean daily feed consumption (g feed/bird/day)		Mean a.s. intake, days 1-5	
	Days 1-5	Days 6-8	mg a.s./bird/day	mg a.s./kg bw/day
Control	48.8	63.4	0.0	0.0
313	50.0	60.2	15.6	84.0
625	44.5	57.5	27.8	163.7
1250	34.6	53.6	43.2	295.1
2500	22.1	53.4	55.3	502.7
5000	15.1	51.3	75.5	871.1

Table CA 8.1.1.2/02-4 Body weights and body weight change of birds after dietary exposure to KWG 4168

Nominal dietary concentration (mg a.s./kg diet)		Body weight (g)			Body weight change (%)	
		Day 0	Day 5	Day 8	Days 0 – 5	Days 5 – 8
Control	Mean *SD*	84.87 *10.87*	200.25 *19.62*	277.90 *22.90*	+135.9	+38.8
313	Mean *SD*	89.75 *11.13*	185.90 *24.42*	245.60 *30.91*	+107.1	+32.1
625	Mean *SD*	88.28 *9.09*	169.88 *20.73*	243.70 *34.22*	+92.4	+43.5
1250	Mean *SD*	98.53 *11.53*	146.39 *18.10*	219.20 *22.77*	+48.6	+49.7
2500	Mean *SD*	88.72 *12.81*	110.01 *19.98*	173.90 *23.56*	+24.0	+58.1
5000	Mean *SD*	86.12 *10.94*	86.64 *18.85*	176.40 *21.98*	+0.6	+103.6

III. Conclusion

Young mallard ducks (*Anas platyrhynchos*) were used to evaluate the dietary toxicity of KWG 4168 over a period of five days.

Groups of ten birds were exposed for five days to dietary concentrations of 313, 625, 1250, 2500 or 5000 mg a.s./kg feed or an untreated control. Following exposure, all groups were held for a three-day observation period on untreated feed.

The LOAEC was 5000 mg a.s./kg feed based on signs of intoxication and mortality. The NOAEC was determined to be 2500 mg a.s./kg feed.

Based on the results of this study the subacute dietary LC_{50} of technical graded KWG 4168 to mallard ducks was determined to be 5000 mg a.s./kg feed (equivalent to 871 mg a.s./kg bw/day).

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline "OECD 205: Avian dietary toxicity test", adopted 4 April 1984 which is still the current version.

Validity criteria according to the OECD 205 (1984) guideline were met:

- Control mortality to not exceed 10% at the end of the test (actual: 0.0%)
- Test substance to remain at least 80% of nominal in the diet throughout the first five days of the test period (actual: 83.4 to 99.9%)
- The lowest treatment level should not result in any compound-related mortality or toxicity (actual: no toxicity at 313 mg a.s./kg diet)

The study is therefore considered acceptable.

Based on the results of this study the subacute dietary LC_{50} of technical graded KWG 4168 to mallard ducks was determined to be 5000 mg a.s./kg feed (equivalent to 871 mg a.s./kg bw/day).

Data Point:	KCA 8.1.1.2/03
Report Author:	[redacted]
Report Year:	1995
Report Title:	KWG 4168 (technical grade): 5-day dietary NOEC to mallard duck
Report No:	VE-007
Document No:	M-008072-01-1
Guideline(s) followed in study:	OECD 205 (1984) U.S. EPA E 71-2 (1982)
Deviations from current test guideline:	None
Previous evaluation:	No, submitted, not evaluated RAR (2010), RAR (2017) Since a NOEC value based on weight data could not be defined, a follow-up study was conducted (report no. VE-007).
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Young mallard ducks (*Anas platyrhynchos*) were used to evaluate the dietary NOEC of KWG 4168 over a period of five days.

Groups of ten birds were exposed for five days to dietary concentrations of 156 or 312 mg a.s./kg feed or an untreated control. Following exposure, all groups were held for a three-day observation period on untreated feed.

Based on the results of this study the NOEC, NOAEC and LOEC were <156, >312 and 156 mg a.s./kg feed, respectively.

I. Materials and Methods

A. Materials

Test Material KWG 4168

Lot/Batch #: 898114002

Purity: 96.6%

Description: Clear liquid

Stability of test compound: Stable under the conditions of this study (recoveries of 105 – 107% after 24 hours)

Reanalysis/Expiry date: 01 August 1994

Density: Not reported

Treatments

Test rates: 156 and 312 mg a.s./kg diet

Solvent/vehicle: 60.0 g peanut oil

Analysis of test concentrations: Yes, mean measured values of a.s. in diet 95.5 – 97.6% of nominal

Test organisms

Species: Mallard ducks (*Anas platyrhynchos*) aged 7 days

Source:	[redacted]
Acclimatisation period:	Acclimated in cages similar to the study cages in size, temperature and lighting
Feeding:	Hatchling starter ration available *ad libitum*
Test design	
Test vessel:	PE-foil coated stainless wire cages of 100 x 70 x 70 cm
Replication:	No replicates
No. of animals/vessel:	Ten unsexed birds
Duration of test:	Eight days
Environmental test conditions	
Mean temperature:	29 – 31°C
Relative humidity:	Not reported
Photoperiod:	16 h light : 8 h dark

B. Study Design

This study was conducted to assess the dietary toxicity of KWG 4168 on mallard duck (*Anas platyrhynchos*). Dietary test concentrations were selected based on the results of a preliminary range-finding study.

All birds were apparently healthy after arrival to the test facility, and were phenotypically similar to wild birds. At six days of age, ten birds of unknown sex weighing 80 to 132 g were randomly allocated to each of the treatment and control diets. Test vessels were PE-foil coated stainless steel wire 100 x 70 x 70 cm cages which were maintained at a mean cage temperature of 29 to 31°C.

Test diets were prepared by mixing the bird feed, KWG 4168 and 60.0 g peanut oil in a glass beaker, then grinding the pre-mix to homogeneity, and finally adding the required amount of feed to obtain the desired nominal concentrations. Nominal test concentrations were 156 and 312 mg a.s./kg diet. Measured concentrations in the test diet ranged from 95.5 to 97.6% of nominal.

Birds were given relevant treated or control feed for five days, after which they received an untreated diet during a three-day observation period. Feed consumption was recorded daily during the exposure period and at the end of the observation period. Food and fresh tap water were available *ad libitum* throughout the study.

Birds were weighed at test initiation, on day 5 and at test termination. Observations for mortality and clinical signs of intoxication were recorded daily throughout the study. Necropsy examinations were conducted on all birds of the 312 mg a.s./kg diet groups. Control birds and those receiving 156 mg a.s./kg diet were not necropsied due to no abnormalities being observed.

Analytical method

Samples of feed were analysed using the validated analytical method VE-006, report reference M-008047-02-1 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria were not assessed in the study report.

The measured concentrations of spiroxamine in the test diets are summarised below.

Table CA 8.1.1.2/03-1 Measured concentration of KWG 4168 in the animal feed

Nominal dietary concentration (mg a.s./kg diet)	Measured concentration of nominal (%)
Control	-
156	97.6
312	95.5

Mortalities and clinical signs of toxicity were not observed in any of the birds during the study. Post-mortem examinations of surviving birds from the 312 mg a.s./kg diet showed no gross lesions or macroscopic organ alterations.

During the 5-day exposure period, a dose-related reduction in feed intake and in body weight gain was noted in the treatment groups. The depression of body weight development was not deemed an adverse treatment-related effect, as the unpalatability of KWG 4168 reduces consumption of contaminated feed.

Table CA 8.1.1.2/03-2 Cumulative mortality of birds after dietary exposure to KWG 4168

Nominal dietary concentration (mg a.s./kg diet)	Cumulative mortality by day									Total (%)
	0	1	2	3	4	5	6	7	8	
Control	0	0	0	0	0	0	0	0	0	0.0
156	0	0	0	0	0	0	0	0	0	0.0
312	0	0	0	0	0	0	0	0	0	0.0

During the five-day exposure period, a dose-related reduction in feed intake and in body weight gain was noted in the treatment groups.

Table CA 8.1.1.2/03-3 Feed consumption and a.s. intake of KWG 4168 during exposure and observation periods

Nominal dietary concentration (mg a.s./kg diet)	Mean daily feed consumption (g feed/bird/day)		Mean a.s. intake, days 1-5	
	Days 1-5	Days 6-8	mg a.s./bird/day	mg a.s./kg bw/day
Control	55.5	57.7	0.0	0.0
156	44.0	48.0	6.9	39.5
312	47.5	55.1	14.8	81.4

Table CA 8.1.1.2/03-4 Body weights and body weight change of birds after dietary exposure to KWG 4168

Nominal dietary concentration (mg a.s./kg diet)		Body weight (g)			Body weight change (%)	
		Day 0	Day 5	Day 8	Days 0 – 5	Days 5 – 8
Control	Mean *SD*	103.60 *13.93*	201.69 *27.09*	285.30 *39.86*	+94.7	+41.5
156	Mean *SD*	100.09 *15.88*	173.72 *23.63*	248.40 *27.27*	+73.6	+43.0
312	Mean *SD*	105.31 *14.52*	182.03 *25.74*	262.80 *29.99*	+72.9	+44.4

III. Conclusion

Young mallard ducks (*Anas platyrhynchos*) were used to evaluate the dietary NOEC of KWG 4168 over a period of five days.

Groups of ten birds were exposed for five days to dietary concentrations of 156 or 312 mg a.s./kg feed or an untreated control. Following exposure, all groups were held for a three-day observation period on untreated feed.

Based on the results of this study the NOEC, NOAEC and LOEC were <156, >312 and 156 mg a.s./kg feed, respectively.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline "OECD 205: Avian dietary toxicity test", adopted 4 April 1984 which is still the current version.

Validity criteria according to the OECD 205 (1984) guideline were met:

- Control mortality to not exceed 10% at the end of the test (actual: 0.0%)
- Test substance to remain at least 80% of nominal in the diet throughout the first five days of the test period (actual: 95.5 to 97.6%)
- The lowest treatment level should not result in any compound-related mortality or toxicity (actual: no toxicity at 156 mg a.s./kg diet)

The study is therefore considered acceptable.

No LC_{50} was reported but, due to the absence of mortality, the LC_{50} is considered to be >312 mg a.s./kg feed (equivalent to >81.4 mg a.s./kg bw/day).

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:	2004
Report Title:	Effects of a subchronic dietary exposure of KWG 4168 techn. on bobwhite quail including effects on reproduction and health
Report No:	SXR/REP/04
Document No:	M-007470-03-1
Guideline(s) followed in study:	OECD Guideline 206 "Avian Reproduction Test" from April 1984 and EPA Pesticide Assessment Guidelines / Subdivision E, § 71-4 from July 1986
Deviations from current test guideline:	Yes Methods: SANCO/3029/99 rev. 4 No correlation coefficient or equation of the line presented Only three levels injected in singlicate for linearity
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

This study was conducted to assess the effects of long-term dietary exposure of KWG 4168 on the health and reproductive performance of adult bobwhite quail (*Colinus virginianus*). Three test concentrations and a control were used, each of which contained 20 pairs of one male and one female bobwhite quail. Nominal concentrations were 30, 77 and 200 mg a.s./kg diet.

After the 21-week dietary exposure period, no dose-dependent and thus treatment-related adverse effects on behaviour, survival rate or body mass changes of adult bobwhite quail were observed.

No evaluation criteria concerning reproductive performance were found to be statistically significantly different to the control in any of the treatment groups. There was, however, a statistically significant difference in the mean body mass of the 14-day old surviving chicks.

The NOEC and LOEC for adult bobwhite quail were determined to be 204 and >204 mg a.s./kg diet, respectively, and for reproductive performance were 29.3 and 78.6 mg a.s./kg diet.

I. Materials and Methods

A. Materials

Test Material	KWG 4168
Lot/Batch #:	898114002
Purity:	97.8% (27 July 1993), 97.5% (21 January 1994), 97.0% (25 July 1994)
Description:	Clear liquid
Stability of test compound:	Measured values after a 14-day storage period showed no inadmissible deviation (87 – 92% of initial)
Reanalysis/Expiry date:	25 January 1995
Density:	Not reported
Treatments	
Test rates:	Nominal: 30, 77 and 200 mg a.s./kg basal diet Measured: 28.3, 78.6 and 204 mg a.s./kg basal diet
Solvent/vehicle:	None
Analysis of test concentrations:	Yes, measured concentrations of the a.s. in prepared diets was 98 – 102% of nominal
Test organisms	
Species:	Bobwhite quail (*Colinus virginianus*) aged 25 weeks. Pen-reared and phenotypically indistinguishable from wild birds.
Source:	[redacted]
Acclimatisation period:	After arrival to the test facility, at least two weeks prior to the initiation of the test, birds were placed in an 18 m^2 aviary for acclimation to climatic conditions
Feeding:	Adults: Fed a full value diet for adult quail (Altromin 0721) *ad libitum* Hatchlings: Fed a commercial game bird feed (Altromin 0711) *ad libitum*
Test design	
Test vessel:	Adults: Stainless steel wire and sheet pens of 75 x 50 x 25 cm Hatchlings: Batteries of brooding pens of 55 x 33 x 18 cm
Replication:	20 pairs per group
No. of animals/vessel:	One male and one female per pen
Duration of test:	21 weeks
Environmental test conditions	

Temperature:	Adults: 20 – 22°C Hatchlings: First week: 35 – 38°C Second week: 30 – 32°C
Relative humidity:	55 – 75%
Photoperiod:	Adults: First 8 weeks: 7 h light : 17 h dark with a 30 minute transition period From week 9: 17 h light : 7 h dark with a 30 minute transition period Hatchlings: Outdoor light/dark cycle

B. Study Design

This study was conducted to assess the effects of long-term dietary exposure of KWG 4168 on the health and reproductive performance of adult bobwhite quail (*Colinus virginianus*).

Test birds were pen-reared, 25-week old bobwhite quail (*Colinus virginianus*) that were healthy and phenotypically indistinguishable from wild birds. After arrival to the test facility, birds were placed in a large 18 m^2 aviary for acclimatisation.

Adult birds were housed indoors in stainless steel wire and sheet pens of 75 x 50 x 25 cm, with a slight slope towards the feeding box. Pens were provided with prepared diets and tap water *ad libitum*, with any uneaten food being removed weekly. Hatchlings were housed in battery brooding pens of 55 x 33 x 18 cm.

Birds were randomly assigned to one of four treatment groups. Each group consisted of 20 pairs, with one male and one female birds per pen. To three of the groups were fed nominal concentrations of 30, 77 and 200 mg a.s./kg diet, with the fourth group receiving untreated control diet. Mean measured concentrations of the diet were 29.3, 78.6 and 204 mg a.s./kg diet, corresponding to 98, 102 and 102% of nominal, respectively.

Fresh batches of diet were prepared in 4-week intervals during the 21-week exposure period. Samples of the control and test diets were taken immediately after preparation of four of the batches, and analysed using gas chromatography.

All adult birds were observed at least once each work day throughout the study for signs of toxicity or behavioural impacts. All birds were necropsied upon death or termination of the study. Body mass of adults was determined at study initiation, every second week until week 8 and after terminal sacrifice. Body mass was not measured during egg laying to prevent any potential adverse effects of handling on egg production. Feed consumption per pen was measured weekly throughout the study.

Eggs were collected daily throughout the course of the study, and stored for a maximum of seven days in a cooler. Mean storage temperature was maintained at 16 ± 1°C. After removal from the cooler, eggs were candled to identify any cracks, and such eggs were discarded. Non-cracked eggs were then placed into an incubator at 37.5 ± 0.5°C, with a relative humidity during incubation of 50 to 65% and during hatch of 70 to 75%. In order to prevent adhesion of the embryo to the shell membrane, eggs were rotated every four hours. Eggs were candled again on day 11 of incubation to determine fertility and on day 18 for determination of embryo viability. On day 21 of incubation, eggs were placed into a hatcher, and all hatchlings, unhatched chicks and eggshells were removed 48 hours after emergence of the first chick.

To determine egg thickness, eggs were opened around the equator, washed out and left to dry with the membrane intact for at least 48 hours at room temperature. The thickness was then measured at four points around the waist using a calibrated micrometre.

Hatchlings were grouped according to parent treatment level and housed for 14 days in batteries of 55 x 33 x 18 cm, at 35 to 38°C. Birds were provided with untreated diet and water *ad libitum*. Grouped hatchling body mass was then recorded by parent treatment group at the end of the 14 days.

Analytical method

Samples of feed were analysed using the validated analytical method 00795, report reference M-007470-03-1 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria were not assessed in the study report.

The measured concentrations of spiroxamine in the test diets are summarised below.

Table CA 8.1.1.3/01-1 Measured dietary concentrations of KWG 4168 in control and test diets

Nominal concentration (mg a.s./kg)	Measured concentration by diet preparation (mg a.s./kg)					
	1st preparation	3rd preparation	5th preparation	6th preparation	Mean	Mean % of nominal
0	0	0	0	0	0	
30	28.3	29.2	30.3	29.4	29.3	98
77	80.1	92.4	71.1	70.7	78.6	102
200	202	222	195	195	204	102

Clinical observations and mortality

During the 21-day study period, a total loss of two of the 160 birds was recorded, one in the 29.3 mg a.s./kg diet group and one in the 204 mg a.s./kg diet group. No test substance-related symptoms were evident prior to death. Three birds in the highest treatment level, 204 mg a.s./kg diet showed short-term, reversible behavioural impacts following injuries on claws and legs. As similar injuries of feet were observed in all test groups and the control, it is assumed that such injuries are attributable to the cage environment rather than to treatment effects.

Gross necropsy

The gross pathological examination of adults at termination of the study showed in particular injuries on the neck and heads of birds as a consequence of cage-mate interactions.

Birds from all treatment groups showed a reduced size of testicles/ovaries. Additionally, greenish discoloured testicles of some males were found throughout all concentration groups. There was, however, no correlation between these findings and the symptoms observed during the exposure period.

Other pathological findings such as enlarged spleen, foot lesions, enlarged kidneys among other findings did not show a clear dose-response, and appeared to be distributed over all test groups at random. It is therefore assumed that the treatment did not adversely affect the body organs of the birds.

Adult body weights and feed consumption

Statistical analyses of body mass and feed consumption of adults revealed a significantly lower male body mass in the 29.3 mg a.s./kg diet ($p \leq 0.05$) treatment group at test initiation and at weeks three and five, however, at test termination no statistically significant difference was recorded. No significant differences in female body mass were observed. Feed consumption was significantly reduced at weeks four, seven and eight.

No significant differences in body mass were found at any time interval in the 78.6 mg a.s./kg diet treatment group. Feed consumption was significantly reduced at weeks four, eight and 11.

No statistically significant differences in male body mass were observed in the 204 mg a.s./kg diet treatment group to the controls, however female body weight at weeks three and five were significantly reduced. Statistically significant reductions in feed consumption were observed at weeks four, eight, 10, 15 and 20.

Table CA 8.1.1.3/01-2 Mean adult body weight after dietary exposure to KWG 4168

Mean measured concentration (mg a.s./kg diet)	Sex	Mean body weight (g)					
		Week 1	Week 3	Week 5	Week 7	Week 9	Week 22
Control	Male	198	202	203	206	209	217
	Female	193	199	202	204	207	247
29.3	Male	189*	194*	195*	199	202	210
	Female	187	193	196	201	204	250
78.6	Male	194	200	202	206	210	216
	Female	190	196	199	203	206	247
204	Male	191	196	197	200	205	212
	Female	186	191*	193*	197	202	233

* Differences between the control were not significant (p>0.05).

Table CA 8.1.1.3/01-3 Mean feed consumption during and after dietary exposure to KWG 4168

Mean measured concentration (mg a.s./kg diet)	Feed consumption (g/pair/day) by week										
	1	2	3	4	5	6	7	8	9	10	11
Control	20.6	24.0	22.3	23.6	21.9	20.6	21.3	22.4	24.5	26.9	28.6
29.3	19.6	23.6	21.3	22.2*	21.3	20.0	19.8*	20.9*	23.2	26.1	27.9
78.6	20.6	23.5	22.2	22.3*	21.2	19.8	20.4	21.3*	23.8	26.3	26.5*
204	20.0	23.3	21.4	22.0*	21.4	20.1	20.3	20.8*	23.1	25.4*	25.4
	12	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**-**
Control	28.5	31.8	34.8	36.8	37.4	39.3	37.2	39.4	38.9	39.4	-
29.3	26.9	30.8	35.1	36.9	37.6	39.4	35.5	38.4	37.0	41.4	-
78.6	27.4	30.0	33.1	36.0	36.7	38.9	36.9	40.7	39.8	43.1	-
204	28.4	29.2	31.8	34.1	35.0	36.4	34.3	37.8	35.1*	39.3	-

* Significantly different from the control at p<0.05

Reproductive results

There were no statistically significant differences observed between the treatment groups and the controls in measured criteria of the reproductive performance (number of laid eggs, number of cracked eggs, eggshell thickness, fertility, hatch rate, body mass of hatchlings) over the 11-week reproductive period of the study.

Juvenile observations and mortality

Observations on the health and vitality of hatchlings during the 14-day post-hatching period showed no statistically significant differences between treatment groups and controls. At the end of the 14-day post-hatching period, mean body mass of hatchlings was statistically significantly reduced in the 78.6 and 204 mg a.s./kg diet treatment groups when compared to the control group.

Table CA 8.1.1.3/01-4 Summary of mallard reproductive performance after dietary exposure to KWG 4168

Parameter	Test concentration (mg a.s./kg diet)			
	Control	29.3	78.6	204
Total eggs laid / hen	51.9	55.1	51.0	45.7
Total eggs set / hen	43.8	48.9	43.9	39.9
Eggshell thickness (mm)	0.21	0.20	0.21	0.20
No. of cracked eggs / hen (% of eggs laid)	8.9	5.0	8.0	5.7

Parameter	Test concentration (mg a.s./kg diet)			
	Control	29.3	78.6	204
No. of fertile eggs on Day 11 / hen (% of eggs set)	94.1	95.3	96.2	86.3
No. of viable embryos on Day 18 / hen (% of eggs set)	90.2	92.8	94.3	85.0
No. of hatchlings / hen (% of fertile eggs)	69.5	68.9	69.9	66.3
No. of 14-day survivors / hen	27.2	30.0	26.8	21.2
No. of 14-day survivors / hen (% eggs set)	62.2	61.4	61.0	53.0
Average body weight of hatchlings (g) (% inhibition compared to control)	-	2.81	0	2.81
Average body weight of 14-day survivors (g) (% inhibition compared to control)	-	2.06	3.83*	8.85*

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

III. Conclusion

During a 21-week dietary exposure to KWG 4168 technical at measured concentrations of 29.3, 78.6 and 204 mg a.s./kg diet, no dose-dependent and thus treatment-related adverse effects on behaviour, survival rate or body mass changes of adult bobwhite quail (*Colinus virginianus*) were observed.

No evaluation criteria concerning reproductive performance were found to be statistically significantly different to the control in any of the treatment groups. There was, however, a statistically significant difference in the mean body mass of the 14-day old surviving chicks.

The NOEC and LOEC for adult bobwhite quail were therefore determined to be 204 and >204 mg a.s./kg diet, respectively, and/or reproductive performance were 29.3 and 78.6 mg a.s./kg diet.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline "OECD 206: Avian reproduction test", adopted 4 April 1984 and meets the requirements set out within. Due to there being only three test concentrations with a wide spacing factor between, the data are not considered suitable for reliable EC_{10} and EC_{20} calculation therefore this has not been conducted.

Validity criteria according to the OECD 206 (1984) guideline were met:

- Control mortality to not exceed 10% at the end of the test (actual: 0.0%)
- Average number of 14-day surviving chicks in the control to be at least 12 (actual: 27.2)
- The average shell thickness for eggs in the control group to be at least 0.19 mm (actual: 0.21 mm)

The study is therefore considered acceptable.

The NOEC determined in the study was 29.3 mg a.s./kg diet (equivalent to 2.02 mg a.s./kg bw/day) and has been based on the statistically significant effects on 14-day survivor body weight at 78.6 mg a.s./kg diet. This NOEC is considered to be very conservative because there was only a 3.8% reduction in body weights, relative to the control, at 78.6 mg a.s./kg diet. Whilst statistically significant, this reduction is not considered to be a true treatment related effect as the reduction is very minor and unlikely to cause an impact at the population level.

It may be a statistical anomaly instead of a substance related effect since over the weeks the body weights of 14 day survivors changed. They were statistically reduced in three of the weeks but in two of the weeks they were reduced without statistical significance. In one of the weeks the body weights were equal to the control, but in three of the weeks they were higher than the control. In the last two weeks for example the mean body weights of 14 day old survivors were 34.3 g and 33.7 g, at 78.8 mg a.s./kg diet, while the control chicks weighed 32.3 g in that period. In contrast to these slight and partially contradictory effects, the results at the next highest test concentration (204 mg a.s./kg diet) were consistent and clear. The reduction of body weight of 14 days survivors compared to the control at this dose group amounted to 8.8 %. This also is not a dramatic decline but the average body weights were reduced over the whole exposure period (6 times statistically significant, 3 times without statistically significance). These findings indicate that at this test concentration (204 mg a.s./kg diet) the effects have to be considered treatment related. It is therefore believed that the LOEC is 204 mg a.s./kg diet and the NOAEC is 78.6 mg a.s./kg diet (equivalent to 5.40 mg a.s./kg bw/day).

Further supporting data has been provided below (KCA 8.1.1.3/05) in the form of historical control data for 14-day old survivors to demonstrate that the mean body weight of 32.6 g achieved at 78.6 mg a.s./kg diet is well within the normal deviation of the historical controls.

Data Point:	KCA 8.1.1.3/02
Report Author:	[redacted]
Report Year:	1997
Report Title:	KWG 4168 Technical - A reproduction study with mallard (Anas platyrhynchos)
Report No:	107913
Document No:	M-008186-01-2
Guideline(s) followed in study:	FIFRA 71-4 (now OCSPP 850.2300) OECD 206 (1984)
Deviations from current test guideline:	Yes Methods: SANCO/3029/99 rev. 4 Accuracy n<5 for some levels, precision not available for 10 mg/kg level
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Mallard ducks (*Anas platyrhynchos*) were exposed to KWG 4168 in the diet at mean measured concentrations of 28.4, 78.8 and 205 mg a.s./kg diet or a control for 20 weeks. Ducks were observed daily for mortality, abnormal behaviour and signs of toxicity. In addition, the effects of adult exposure on the number of eggs laid, normal development of eggs, embryo viability, percent hatchability, offspring survival and eggshell thickness were evaluated.

No mortalities, overt signs of toxicity or other treatment-related effects on body weight were observed at any of the concentrations tested. There were also no treatment-related effects upon feed consumption or reproductive performance at the 28.4 and 78.8 mg a.s./kg diet test concentrations.

At the 205 mg a.s./kg diet test concentration there was a treatment-related reduction in feed consumption and an increase in the number of hens displaying lesions of egg yolk peritonitis at terminal necropsy. Additionally, there were reductions in egg production, viable eggs and embryo survival that were reflected as reductions in the numbers of hatchlings and 14-day old survivors. There was also a treatment-related reduction in the body weight of hatchlings in the 205 mg a.s./kg diet treatment group.

Based upon the multiple effects seen at the highest test concentration, the NOEC and LOEC for this study were determined to be 78.8 and 205 mg a.s./kg diet, respectively.

I. Materials and Methods

A. Materials

Test Material	KWG 4168
Lot/Batch #:	603-0152
Purity:	100% (reported)
Description:	Clear yellow liquid
Stability of test compound:	Analysis of diet samples collected from feeders after being held at ambient temperatures for 7 days were 95, 104 and 92% of nominal for the 30, 77 and 200 mg a.s./kg diet test concentrations
Reanalysis/Expiry date:	Not available
Density:	Not available
Treatments	
Test rates:	Nominal: 0, 30, 77 and 200 mg a.s./kg diet Mean measured: 0, 28.4, 78.8 and 205 mg a.s./kg diet
Solvent/vehicle:	100 mL acetone and 180 mL corn oil
Analysis of test concentrations:	Yes, mean measured concentrations 95 – 103% of nominal
Test organisms	
Species:	Mallard ducks (*Anas platyrhynchos*) aged 23 weeks
Source:	[redacted]
Acclimatisation period:	Three-week acclimation period
Feeding:	Feed formulated to test facility specifications
Test design	
Test vessel:	Adults: Vinyl-coated wire mesh batteries of 75 x 90 x 45 cm Hatchlings: Vinyl-coated wire mesh and stainless steel sheeting pens of 62 x 92 x 25.5 cm
Replication:	Eight pairs per group
No. of animals/vessel:	One male and one female per pen
Duration of test:	Acclimation: 3 weeks Pre-photostimulation: 8 weeks Pre-laying (with photostimulation): 1 week Egg laying: 10 weeks Post-adult termination (incubation, hatching and 14-day offspring rearing period): 5 weeks
Environmental test conditions	

Temperature:	Adults: 21.7 °C (average) Hatchlings: 38 °C until aged 5-7, then 29 °C
Relative humidity:	36% (average)
Photoperiod:	First eight weeks: 8 h light : 16 h dark at approx. 275 lux. From week nine: 17 h light : 7 h dark at approx. 275 lux.

B. Study Design

This study was conducted in order to evaluate the effects of dietary exposure of KWG 4168 to adult mallard ducks (*Anas platyrhynchos*) over a 20 week period. Test concentrations were based on expected environmental concentrations.

Test birds were pen-reared mallard ducks (*Anas platyrhynchos*) that were apparently healthy and phenotypically indistinguishable from wild birds. All birds were from the same hatch and were 23 weeks of age at test initiation.

All birds were given feed and water *ad libitum* during acclimation and testing. The basal diet was formulated to contain at least 27% protein and 2.5 % fat, with no more than 5% fibre. Pulverised limestone was added to the adult diets at 5% w/w in order to provide calcium for eggshell formation.

Test diets were prepared by mixing relevant amounts of KWG 4168 with 50 mL acetone and 180 mL corn oil, and then adding half the required amount of basal diet. The mixing beaker was rinsed with a further 50 mL acetone, and the premix was then added to the remaining half of the basal ration and mixed. Nominal test concentrations were a control 30, 77 and 200 mg a.s./kg diet. Mean measured test concentrations were 0, 28.4, 78.8 and 205 mg a.s./kg diet, corresponding to ~, 95, 102 and 103% of nominal, respectively.

Each test concentration contained eight pairs of one male and one female duck, housed indoors in vinyl-coated wire mesh batteries of 75 x 90 x 45 cm. These were maintained at an average temperature of 21.7 ± 1.2 °C (SD), with an average relative humidity of 36 ± 17 % (SD). The air handling system was designed to vent up to fifteen room volumes every hour. The photoperiod during acclimation and for the first eight weeks of the test was eight hours or less of light per 24 hours. From the beginning of week 9, the photoperiod was increased to 17 hours of light per day to induce egg laying. Illumination was provided by fluorescent lights at a mean of 275 lux.

Mallard ducks were observed daily for mortality, abnormal behaviour and signs of toxicity. Adult body weights were measured at test initiation, on weeks 2, 4, 6, 8 and at test termination. Feed consumption was measured weekly for each pen. Necropsies were performed on all adults surviving to test termination.

Weekly throughout the laying period, eggs were collected from alternate pens for measurements of eggshell thickness. Eggs were opened at the waist and the contents removed and rinsed before being allowed to air dry for at least one week. Measurements of the dried shell plus the membrane was determined by measuring five points around the waist of the egg using a micrometre.

Effects of adult exposure on the number of eggs laid, normal development of eggs, embryo viability, percent hatchability, offspring survival and eggshell thickness were evaluated.

Hatchlings were placed in vinyl-coated wire mesh and stainless steel sheeting pens of 62 x 92 x 25.5 cm. These were maintained at approximately 38 °C from the time of hatching until the birds were five to seven days of age, after which the temperature was set to maintain an average temperature of approximately 29 °C.

Statistical analyses were conducted using Dunnett's method following arcsine square root transformation.

Analytical method

Samples of feed were analysed using the validated analytical method M-008186-01-1, report reference M-008186-01-1 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria were not assessed in the study report.

The measured concentrations of spiroxamine in the test diets are summarised below. Mean measured test concentrations were 0, 28.4, 78.8 and 205 mg a.s./kg diet.

Table CA 8.1.1.3/02-1 Measured concentration of KWG 4168 in the animal feed

Nominal dietary concentration (mg a.s./kg diet)	Mean measured concentration (% of nominal)
Control	-
30	95
77	102
200	103

Mortalities and clinical observations

There were no mortalities or overt signs of toxicity observed at any of the concentrations tested. Some incidental clinical observations and injuries were noted, however were not deemed to be treatment-related.

All surviving adults were subjected to gross necropsy following test termination. All necropsy findings in the 28.4 and 78.8 mg a.s./kg diet treatment groups were considered incidental to treatment. While there were no treatment related findings among drakes in the 205 mg a.s./kg diet treatment group, there may have been a treatment-related increase in the incidence of hens that exhibited lesions pathognomonic of egg yolk peritonitis.

Adult body weight

There were no apparent treatment-related effects on adult body weight at any of the concentrations tested.

Table CA 8.1.1.3/02-2 Mean adult body weight after dietary exposure to KWG 4168

Mean measured concentration (mg a.s./kg diet)	Sex	Mean body weight (g)			
		Test initiation	Week 8	Test termination	Total change
Control	Male Female	1180 1052	1145 1049	1204 1154	24 101
28.4	Male Female	1184 1055	1165 1029	1197 1130	12 74
78.8	Male Female	1183 1059	1196 1081	1188 1156	5 97
205	Male Female	1183 1052	1204 1076	1211 1152	28 100

Differences between the control and each treatment group were not significant (p>0.05)
Only surviving birds were included in the calculations for each body weight interval

Adult feed consumption

There were no treatment-related effects on feed consumption in the 28.4 and 78.8 mg a.s./kg diet treatment groups. Slight and inconsistent significant reductions in feed consumption were occasionally observed, however were not considered to be treatment related.

At the 205 mg a.s./kg diet test concentration there were statistically significant treatment-related reductions in feed consumption during weeks 11 to 17. While not always significantly different, feed consumption for the 205 mg a.s./kg diet was consistently lower than that of the control group from week five until the end of the adult test period.

Table CA 8.1.1.3/02-3 Mean feed consumption during and after dietary exposure to KWG 4168

Mean measured concentration (mg a.s./kg diet)	Feed consumption (g/bird/day) by week									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Control	105	134	123	106	112	104	124	109	114	154
28.4	106	143	126	109	108	103	122	115	104	149
78.8	119	161*	141	131*	120	105	132	118	117	159
205	119	147	126	108	110	99	118	107	92	139
	11	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Control	178	173	183	182	184	188	176	190	187	195
28.4	153	159	161	155*	161	155**	163	169	175	176
78.8	161	162	171	166	184	177	164	183	197	185
205	141**	138**	149**	148**	154*	142**	146*	163	163	163*

* Significantly different from the control at $p<0.05$
** Significantly different from the control at $p<0.01$

Reproductive results

There were no apparent treatment-related effects on reproductive performance at the 28.4 and 78.8 mg a.s./kg diet test concentrations.

At the 205 mg a.s./kg diet test concentration there were slight reductions in a number of parameters that indicated effects on egg production, egg viability and embryo survival. The reductions were also reflected as reductions in the numbers of hatchlings and 14-day old survivors. While none of the differences from the control were statistically significant, they may nonetheless have been treatment-related.

Table CA 8.1.1.3/02-4 Summary of mallard reproductive performance after dietary exposure to KWG 4168

Parameter	Test concentration (mg a.s./kg diet)			
	Control	**28.4**	**78.8**	**205**
Total eggs laid	751	783	767	642
Eggs cracked	18	5	7	15
Eggs set	634	698	684	546
Viable embryos	605	638	631	455
Live 3-week embryos	581	626	610	423
Hatchlings	397	482	422	288
14-day survivors	391	474	411	285
Eggs laid/hen	47	49	48	40
Eggs laid/hen/day[1]	0.59	0.61	0.60	0.50
14-day survivors/hen	24	30	26	18

Differences between the control and each treatment group were not significant ($p>0.05$)
[1] Based on 80 days of egg production

Egg shell thickness

There were no apparent treatment-related effects on eggshell thickness.

Table CA 8.1.1.3/02-5 Mean eggshell thickness after dietary exposure to KWG 4168

Mean measured concentration (mg a.s./kg diet)	Mean shell thickness (±SD)
Control	0.382 ± 0.023
28.4	0.385 ± 0.020
78.8	0.372 ± 0.025
205	0.363 ± 0.020

Differences between the control and each treatment group were not significant (p>0.05)

Offspring body weight

There were no apparent treatment-related effects on the body weights of hatchlings or 14-day old survivors at the 28.4 mg a.s./kg diet test concentration. While there were no treatment-related effects on the weight of hatchlings or 14-day old survivors at the 78.8 mg a.s./kg diet test concentration, there was a slight but statistically significant (p<0.05) decrease in the mean body weight of hatchlings. The difference between the mean of the control group (38 ± 2 g) and the mean of the 78.8 mg a.s./kg diet group (36 ± 2 g) was only about 5%, with both means well within historical control values for the parameter. This slight difference in mean hatchling weight is therefore not considered to be biologically significant.

However, in the 205 mg a.s./kg diet test concentration there was a statistically significant (p<0.01), treatment-related reduction in hatchling body weight. The difference between the test group and the control was greater than 10%, and the mean body weight (33 g) was outside the range of historical control values.

Table CA 8.1.1.3/02-6 Mean body weight of hatchlings and 14-day survivors after parental dietary exposure to KWG 4168

Mean measured concentration (mg a.s./kg diet)	Mean body weight (g) (±SD)	
	Hatchlings	14-day survivors
Control	38 ± 2	289 ± 20
28.4	38 ± 2	291 ± 26
78.8	36* ± 2	290 ± 21
205	33** ± 3	276 ± 32

Only surviving hatchlings were weighed
* Significantly different from the control at p<0.05
** Significantly different from the control at p<0.01

III. Conclusion

Mallard ducks (*Anas platyrhynchos*) were exposed to KWG 4168 at mean measured dietary concentrations of 0, 28.4, 78.8 or 205 mg a.s./kg diet for 20 weeks. There were no mortalities, overt signs of toxicity or other treatment-related effects on body weight at any of the concentrations tested. There were also no treatment-related effects upon feed consumption or reproductive performance at the 28.4 and 78.8 mg a.s./kg diet test concentrations.

At the 205 mg a.s./kg diet test concentration there was a treatment-related reduction in feed consumption and an increase in the number of hens displaying lesions of egg yolk peritonitis at terminal necropsy. Additionally, there were reductions in egg production, viable eggs and embryo survival that were reflected as reductions in the numbers of hatchlings and 14-day old survivors. There was also a treatment-related reduction in the body weight of hatchlings in the 205 mg a.s./kg diet treatment group.

Based upon the multiple effects seen at the highest test concentration, the NOEC and LOEC for this study were determined to be 78.8 and 205 mg a.s./kg diet, respectively.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline "OECD 206: Avian reproduction test", adopted 4 April 1984 and meets the requirements set out within. Due to there being only three test

concentrations with a wide spacing factor between, the data are not considered suitable for reliable EC_{10} and EC_{20} calculation therefore this has not been conducted.

Validity criteria according to the OECD 206 (1984) guideline were met:

- Control mortality to not exceed 10% at the end of the test (actual: 0.0%)
- Average number of 14-day-old survivors per hen in the controls to be at least 14 (actual: 24)
- Average eggshell thickness in the control group to be at least 0.34 mm (actual: 0.382 mm)

The study is therefore considered acceptable.

The NOEC determined from this study was 78.8 mg a.s./kg diet which is equivalent to 10.6 mg a.s./kg bw/day based on a mean body mass of 1138.1 g/bird and a mean feed consumption rate of 152.5 g/bird/d.

Data Point:	KCA 8.1.1.3/03
Report Author:	[redacted]
Report Year:	1998
Report Title:	Results from the KWG 4168 northern bobwhite pilot reproduction study
Report No:	108264
Document No:	M-008191-01-1
Guideline(s) followed in study:	none
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

The toxicity of technical KWG 4168 was evaluated in a northern bobwhite pilot reproduction study using an 8-week exposure during egg laying. Nominal dietary levels were set at 0 (Control), 100, 447, and 1000 ppm a.i.. The results from this pilot study were used in setting test levels for the definitive northern bobwhite and mallard reproduction studies.

Based on all parameters measured, the no observed effect concentration (NOEC) for this study was <100 ppm and the lowest observed effect concentration (LOEC) was 100 ppm.

I. Materials and Methods

A. Materials

Test Material: KWG 4168

Lot/Batch #: Not reported

Purity: Not reported

Description: Not reported

Stability of test compound: Not reported

Reanalysis/Expiry date: Not reported

Density: Not reported

Treatments

Test rates:	0, 100, 447 and 1000 ppm
Solvent/vehicle:	Not reported
Analysis of test concentrations:	None

Test organisms

Species:	Northern Bobwhite
Source:	[redacted]
Acclimatisation period:	21 days - Upon arrival, the birds were housed as pairs. The photoperiod in the room was 17 hours light/7 hours dark per day to bring the birds into the reproductive state.
Feeding:	Not reported

Test design

Test vessel:	Not reported
Replication:	Not reported
No. of animals/vessel:	Not reported
Duration of test:	8 weeks

Environmental test conditions

Temperature:	Not reported
Relative humidity:	Not reported
Photoperiod:	17 hour light and 7 hours dark

B. Study Design

The toxicity of technical KWG 4168 was evaluated in a northern bobwhite pilot reproduction study using an 8-week exposure during egg laying. Nominal dietary levels were set at 0 (Control), 100, 447, and 1000 ppm a.i.. The results from this pilot study were used in setting test levels for the definitive northern bobwhite and mallard reproduction studies.

Adult northern bobwhites (approximately 22 weeks old) were obtained from Barrett's Quail Farm and were held for approximately 21 days prior to initiation of the study. Upon arrival, the birds were housed as pairs. The photoperiod in the room was 17 hours light/7 hours dark per day to bring the birds into the reproductive state. Prior to study initiation the birds were randomised into four test levels (control, 100, 447 and 1000 ppm a.i. KWG 4168) of twelve pairs each. The appropriate feed was then presented to the birds for an eight-week exposure period. During this time adult body weight, feed consumption, survival, and reproductive success were monitored.

The monitored study end-points for the adult birds were body weight, feed consumption and survival, whereas, the reproduction end-points were number of eggs laid per hen, number of cracked eggs per hen, number of fertile eggs, number of vible embryos, number of nominal hatchlings and hatchling body weights.

II. Results and Discussion

There was a statistically significant reduction ($p < 0.05$) in female body weight in the 447 ppm treatment group at weeks 1, 2 and 3 and at study termination. This was not considered a treatment related reduction for three reasons: 1) the mean body weight of this group was 19 g lighter than controls at the initiation of the study and stayed approximately 30 g lighter than controls during the remainder; 2) the 1000 ppm group did not differ significantly from the control; and 3) there was no trend for body weight reduction. There were no trends or statistically significant effects noted in male body weights.

Table CA 8.1.1.3/03-1 Body weight (g) for Female Northern Bobwhite Fed KWG 4168 in the Diet for Eight Weeks

Study week	Nominal Dietary Concentration (ppm)			
	Control	**100**	**447**	**1000**
Initial	276 ± 22	263 ± 21	257 ± 20	281 ± 34
1	286 ± 15	271 ± 20	256[d] ± 15	282 ± 28
2	285 ± 14	276 ± 19	255[d] ± 19	282 ± 28
3	293 ± 18	280 ± 22	261[d] ± 16	288 ± 28
Terminal	298 ± 17	288 ± 39	270[d] ± 16	295 ± 27
Change	21 ± 14	25 ± 39	13 ± 12	14 ± 18

[d]Statistically significant from control (Dunnett's one-tailed test; $p \leq 0.05$).

Table CA 8.1.1.3/03-2 Body weight (g) for Male Northern Bobwhite Fed KWG 4168 in the Diet for Eight Weeks

Study week	Nominal Dietary Concentration (ppm)			
	Control	**100**	**447**	**1000**
Initial	263 ± 25	252 ± 19	257 ± 21	255 ± 16
1	269 ± 26	258 ± 21	260 ± 20	259 ± 16
2	270 ± 27	258 ± 21	261 ± 21	258 ± 18
3	277 ± 28	262 ± 21	266 ± 21	260 ± 17
Terminal	271 ± 36	269 ± 21	272 ± 21	254 ± 41
Change	8 ± 27	17 ± 8	15 ± 10	-1.3 ± 37

Feed consumption was significantly reduced ($p < 0.05$) in the 447 and 1000 ppm treatment groups compared to the control group (Table 3). This was considered to be treatment related as there was a dose-response trend for reduced feed consumption over treatment groups.

Table CA 8.1.1.3/03-3 Overall Mean Feed Consumption (g/bird/day) for Northern Bobwhite Fed KWG 4168 in the Diet for Eight Weeks

Nominal Dietary Concentration (ppm)	Mean Feed Consumption
Control	22.8 ± 1.3
100	21.8 ± 1.8
447	20.8[d] ± 1.6
1000	19.3[d] ± 1.5

[d]Statistically significant from control (Dunnett's one-tailed test; $p \leq 0.05$)

Reproductive totals show a clear dose response effect. There was a 36%, 54%, and 82% reduction in number of eggs laid compared to controls for the 100, 447, and 1000 ppm treatment groups, respectively. As expected, this trend is seen in the mean number of eggs laid per hen, the mean eggs set per hen, and the mean number of hatchlings per hen, where all three treatment groups were significantly reduced compared to the control group. Fertility of the eggs was significantly reduced in the 1000 ppm treatment group. Only 46% of the 1000 ppm eggs set were fertile compared to 90% for the control group. Although only the 447 ppm treatment group had a significantly reduced percentage of hatchlings of live three-week embryos, there appeared to be a trend for a treatment effect on all treatment levels for this parameter and for percentage of hatchlings of fertile eggs per hen.

Table CA 8.1.1.3/03-4 Reproductive Totals for Northern Bobwhite fed KWG 4168 in the Diet for Eight Weeks

Reproductive Parameter	Nominal Dietary Concentration (ppm)			
	Control	100	447	1000
Eggs laid	540	345	251	95
Eggs cracked	3	3	6	2
Eggs defective	13	11	15	19
Eggs set	524	331	230	74
Fertile eggs	469	312	203	39
3-Week viable embryos	465	309	193	39
Normal hatchlings	410	246	137	25

Table CA 8.1.1.3/03-5 Reproductive Success for Northern Bobwhite fed KWG 4168 in the Diet for Eight Weeks

Reproductive Parameter	Nominal Dietary Concentration (ppm)			
	Control	100	447	1000
Eggs laid per hen in 8 weeks ($\bar{x}$)	45.0 ± 14.8	28.8[d] ± 18.3	20.9[d] ± 11.7	7.9[d] ± 8.4
Eggs cracked of eggs laid per cage (%)	0.5 ± 1.0	0.6 ± 1.1	2.8 ± 4.4	2.3 ± 7.5
Eggs defective of eggs laid per cage (%)	2.1 ± 2.9	5.2 ± 9.5	7.9 ± 9.1	23.3 ± 27.0
Eggs set per hen ($\bar{x}$)	43.7 ± 14.3	27.6[d] ± 17.4	19.2[d] ± 11.7	6.2[d] ± 7.7
Fertile eggs of eggs set per hen (%)	90.2 ± 11.9	94.5 ± 6.8	84.2 ± 15.0	45.9[d] ± 43.6
Live three-week embryos of fertile eggs per hen (%)	99.0 ± 1.4	99.2 ± 2.0	93.8[d] ± 10.0	100.0 ± 0.0
Normal hatchlings per hen ($\bar{x}$)	34.2 ± 13.1	20.4[d] ± 14.4	11.4[d] ± 10.4	2.1[d] ± 4.5
Normal hatchlings of fertile eggs per hen (%)	88.3 ± 8.3	78.7 ± 25.7	67.1 ± 30.3	73.6 ± 35.6
Normal hatchlings of live three-week embryos per hen (%)	89.1 ± 8.1	79.3 ± 29.7	69.9 ± 29.0	73.6 ± 35.6

[d]Statistically significant from control (Dunnett's one-tailed test, $p \leq 0.05$)

There was no trend or statistically significant effect for reduced body weight in hatchlings.

Table CA 8.1.1.3/03-6 Hatch Body Weight Data for offspring of Northern Bobwhite fed KWG 4168 in the diet for eight weeks

Reproductive Parameter	Nominal Dietary Concentration (ppm)			
	Control	100	447	1000
Hatchling body weight				
Mean Hatch Weight (g)	6.5	6.2	5.8	6.2
Standard deviation	0.6	0.6	0.5	0.6

III. Conclusion

Based on all parameters measured, the no observed effect concentration (NOEC) for this study was <100 ppm and the lowest observed effect concentration (LOEC) was 100 ppm.

Assessment and conclusion by applicant:

The study was not conducted to a specific test guideline but clearly followed the general principles of OECD 206. The study was conducted as a pilot study in light of the definitive mallard duck and bobwhite quail studies and was non-GLP. The results are deemed valid but the study has been submitted as supporting information only.

Data Point:	KCA 8.1.1.3/04
Report Author:	[redacted]
Report Year:	2006
Report Title:	Comment on study SXR/REP 04 (GLP-No.: E 298 0738-7) by [redacted]; B. (1995): Effects of subchronic dietary exposure of KWG 4168 techn. on bobwhite quail including effects on reproduction and health, [redacted]
Report No:	M-279402-01-1
Document No:	M-279402-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary

Comment on study SXR/REP 04 (GLP-No.: E 298 0738-7) by [redacted] (1995).

The statistical re-evaluation of the body weight of 14-day old survivors revealed a NOEC at 77 mg a.s./kg food for this parameter.

The reproductive NOEC, cited in the report, is based on this parameter. There were no further endpoints which showed significant differences to the control at that concentration. Therefore it is justified to define the NOEC at 77 mg a.s./kg food (nominal) or 78.6 mg a.s./kg food (measured).

I. Results and Discussion

Comments on the NOEC

The Test Results, cited on page 8 in the report, read: "Based on the results of this study, the NOEC for adult Bobwhite Quail is 204 ppm KWG 4168 technical a.i., the highest test concentration. Accordingly, the LOEC is > 204 ppm.

The reproductive NOEC was 29.3 ppm. The reproductive LOEC was 78.6 ppm, based on body mass development of the hatching during the 14 day post – hatch observation period."

The absolute differences of the mean body weights between the treatment groups and the control were rather small. The body weight amounted to 96.2 % of the control at 77 ppm, and to 91.2 % at 200 ppm. Due to this subtle distinction, it was decided to subject the respective data to a new statistical analysis to validate the results.

Comment on the statistical analysis of the endpoint of concern

The relevant data for the NOEC setting, the body mass data of 14-day old chicks, were displayed in Table 5 on page 37 of the report:

Table CA 8.1.1.3/04-1 Body mass of 14-day old chicks from bobwhite quail fed KWG 4168 (technical a.s.) in diets for a 21-week period

	Study week number										
Control	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1 – 10**
Mean body mass (g):	32.6	34.4	30.2	35.6	36.5	33.8	34.8	34.6	32.3	32.3	33.9
Standard deviation:	2.1	4.1	5.8	4.7	4.0	5.0	4.4	4.3	5.7	6.8	5.3
Total no. of survivors:	3	27	49	66	56	78	67	67	71	60	544

	Study week number										
29.3 ppm	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1 - 10**
Mean body mass (g):	32.1	30.9*	29.6	32.8*	33.4*	32.9	34.6	33.5	35.3	34.1	33.2
Standard deviation:		6.8	5.2	5.7	4.7	5.2	4.6	5.2	4.9	5.0	5.4
Total no. of survivors:	1	37	56	76	70	72	75	78	61	74	600
78.6 ppm	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1 - 10**
Mean body mass (g):		32.6	31.5	34.1	30.1*	33.7	30.7*	31.4*	34.3	33.7	32.6*
Standard deviation:		4.9	4.0	3.8	6.2	3.6	4.6	4.4	4.3	3.9	4.6
Total no. of survivors:	0	23	41	71	51	66	61	80	60	83	536
204 ppm	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1 - 10**
Mean body mass (g):		28.6*	29.7	30.3*	30.8*	30.4*	31.6*	31.3*	32.3	31.7	30.9*
Standard deviation:		7.8	3.9	4.6	4.0	4.4	4.3	5.1	4.0	3.9	4.7
Total no. of survivors:	0	22	31	54	43	62	44	64	56	47	423

*statistically significant negative effect (5% level)

Reported are the mean body weights of all chicks per test group on a weekly basis (column 1, 2, 3 … 10). The value of the column 1-10 is not the mean of all weekly data, but the mean body weight over all chicks per concentration, which were produced in this study (verified by the original excel spreadsheet). This overall mean gives a good impression of the quality of the chicks of the control and the treatment groups. But this way of condensing the data results in only one value per group. With such a data set, a statistical analysis is not possible.

It remained unclear, which data were subjected to statistical analysis. In principal 3 methods may be considered:

1. Statistic with the weekly means

The way the data are presented in the study suggests that this may have been the choice of the author. The number of replicates would be in our example 9-10. This procedure cannot be recommended since in many cases the reproductive success is weaker during the first weeks (including the control). This can lead to quite high standard deviation in all groups, which reduces the statistical power of the test, so that possible effects can easily be masked.

2. Statistic with the body weights of all single chicks

The number of replicates would amount to some hundreds. But since chicks of one cohort are reared together, the request of independent replicates is not obeyed. Another argument against this procedure is that results cannot be related to the exposure of the distinct pairs.

3. Statistic with mean body weight data per pair

The most often used procedure is to calculate the mean body weight of all the 14 day old chicks, which were produced by each single pair. This method provides 18-20 replicates per group, which can be submitted to statistical analysis. A problem of this procedure is the way how prematurely adult birds should be considered – in particular if there is no indication of a treatment related mortality but more a housing artefact. It is a common practise to exclude them, to avoid an unreasonable increase of standard deviations and to eliminate statistical effects, caused by artefacts. But in general this method is the most appropriate and most accepted one. It is the way, US- EPA normally performs the statistic evaluation of bird reproduction studies, and the author of our study described this method as the one used. Unfortunately the underlying data sets are not displayed in the study.

Re-evaluation of the body mass of 14 day old survivor

Since it was not completely clear how the statistic was performed, it was decided to re-evaluate the data on a per pair basis (method 3). We excluded one pair at 30 ppm and one pair at 200 ppm. In both cases one bird of these pairs died prematurely during the run of the study. At 200 ppm one pair produced no chick at all, therefore no analysable data exist for this pair and this endpoint (body weight of chicks).

Hence, the number of replicates amounted to 20 for the control, 19 for the 27 ppm group, 20 for the 70 ppm group and 18 for the 200 ppm group. The data were analysed on equal variance with the Bartlett's test. It revealed that homogeneity had to be rejected.

Then the Welch-t test for inhomogeneous variances with Bonferroni adjustment was performed. The program suggested a NOEC of 77.0 mg a.s./kg food (see below).

Statistical Evaluation of a Metric Response: 'No. Weight of survived

Relation of Measured Response on Concentration at 0,0 d

Bartlett´s Test Procedure on Variance Homogeneity

Homogeneity of variance was tested (Alpha = 0,05); var: variance; df: degrees of freedom per variance; c: Bartlett correction; dfm: deg. of freedom multiple test; p(Chi²): probability of Chi², if Ho: var1 = var2 =...= vark is true.

Treatm. [mg a.s./k	var	df	df*var	log(var)	df*log(var)
Control	16,7368	19	318,00	1,2237	23,2498
30,0	3,6173	18	65,11	0,5584	10,0510
77,0	2,5065	19	47,62	0,3991	7,5821
200,0	20,2894	17	344,92	1,3073	22,2236
	Total:	73	775,65		63,1064

Chi² = 26,600
c = 1,023
dfm = 3
p(Chi²) < 0.001
p(Chi²) <= Alpha, homogeneity hypothesis is rejected

Welch-t test for Inhomogeneous Variances with Bonferroni Adjustment

Multiple sequentially rejective comparisons after Welch of treatments with "Control" by the t test procedure. Significance was Alpha = 0,05; two-sided; Mean: arithmetic mean; n: sample size; s²: variance; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; p(t): probability of sample t for Ho: μ1 = μ2; Alpha(i): adjusted significance levels; the differences are significant in case p(i) <= Alpha(i) ; dfm: modified degrees of freedom due to heteroscedascity. (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments)

Treatm. [mg a.s./k	Mean	s²	df	%MDD	t	p(t)	Alpha(i)	Sign.
Control	33,224	10,625						
30,0	33,065	10,625	68	6,3	-0,15	0,880	0,050	-
77,0	32,497	10,625	93	7,1	-0,71	0,482	0,025	-
200,0	30,050	10,625	41	8,0	-3	0,005	0,017	+

+: significant; -: non-significant
A NOEC of 77,0 mg a.s./kg is suggested by the program.

II. Conclusion

The statistical re-evaluation of the body weight of 14 day old survivors revealed a NOEC at 77 mg a.s./kg food for this parameter.

The reproductive NOEC, cited in the report, is based on this parameter. There were no further endpoints which showed significant differences to the control at that concentration. Therefore it is justified to define the NOEC at 77 mg a.s./kg food (nominal) or 78.6 mg a.s./kg food (measured).

Assessment and conclusion by applicant:

This non-GLP statistical re-analysis report has been submitted in order to justify the use of the NOAEC of 78.6 mg a.s./kg food (equivalent to 5.40 mg a.s./kg bw/day) derived in the bobwhite quail reproduction study (M-007470-03-1).

The report is considered acceptable and has been used to help justify the use of a NOAEL of 5.40 mg a.s./kg bw/day in the Tier I risk assessment.

Data Point:	KCA 8.1.1.3/05
Report Author:	[redacted]
Report Year:	2008
Report Title:	Evaluation of historical control data on bobwhite quail 14-d chick body weights to establish the NOAEL in the study SXR/REP 04 with spiroxamine
Report No:	M-304591-01-1
Document No:	M-304591-01-1
Guideline(s) followed in study:	OECD 206
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary

The effect of KWG 4168 (spiroxamine) on the reproduction of Bobwhite Quail has been investigated by [redacted] (1995) according to OECD guideline 206 (1984) and EPA FIFRA guideline 71-4 (study M- M-007470-03-1).

In this study, adult Bobwhite quail were exposed over 21 weeks to nominal dietary concentrations of 0 (control), 30, 77 and 200 ppm in the diet (equivalent to mean measured concentrations of 0, 29.3, 78.6 and 204 ppm in the diet).

Effects of exposure on the reproductive performance of the birds were evaluated based on the egg production over 11 weeks, hatching success and body weight of the chicks at hatch and 14-day post-hatch.

The study author concluded that "The NOEC for adult Bobwhite Quail was 204 ppm KWG 4168 technical a.s., the highest test concentration. Accordingly, the LOEC is > 204 ppm. The reproductive NOEC was 29.3 ppm. The reproductive LOEC was 78.6 ppm, based on body mass development of the hatching during the 14 day post-hatch observations period."

The mean 14-d chick body weight in this study at the different treatment levels, and relative differences to the control is presented in the table below.

Table CA 8.1.1.3/05-1 Mean 14-d chick body weight in study SXR/REP 04

Treatment level (ppm)	14-d chick bw (g)	Relative to control (%)	Difference (%)
0 (control)	33.9	100	-
29.3	33.2	97.8	2.2
78.6	32.6	96.3	3.7
204	30.9	91.1	8.9

The differences of the chick body weights between the treatment groups and the control were rather small (< 5% in the 78.6 ppm group), and the statistical significance of the differnce varies according to the various methods used (*e.g.* [redacted] 2006).

It is toxicological practise to consider differences of ≤ 5 % in sublethal parameter compared to the control to be usually within natural variation and without toxicolgoical relevances (NOAEL).

I. Results and Discussion

Historical control data for the endpoint of concern in the study of [redacted] (1995) were obtained from 59 regulatory studies reported by various laboratories which met the above defined criteria.

These studies include 13 results of the "on-site historical control", i.e. the Bobwhite quail reproduction studies conducted to the same guidelines in the laboratory of [redacted]

From any other laboratory, not more than a maximum of 10 studies was included in this exercise, in order to avoid any potentially undue influence of a few more frequently encountered report sources in the archive of [redacted]

An overview over some descriptive statistics for this historical control is presented in the table below.

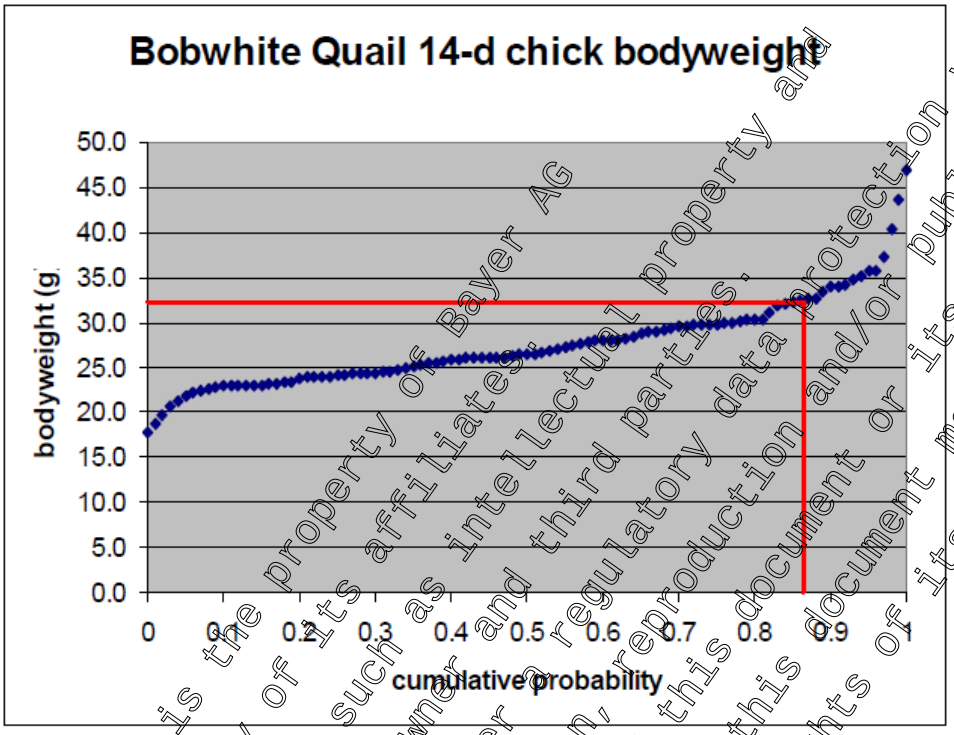
Table CA 8.1.1.3/05-2 Body mass of 14-day old chicks from bobwhite quail fed KWG 4168 (technical a.s.) in diets for a 21-week period

Lab code	# of studies	14-d chick bodyweight (g)				
		Arithmetic mean	Standard deviation	25th percentile	50th percentile	75th percentile
BCS DE	13	28.7	7.1	22.6	29.4	22.6
BCS US	10	30.7	6.8	29.1	29.7	30.2
BLAL	4	30.9	2.0	29.9	31.3	32.3
EBA	4	26	1.8	25.9	26.4	26.6
EPT	1				32.5	
HLS	7	25.0	2.5	23.4	26.0	26.6
LPT	1				31.7	
NOTOX	4	25.5	0.8	25.2	25.4	25.7
ProTox	5	25.4	1.6	24.3	25.0	25.8
Wildlife	10	24.9	2.2	23.0	24.0	26.0
Total	**59**	**27.5**	**5.1**	**24.1**	**26.5**	**29.8**

As displayed in Fig. 1, the body weight of 32.6 g as reported from the 78.6 ppm treatment level in the study of [redacted] is corresponding with the 87th percentile in the distribution of the historical control of 14-d chick body weights. The control group of this study represented the 90th percentile in the historical control data distribution.

Figure CA 8.1.1.3/05-1

Bobwhite quail 14-d chick body weight distribution



Evaluation

The mean body weight of 14-d old chicks at the 78.6 ppm treatment level in the Bobwhite quail reproduction study by Schmuck (1995) was 32.6 g (3.7% different from the concurrent control).

The mean body weight of 14-d old chicks of historical controls is 27.5 g, with a standard deviation of ± 5.1 g.

Thus, the 14-d body weight of 32.6 g obtained at 78.6 ppm spiroxamine is within the standard deviation of the historical controls.

Also the presented distribution of the historical control suggests that a body weight of 32.6 g is not to be considered biologically significantly reduced, since it is corresponding with the 87th percentile body weight of chicks from (untreated) controls reported from 59 studies conducted according to the same guidelines.

Since the 14-d chick body weights at 78.6 ppm in the avian reproduction study with spiroxamine do not differ by more 3.7% of the concurrent control, and are well within the normal range of the historical control, this difference should not be considered as adverse, being within normal variability.

Therefore Bayer CropScience propose that the 78.6 ppm level of the Bobwhite quail reproduction study ([redacted] 1995) should be considered as ecologically acceptable no (observed) adverse effect level (NOAEL) for the reproductive risk assessment for spiroxamine.

II. Conclusion

The historical control evaluation of the body weight of 14 day old survivors consisting of 59 comparable studies with Bobwhite quail suggest that the magnitude of effects (3.7%) observed at the 78.6 ppm level can be considered as being within the normal variation of untreated birds and as such not adverse.

There were no further observations with significant differences to the concurrent control at that concentration or below.

Therefore it is considered justified to define the NOAEC at 77 mg a.s./kg food (nominal) or 78.6 mg a.s./kg food (measured).

Assessment and conclusion by applicant:

This non-GLP historical control analysis report has been submitted in order to justify the use of the NOAEC of 78.6 mg a.s./kg food (equivalent to 5.40 mg a.s./kg bw/day) derived in the bobwhite quail reproduction study (M-007470-03-1). The results demonstrate that the mean body weight of 32.6 g achieved at the 78.6 mg a.s./kg diet dose group was well within the normal deviation of the historical control data from 59 regulatory studies and is not therefore a biologically relevant reduction.

The report is considered acceptable and has been used to help justify the use of a NOAEL of 5.40 mg a.s./kg bw/day in the Tier I risk assessment.

Relevant literature on birds

No relevant scientifically peer-reviewed open literature could be found on spiroxamine or its major metabolites, from an ecotoxicological perspective, on birds. Details of the literature search undertaken can be found in the M-CA Section 9 of the current submission.

CA 8.1.2 Effects on terrestrial vertebrates other than birds

A summary of essential data required for compliance with data point CA 8.1.2 is presented in the table below.

Table CA 8.1.2-1 Summary of mammalian toxicity studies with spiroxamine

Organism	Test item	Test type	Endpoints		Reference
Rat	Spiroxamine	Acute oral toxicity	LD_{50} 595 mg a.s./kg bw (male) LD_{50} >500<560 mg a.s./kg bw (female)	EU	M-007791-01-1
Mouse	Spiroxamine	Acute oral toxicity	LD_{50} 460 mg a.s./kg bw (male) LD_{50} 561 mg a.s./kg bw (female)	EU	M-007804-01-1
Rat	Spiroxamine	Chronic 2-generation	NOAEL (parental) ♂/♀ 5.5 / 6.7 mg a.s./kg bw/day NOAEL (reproductive) ♂/♀ 21.0 / 21.2 mg a.s./kg bw/day NOAEL (offspring) ♂/♀ 6.5 / 6.7 mg a.s./kg bw/day	EU	M-304231-01-1

EU: previously evaluated as part of the original EU review and listed in EFSA conclusion and DAR

CA 8.1.2.1 Acute oral toxicity to mammals

Summaries of the available acute oral data used to derive endpoints for the wild mammal risk assessment are provided in Document M-CA Section 5.2.1 and Section 5.8.1.4.

CA 8.1.2.2 Long-term and reproduction toxicity to mammals

Summaries of the available reproductive data used to derive endpoints for the wild mammal risk assessment are provided in Document M-CA Section 5.2.1.

Relevant Literature on Terrestrial Vertebrates other than Birds

No relevant scientifically peer-reviewed open literature could be found on spiroxamine or its major metabolites, from an ecotoxicological perspective on wild mammals. Please refer to the M-CA Section 5 for details of the review of literature on mammals from a human toxicological perspective. Details of the literature search undertaken can be found in the M-CA Section 9 of the current submission.

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

According to the EFSA Guidance Document on Risk Assessment for Birds and Mammals (2009), for active substances with a Log P_{ow} >3, an assessment of the risk of bioconcentration of the substance in the prey of birds and mammals (secondary poisoning) shall be provided in the document M-CP Section 10. Spiroxamine has a Log P_{ow} of 2.79 and 2.98 at pH 7 for diastomers A and B, respectively but at pH 9 these value are 4.88 and 5.08, respectively. Thus, the potential risk from bioconcentration needs to be addressed in the risk assessment.

The Log P_{ow} of spiroxamine-desethyl (M01) is 2.41, 1.97 and >3.64 at pH 4, 7 and 9, respectively. The Log P_{ow} of spiroxamine-despropyl (M02) is 1.95, 1.41 and >3.44 at pH 4, 7 and 9, respectively. The Log P_{ow} of spiroxamine-N-oxide (M03) is 2.82, 2.59 and 2.63 at pH 4, 7 and 9, respectively. The Log P_{ow} of spiroxamine-carboxylic acid (M06) is 0.45, -0.25 and 0.10 at pH 4, 7 and 9, respectively. Thus, an assessment of the potential risk from bioconcentration of spiroxamine-desethyl (M01) and spiroxamine-despropyl (M02) also needs to be addressed in the risk assessment.

An earthworm bioconcentration study using spiroxamine technical is available and has been summarised below.

Data Point:	KCA 8.1.3/01
Report Author:	[redacted]
Report Year:	2011
Report Title:	Study on the bioaccumulation of [1,3-dioxolane-4-14C] spiroxamine on the earthworm Eisenia fetida, tested in artificial soil with 5 percent peat
Report No:	LRT-BG-B-01/11
Document No:	M-411910-01-1
Guideline(s) followed in study:	Proposal for a new Guideline for OECD Guidelines for the Testing of Chemicals Bioaccumulation in terrestrial Oligochaetes, Draft Document, November 2009
Deviations from current test guideline:	Yes OECD 317 (2010) pH value was not recorded for the separately produced artificial soil pH on day 0 of soil was 6.63, which is slightly outside of the specified range of 6.0 – 6.5 Due to the equal composition of the artificial soil, the result of the TOC – content determination for the first run was also used for the metabolism investigation part and the 2nd run of the study. These deviations were not considered to have any influence on 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

The purpose of this study was to determine the bioaccumulation of spiroxamine in earthworms, *Eisenia fetida*. Adult worms were exposed to soil, spiked with a mixture of ^{14}C-labelled and non-labelled spiroxamine at a concentration of 5.09 mg spiroxamine / kg dry weight soil.

Test vessels without the addition of the test item served as controls. The test animals (one per test vessel, 3 replicates per treatment group) were exposed to the spiked soil for 21 days to assess the bioaccumulation kinetics of the test item in the test organisms (uptake phase). After the uptake phase the test animals were exposed to untreated control soil for a period of 21 days (elimination phase).

The calculated bioaccumulation factor (BAF) was 1.56. It can be concluded, that the bioaccumulation of spiroxamine in earthworms is considered to be low.

I. Materials and Methods

A. Materials

	Cold	Hot
Test Material	KWG 4168	[1,3-dioxolane-4-^{14}C] KWG 4168
Lot/Batch #:	AE 1344293-01-02	
Content of a.s. analysed:	96.5% w/w	>98 % (sum of isomers, method: HPLC-radioactivity-detector) 97.9 % (sum of isomers, method: TLC-scan)
Description:	Light pink liquid	liquid
Stability of test compound:	Not reported	Not reported
Reanalysis/Expiry date:	2011-07-07	Not reported
Density:	Not reported	Not reported

Treatments

Test rates: Nominal: µg test item (= 250 Bq) / g artificial soil. Measured: 5.09 µg test item (= 241 Bq (see Table 12) / g artificial soil was reached

Solvent/vehicle: Quartz sand

Analysis of test concentrations: Radioactivity measured in soil and worms

Test organisms

Species: *Eisenia fetida*

Source: In-house culture

Test design

Test vessel: Not reported

Test soil: 5% sphagnum peat (shredded), 20% kaolinite clay, 72.7% industrial quartz sand, 0.3% calcium carbonate, 2% dried ground cow manure (food)

Replication: Triplicate

Number of organisms per vessel:	One
Duration of test:	Exposure: 21 days
Environmental test conditions	
Temperature:	20 ± 2°C
pH:	6.00 – 6.63
Water content:	Then, the soil was moistened with deionised water to reach a water content of 58% of the maximum water holding capacity
Photoperiod:	16-hour light to 8-hour darkness photoperiod and a light intensity of 429 – 799 Lux

B. Study Design

The purpose of this study was to determine the bioaccumulation of spiroxamine in earthworms, *Eisenia fetida*. Adult worms were exposed to soil spiked with a mixture of ^{14}C-labelled and non-labelled spiroxamine at a concentration of 5.09 mg Spiroxamine / kg dry weight soil.

Test vessels without the addition of the test item served as controls. The test animals (one per test vessel, 3 replicates per treatment group) were exposed to the spiked soil for 21 days to assess the bioaccumulation kinetics of the test item in the test organisms (uptake phase). After the uptake phase, the test animals were exposed to untreated control soil for a period of 21 days (elimination phase).

In addition other parameters (*e.g.* mortality, weight, lipid content and total carbon content) were recorded for the earthworms during the study. Bioaccumulation factor (BAF) is based on the parent compound.

Three days prior to the start of exposure, the test organisms were acclimated to the artificial soil and test temperature. The body wet weight of the test organisms at the start of the test ranged from 0.25 g to 0.44 g per worm. The worms were adult with a well developed clitellum and approximately 5 months old. The age of the worms from the synchronised culture differed by not more than 4 weeks.

The ^{14}C - labelled test item was dissolved in acetonitrile. The stock solution was prepared by weighing 0.0887 g of non - labelled test item into a 10 mL volumetric flask, adding 4.25 ml ^{14}C - labelled test item solution (= 1.039 mg KML 9032; 4.25 MBq) and dissolving this mixture in enough acetonitrile to achieve a total of 10 mL. All concentrations are based on the content of test item spiroxamine.

The stock solution was used to soulden 170 g quartz sand. After evaporation of the solvent under a fume hood (overnight), the quartz sand was well mixed to ensure homogeneous distribution of test item.

160 g of the quartz sand / test item mixture was added to 1110.48 g (wet weight) of the artificial soil (pre–mixture). Subsequently, the remaining artificial soil (16.70 Kg wet weight) was divided by two and each of these portions was treated with equal amounts of the pre-mixture. Hence, the total wet weight amount of treated soil was 17.81 Kg (nominal 16 Kg dry weight). To ensure equal distribution of test item in the artificial soil, treatments were carried out by using a gyro wheel mixer (Elte 650, J. Engelsmann AG, Ludwigshafen, Germany), mixing terms: 15 min at 50 rpm. Subsequently both portions were thoroughly mixed together.

Approx. 200 g dws (dry weight artificial soil) of the spiked artificial soil was filled into the test vessels to determine biological effects. This soil was equilibrated three days under test conditions. Approx. 500 g dws (dry weight artificial soil) per vessel was used for metabolism investigations.

Three days prior to the start (2nd run) of the uptake/elimination test, required worms were held in untreated artificial soil for acclimatisation. At the start of the study, the worms were quickly washed

with water. Surplus water was absorbed on filter paper. Subsequently the worms were weighed and directly placed into the corresponding test vessels.

During the uptake phase, samples were taken at days 0, 1, 2, 4, 8, 10, 14, 17 and 21, during the elimination phase samples were taken at days 0.25, 1, 2, 4, 7, 10, 14, 17 and 21 after introduction of the worms into the soil. These measured values were used to determine the uptake and elimination of the test item in the worms, to calculate the uptake rate constant, the clearance rate constant and the steady-state bioaccumulation factor (BAF).

Samples for the uptake or elimination phase, respectively, were incubated at a light intensity between 402 and 658 Lux (mean: 482 Lux, 16:8 light / dark frequency) and a temperature of 20 ± 2°C.

At the end of the incubation phase for metabolism investigations the worms were purged, washed, weighed and frozen until the corresponding investigations. The worms remain in the substrate for 21 days.

Due to partly incomplete combustion of worms within a first test, the obtained data were not suitable for further analysis. The corresponding data were archived with the raw data and were not presented in the report. Results of the metabolism investigation part of the first run were not affected by this technical error and therefore described in the report. The uptake and the elimination part of the study were repeated and the corresponding results were presented in the report.

II. Results and Discussion

The study was assessed against the validity criteria in the draft (2009) guideline OECD Guidelines for the Testing of Chemicals – Bioaccumulation in terrestrial Oligochaetes:

- Overall mortality in the control during the uptake and elimination phase ≤ 10 % (actual: 0 %)
- Overall mortality in the treatment group during the uptake and elimination phase ≤ 10 % (actual: 0 %)
- Mean mass loss at the end of the uptake phase, compared to the initial fresh weight ≤ 20 % (actual: none)
- Mean mass loss at the end of the elimination phase, compared to the initial fresh weight ≤ 20 % (actual: none)

The study was therefore, considered acceptable.

During the study earthworms did not show any mortality or signs of intoxication. The weight development was similar between earthworms exposed to spiroxamine and control soils.

Spiroxamine was mixed into the artificial soil thoroughly and the concentration of spiroxamine in soil was confirmed by radiochemical analysis. The mean value measured during the uptake phase was 5.06 mg t.i. / kg dry weight soil, corresponding to 101.1 % of the nominal concentration.

During the elimination phase the mean value for the concentration of spiroxamine in the soil was lower than the natural background level radioactive radiation.

No test item was detected in the control soil (mean of the natural radioactive radiation of control soil from uptake or elimination phase, respectively: 0.32 Bq).

After an initial increase of the spiroxamine concentration during the first two sampling dates (day 1 and 2) of the uptake phase, the concentration in earthworms did not further increase over the testing period of 21 days (uptake phase). The average value measured in earthworms was 7.90 mg spiroxamine / kg dry weight of worm in the treated group.

The calculated bioaccumulation factor (BAF) was: 1.56.

The calculated kinetic bioaccumulation factor (BAFk) was: 1.68 g soil/kg of worm.

Based on the lipid content of the worms and the total organic carbon content (TOC) of the artificial soil, a biota soil accumulation factor (BSAF) was calculated:

BSAF: 0.72 kg TOC/kg lipid

Uptake of test item from the treated substrate: day 1 = 0.47 %, day 21 = 0.93 %

Remaining test item in worm after elimination: day 0.25 = 51.0 %, day 21 = 15.6 %

After extraction, the TRR (Total Radioactive Residue) in the earthworm sample was calculated from the sum of radioactivity in the extracts and remaining solids (PES – post extraction solids). The TRR amounted to 1.071 mg/kg. Approximately 80% of the TRR was extractable. The main part of the extracted residues in earthworms were parent compound (0.548 mg/kg, 51.1% of the TRR).

III. Conclusion

It can be concluded that the bioaccumulation of spiroxamine in earthworms is considered to be low.

The calculated bioaccumulation factor (BAF) was: 1.56.

Assessment and conclusion by applicant:

The study has been assessed against the validity criteria of the current OECD 317 test guideline – Bioaccumulation in terrestrial Oligochaetes:

- At the end of the test, the overall mortality during uptake and elimination phase should not exceed 10% of the total number of the introduced worms (actual: 0%)
- For *Eisenia fetida* the mean mass loss as measured at the end of the uptake and at the end of the elimination phase should not exceed 20% compared to the initial fresh weight (f.w.) at start of each phase (actual: none)

The validity criteria of the current test guideline have been met therefore the study is considered acceptable.

The calculated bioaccumulation factor (BAF) was: 1.56.

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

No additional studies on other terrestrial vertebrates are required in accordance with Commission Regulation (EU) No 283/2013.

In the supporting publication by EFSA (2017)[1] to review the biological relevance of the magnitude of effects observed in studies with amphibians and reptiles, it is noted that fish-generated toxicity data seem to be appropriate to cover aquatic amphibians. For terrestrial organisms typically birds and mammals are shown to be more sensitive than amphibians and reptiles to a higher number of substances. Currently data do not allow for extrapolating between groups, however the frequency of cases in which amphibians or reptiles are more sensitive than birds or mammals is around 30%. In the absence of further available data with reptiles and amphibians it can be reasonably assumed therefore that the risk assessment for fish, birds and mammals is likely to be protective of the risk to amphibians and reptiles.

Relevant literature on other terrestrial vertebrate wildlife

No relevant scientifically peer-reviewed open literature could be found on spiroxamine or its major metabolites, from an ecotoxicological perspective, on other terrestrial wildlife. Details of the literature search undertaken can be found in the M-CA Section 9 of the current submission.

[1] EFSA supporting publication 2017; EN-1251. Biological relevance of the magnitude of effects (considering mortality, sub-lethal and reproductive effects) observed in studies with amphibians and reptiles in view of population level impacts on amphibians and reptiles.

CA 8.1.5 Endocrine disrupting properties

A full assessment of the endocrine disrupting potential of spiroxamine, in accordance with the EFSA and ECHA Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 (adopted 05 June 2018) and the OECD conceptual framework as described in OECD TG 150 (2018), will be submitted.

CA 8.2 Effects on aquatic organisms

The aquatic studies conducted with spiroxamine and its metabolites are summarised in the following table. The data include studies previously reviewed and included in the DAR and EFSA conclusion for spiroxamine as well as any previously unsubmitted or new studies which have also been conducted.

Table CA 8.2-1 Summary of endpoints for toxicity of spiroxamine and metabolites to aquatic organisms

Organism	Test item	Test type	Endpoints		Reference
Fish					
Oncorhynchus mykiss (Rainbow trout)	Spiroxamine	Acute toxicity 96 h (static)	96-hour LC_{50} 18,500 µg a.s./L (mm)	EU	M-006243-01-1
Lepomis macrochirus (Bluegill sunfish)	Spiroxamine	Acute toxicity 96 h (static)	96-hour LC_{50} > 1130 µg a.s./L (mm)	EU	M-006229-01-1
Danio rerio (Zebra fish)	Spiroxamine	Acute toxicity 96 h (static)	96-hour LC_{50} 2,410 µg a.s./L (nm)	EU	M-303809-02-1
Oncorhynchus mykiss (Rainbow trout)	Spiroxamine	Chronic toxicity (ELS) 93 d (flow through)	NOEC <62.5 µg a.s./L (nom) (EC_0) 14 µg a.s./L (nom)	EU	M-006232-01-1
		Statistical Re-analysis	EC_{10} <62.5 µg a.s./L (nom)	NEW	M-760407-01-1
Oncorhynchus mykiss (Rainbow trout)	Spiroxamine	Chronic toxicity (ELS; radiolabelled) 96 d (flow through)	NOEC 14.2 µg a.s./L (mm)	EU	M-006449-02-1
		Statistical Re-analysis	EC_{10} 91.5 µg a.s./L (mm) EC_{20} 195 µg a.s./L (mm)	NEW	M-760405-01-1
Oncorhynchus mykiss (Rainbow trout)	Spiroxamine	Chronic toxicity (ELS; sediment system; pulsed exposure) 56 d	NOEC 3 x 60 µg a.s./L (mm)	EU	M-304369-01-1
Danio rerio (Zebra fish)	Spiroxamine	Chronic toxicity (FFLC) 230 d (flow through)	NOEC 2.6 µg a.s./L (nom)	EU	M-304458-02-1
		Statistical Re-analysis	EC_{10} 1.88 µg a.s./L (nom) EC_{20} 4.46 µg a.s./L (nom)	NEW	M-760413-01-1

Organism	Test item	Test type	Endpoints		Reference
Danio rerio (Zebra fish)	Spiroxamine	Chronic toxicity (FFLC; sediment system; pulsed exposure) 56 d	EC_{10} (survival) 23.3 µg a.s./L (im) NOEC (biomarker VTG) 15.8 µg a.s./L (im)	EU	M-467979-03-1
		Statistical Re-analysis	EC_{10} not determinable	NEW	M-760412-01-1
Pimephales promelas (Fathead minnow)	Spiroxamine	Fish screening assay	Growth and fertility not affected at up to and including 58.8 µg a.s./L (mm) No effects on endocrine specific biomarker endpoints at up to and including 18.9 µg a.s./L (mm)	EU	M-304833-01-1
Lepomis macrochirus (Bluegill sunfish)	Spiroxamine	BCF	$BCF_{(whole\ fish)}$ 87 CT_{50} 13 - 19 hours	EU	M-006479-01-1
Aquatic invertebrates					
Daphnia magna	Spiroxamine	Acute toxicity 48 h (static)	48-hour EC_{50} 6,100 µg a.s./L (im)	EU	M-006245-01-1
Daphnia magna	Spiroxamine	Acute toxicity 48 h (radiolabelled; static)	48-hour EC_{50} 6,800 µg a.s./L (mm)	EU	M-006476-01-1
Daphnia magna	Spiroxamine	Acute toxicity 48 h (radiolabelled; flow-through)	48-hour EC_{50} 3,000 µg a.s./L (mm)	EU	M-006523-01-1
Daphnia magna	KWG 4168 N-oxide (M03)	Acute toxicity 48 h (static)	48-hour EC_{50} >100,000 µg/L (nom)	EU	M-006702-01-1
Daphnia magna	Spiroxamine	Chronic toxicity 21 d (static-renewal)	NOEC 100 µg a.s./L (nom)	EU	M-006401-01-1
		Statistical Re-analysis	EC_{10} 120 µg a.s./L (nom) EC_{20} 200 µg a.s./L (nom)	NEW	M-761546-01-1
Daphnia magna	Spiroxamine	Chronic toxicity 21 d (radiolabeled; flow-through)	NOEC 34 µg a.s./L (mm)	EU	M-006555-01-1

Organism	Test item	Test type	Endpoints		Reference
		Statistical Re-analysis	EC_{10} 32 µg a.s./L (mm)* EC_{20} 68 µg a.s./L (mm)*	NEW	M-760409-01-1
Daphnia magna	Spiroxamine	Chronic toxicity 21 d (radiolabeled; static-renewal)	NOEC 47 µg a.s./L (mm)	EU	M-006466-01-1
		Statistical Re-analysis	EC_{10} 39 µg a.s./L (mm) EC_{20} 65 µg a.s./L (mm)	NEW	M-761504-01-1
Sediment-dwelling organisms					
Chironomus riparius	Spiroxamine	Chronic toxicity 28 d (static; radiolabelled)	EC_{15} (development time) ~5,600 µg a.s./L (nom) NOEC (emergence) 5,600 µg a.s./L (nom)	EU	M-006549-01-1
		Statistical Re-analysis	EC_{10}/EC_{20} not determinable	NEW	M-760403-01-1
Lumbriculus variegatus	Spiroxamine	Chronic toxicity 28 d (static)	EC_{10} 7,120 µg a.s./kg sediment (dm) NOEC 16,700 µg a.s./kg sediment (mm)	NEW	M-688127-01-1
Amphibia					
Xenopus laevis	Spiroxamine	XETA	No indication of endocrine activity on the thyroid axis concluded. A statistically significant increase in flouresence was observed at the 1.6 mg/L treatment but this concentration was above the MTC	NEW	M-762327-01-1
Algae					
Scenedesmus subspicatus	Spiroxamine	72 h (static)	E_rC_{50} 12 µg a.s./L (mm) E_bC_{50} 3.2 µg a.s./L (mm)	EU	M-006228-01-1

Organism	Test item	Test type	Endpoints		Reference
		Statistical Re-analysis	E_rC_{10} 1.56 µg a.s./L (mm) E_rC_{20} 3.51 µg a.s./L (mm) E_rC_{50} 11.9 µg a.s./L (mm) E_yC_{10} 0.84 µg a.s./L (mm) E_yC_{20} 1.44 µg a.s./L (mm) E_yC_{50} 3.28 µg a.s./L (mm)	NEW	M-761401-01-1
Selenastrum capricornutum	Spiroxamine	120 h (static)	E_rC_{50} 19.43 µg a.s./L (nom) E_bC_{50} 5.42 µg a.s./L (nom)	EU	M-006518-01-1
		Statistical Re-analysis	E_rC_{10} 9.20 µg a.s./L (nom) E_rC_{20} 10.9 µg a.s./L (nom) E_rC_{50} 15.2 µg a.s./L (nom) E_yC_{10} 3.60 µg a.s./L (nom) E_yC_{20} 4.73 µg a.s./L (nom) E_yC_{50} 7.99 µg a.s./L (nom)	NEW	M-761402-01-1
Selenastrum capricornutum	Spiroxamine	96 h (static)	E_rC_{50} >8.14 µg a.s./L (im) E_bC_{50} 5.5 µg a.s./L (im) EC_{50} (cell density) 5.7 µg a.s./L (im)	EU	M-006533-01-1
		Statistical Re-analysis	E_rC_{10} 4.93 µg a.s./L (im) E_rC_{20} 10.5 µg a.s./L (im) E_rC_{50} >8.14 µg a.s./L (im) E_yC_{10} 1.29 µg a.s./L (im) E_yC_{20} 2.18 µg a.s./L (im) E_yC_{50} 5.90 µg a.s./L (im)	NEW	M-761427-01-1
Desmodesmus subspicatus	Spiroxamine	72 h (static)	E_rC_{10} <9.53 µg a.s./L (nom) E_rC_{20} 11.4 µg a.s./L (nom) E_rC_{50} 175 µg a.s./L (nom)	EU	M-273962-01-1

Organism	Test item	Test type	Endpoints		Reference
		Statistical Re-analysis	E_yC_{10} not determinable E_yC_{20} not determinable E_yC_{50} 10.5 µg a.s./L (nom)	NEW	M-761457-01-1
Skeletonema costatum	Spiroxamine	96 h (static)	E_rC_{50} 6.3 µg a.s./L (im)	EU	M-006512-01-1
		Statistical Re-analysis	E_rC_{10} not determinable E_rC_{20} not determinable E_rC_{50} 6.33 µg a.s./L (im) E_yC_{10} not determinable E_yC_{20} not determinable E_yC_{50} 1.29 µg a.s./L (im)	NEW	M-761414-01-1
Anabaena flos-aquae	Spiroxamine	96 h (static)	EC_{50} (cell density) > 990 µg a.s./L (mm)	EU	M-006537-01-1
Navicula pelliculosa	Spiroxamine	96 h (static)	E_rC_{50} 11.85 µg a.s./L (mm)	EU	M-006542-01-1
		Statistical Re-analysis	72-hour E_rC_{10} 8.36 µg a.s./L (mm) E_rC_{20} 9.44 µg a.s./L (mm) E_rC_{50} 11.9 µg a.s./L (mm)	EU	M-280532-01-1
		Statistical Re-analysis	72-hour E_yC_{10} 6.83 µg a.s./L (mm) E_yC_{20} 7.60 µg a.s./L (mm) E_yC_{50} 9.32 µg a.s./L (mm)	NEW	M-761458-01-1
Desmodesmus subspicatus	CGA 414168-desethyl (M01)	72 h (static)	E_rC_{10} not determinable E_rC_{20} 42.9 µg/L (nom) E_rC_{50} 737 µg/L (nom)	EU	M-288232-01-1
		Statistical Re-analysis	E_yC_{10} not determinable E_yC_{20} not determinable E_yC_{50} 30.6 µg/L (nom)	NEW	M-761465-01-1

Organism	Test item	Test type	Endpoints		Reference
Pseudokirchneriella subcapitata	KWG 4168-despropyl (M02)	72 h (static)	E_rC_{10} 20.3 µg/L (im) E_rC_{20} 55.7 µg/L (im) E_rC_{50} 383 µg/L (im) E_yC_{10} n.d. E_yC_{20} 14.8 µg/L (im) E_yC_{50} 42.3 µg/L (im)	NEW	M-680695-01-2
Desmodesmus subspicatus	KWG 4168-N-oxide (M03)	72 h (static)	E_rC_{10} 658 µg/L (nom) E_rC_{20} 2,300 µg/L (nom) E_rC_{50} 31,700 µg/L (nom)	EU	M-288235-01-1
		Statistical Re-analysis	E_yC_{10} 218 µg/L (nom) E_yC_{20} 526 µg/L (nom) E_yC_{50} 2835 µg/L (nom)	NEW	M-761467-01-1
Desmodesmus subspicatus	KWG 4168-acid (M06)	72 h (static)	E_rC_{10} >3,200 µg/L (nom) E_rC_{20} >3,200 µg/L (nom) E_rC_{50} >3,200 µg/L (nom)	EU	M-309818-01-1
		Statistical Re-analysis	Not determinable. E_rC_{50} considered to be >3,200 µg/L (nom)	NEW	M-761469-01-1
Aquatic plants					
Lemna gibba	Spiroxamine	14 d (static)	14-day EC_{50} (frond counts) 1,910 µg a.s./L (mm) 14-day E_rC_{50} 2,650 µg a.s./L (mm)	EU	M-006497-01-1
		Statistical Re-analysis	frond number 7-day E_rC_{10} 2,060 µg a.s./L (mm) 7-day E_rC_{20} 3,110 µg a.s./L (mm) 7-day E_rC_{50} 6,780 µg a.s./L (mm)	EU	M-303421-01-1

Organism	Test item	Test type	Endpoints		Reference
		Statistical Re-analysis	frond number 14-day E_rC_{10} 1,260 µg a.s./L (mm) 14-day E_rC_{20} 1,820 µg a.s./L (mm) 14-day E_rC_{50} 3,170 µg a.s./L (mm) 7-day E_yC_{10} 220 µg a.s./L (mm) 7-day E_yC_{20} 620 µg a.s./L (mm) 7-day E_yC_{50} 3,020 µg a.s./L (mm) 14-day E_yC_{10} 560 µg a.s./L (mm) 14-day E_yC_{20} 930 µg a.s./L (mm) 14-day E_yC_{50} 1,990 µg a.s./L (mm)	NEW	M-760417-01-1
		14 d (static)	14-day EC_{50} (frond number) 2,760 µg a.s./L (mm) 14-day EC_{50} (biomass) 9,380 µg a.s./L (mm)	EU	M-006540-01-1
Lemna gibba	Spiroxamine	Statistical Re-analysis	frond number 7-day E_rC_{10} 3,510 µg a.s./L (mm) 7-day E_rC_{20} 4,130 µg a.s./L (mm) 7-day E_rC_{50} 5,600 µg a.s./L (mm) dry weight 14-day E_rC_{10} 4,760 µg a.s./L (mm) 14-day E_rC_{20} 7,960 µg a.s./L (mm) 14-day E_rC_{50} 21,200 µg a.s./L (mm)	EU	M-303443-01-1

Organism	Test item	Test type	Endpoints		Reference
		Statistical Re-analysis	frond number 14-day E_rC_{10} 2,530 µg a.s./L (mm) 14-day E_rC_{20} 2,790 µg a.s./L (mm) 14-day E_rC_{50} 3,670 µg a.s./L (mm) 7-day E_yC_{10} 2,340 µg a.s./L (mm) 7-day E_yC_{20} 2,860 µg a.s./L (mm) 7-day E_yC_{50} 4,230 µg a.s./L (mm) 14-day E_yC_{10} 1,290 µg a.s./L (mm) 14-day E_yC_{20} 1,770 µg a.s./L (mm) 14-day E_yC_{50} 2,860 µg a.s./L (mm)	NEW	M-760416-01-1

EU: previously evaluated as part of the original EU review and listed in EFSA conclusion and DAR
NEW: new study or data generated since the previous EU review or previously not submitted
mm = Results based on mean measured test concentrations
nom = Results based on nominal test concentrations
im = Results based on initial measured test concentrations
* EC_{10} considered unreliable therefore not used in risk assessment

CA 8.2.1 Acute toxicity to fish

Data Point:	KCA 8.2.1/01
Report Author:	[redacted]
Report Year:	1994
Report Title:	KWG 4168 techn.- Acute toxicity (96h) to rainbow trout in a static test
Report No:	DOM 93062
Document No:	M-006243-01-1
Guideline(s) followed in study:	OECD 203 (1992)
Deviations from current test guideline:	Yes OECD 203 (2019) Conditions during the acclimation period were not reported (however <5% of fish died). A dissolved oxygen concentration <60% was observed in one individual measurement in the lowest test concentration, however later readings were above 60%.
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The acute toxicity of KWG 4168 to rainbow trout (*Oncorhynchus mykiss*) was determined in a static 96-hour test. Seven test concentrations were assessed along with a control and solvent control. Each treatment group contained 20 fish.

At test termination, mortalities of 0, 0, 0, 0, 0, 0, 0, 45 and 100% were observed in the control, solvent control and 0.78, 1.48, 3.13, 4.33, 8.80, 18.0 and 26.2 mg a.s./L test concentrations. Sub-lethal signs of intoxication were observed from the 8.80 mg a.s./L test concentration onwards, and included lying on back/side, tumbling during swimming and fish staying mainly on the bottom of the tank.

The resulting 96-hour LC_{50} was determined to be 18.5 mg a.s./L, with 95% confidence intervals of 8.80 to 26.2 mg a.s./L. The LOEC and NOEC were 8.80 and 4.33 mg a.s./L, respectively.

I. Materials and Methods

A. Materials

Test Material KWG 4168

Lot/Batch #: 898114002

Purity: 97.8%

Description: Colourless liquid

Stability of test compound: Stable as shown by percent ranges for measured analytical values

Reanalysis/Expiry date: 27 January 1994

Density: Not reported

Treatments

Test rates: Nominal: 1.0, 1.78, 3.16, 5.62, 10.0, 17.8 and 31.6 mg a.s./L
Mean measured: 0.78, 1.48, 3.13, 4.33, 8.80, 18.0 and 26.2 mg/L

Solvent/vehicle: 100 µL acetone/L test water

Analysis of test concentrations:	Yes, mean measured concentrations 78 – 101% of nominal
Test organisms	
Species:	Rainbow trout (*Oncorhynchus mykiss*)
Source:	[redacted]
Acclimatisation period:	48 hours
Feeding:	Not fed for 48-hours prior to or during the test
Treatment for disease:	None
Test design	
Test vessel:	Glass aquaria of 32 x 36 x 38 cm containing 40 L test solution
Test medium:	Reconstituted water
Replication:	No replicates
No. of animals/vessel:	20 fish
Duration of test:	96 hours
Environmental test conditions	
Temperature:	11°C
Dissolved oxygen:	6.5 – 10.3 mg/L (approx. 58.93 – 93.38% of saturation)
pH:	6.9 – 7.4
Photoperiod:	16 h light – 8 h dark

B. Study Design

This study was conducted in order to assess the acute toxicity of KWG 4168 to rainbow trout (*Oncorhynchus mykiss*) in a 96-hour static test. Test concentrations were determined based on the results of a preliminary range-finding test.

The mean body weight of fish at the beginning of the test was 2.3 ± 0.5 g (±SD), and the mean length was 6.2 ± 0.4 cm (±SD). Loading was 1.15 g fish/L test medium.

Nominal test concentrations were 1.0, 1.78, 3.16, 5.62, 10.0, 17.8 and 31.6 mg a.s./L. Mean measured concentrations were 78, 83, 99, 77, 88, 101 and 83% of nominal, corresponding to 0.78, 1.48, 3.13, 4.33, 8.80, 18.0 and 26.2 mg a.s./L, respectively.

Test vessels were glass aquaria of 32 x 36 x 38 cm containing 40 L test solution. To each vessel were added 20 fish, which were observed after four hours and then daily for mortalities and symptoms of intoxication.

Dissolved oxygen and pH values were determined daily in each aquarium, and the water temperature of the control aquarium was recorded hourly.

Analytical determinations of the active substance were made in the test medium at test initiation and termination.

Analytical method

Samples of water were analysed using the validated analytical method 00252 M001, report reference M-008490-02-2 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the OECD 203 guideline were met:

- Control mortality to not exceed 10% at the end of the test (actual: 0.0 and 0.0% for the control and solvent control, respectively)

One of the criteria was not met:

- Dissolved oxygen concentration to be ≥60% of the air saturation value (actual: 58.93 to 93.38%)

A dissolved oxygen concentration <60% was observed in only one individual measurement in the lowest test concentration, and later readings were above 60%.

Table CA 8.2.1/01-1 Measured concentrations of KWG 4168 in test medium

Nominal test concentration (mg a.s./L)	Measured concentration (% of nominal) at test initiation	Measured concentration (% of nominal) at test termination[1]	Mean measured test concentration (% of nominal)	Mean measured test concentration (mg a.s./L)
Control	-	-	-	-
Solvent control	-	-	-	-
1.00	86	71	78	0.78
1.78	80	86	83	1.48
3.16	97	100	99	3.13
5.62	73	80	77	4.33
10.0	87	89	88	8.80
17.8	98	103	101	18.0
31.6	78	87[a]	83[b]	26.2

[1] Mean of two replicates , corrected for recoveries
[a] Measured concentration at day 2
[b] Mean calculated concentration between day 0 and day 2

The results have been presented based on the mean measured concentrations.

At test termination, mortalities of 0, 0, 0, 0, 0, 0, 45 and 100% were observed in the control, solvent control and 0.78, 1.48, 3.13, 4.33, 8.80, 18.0 and 26.2 mg a.s./L test concentrations. Sub-lethal signs of intoxication were observed from the 8.80 mg a.s./L test concentration onwards, and included irregular swimming behaviour lying on back/side, tumbling during swimming and fish staying mainly on the bottom or surface of the tank. In the 3.13 mg a.s./L test concentration at 96 hours, 10% of fish showed slight tumbling during swimming, however in the next higher test level all 20 fish were without symptoms. This observation is therefore not considered to be treatment-related.

Table CA 8.2.1/01-2 Mortalities and symptoms of intoxication of rainbow trout during exposure to KWG 4168

Mean measured concentration (mg a.s./L)	Number of mortalities / number with symptoms of intoxication[1]				
	4 h	24 h	48 h	72 h	96 h
Control	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
Solvent control	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
0.78	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
1.48	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
3.13	0 / 0	0 / 0	0 / 0	0 / 0	0 / 2
4.33	0 / 20	0 / 0	0 / 0	0 / 0	0 / 0
8.80	0 / 20	0 / 20	0 / 20	0 / 20	0 / 20

Mean measured concentration (mg a.s./L)	Number of mortalities / number with symptoms of intoxication[1]				
	4 h	24 h	48 h	72 h	96 h
18.0	2 / 20	9 / 20	9 / 20	9 / 20	9 / 20
26.2	5 / 20	19 / 20	20 / 20	-	-
LC_{50} (mg a.s./L) (95% CI)	-	18.5[a] (15.6 – 20.5)	18.5[b] (8.8 – 26.2)	18.5[b] (8.8 – 26.2)	18.5[b] (8.8 – 26.2)

[1] 20 fish were introduced per aquarium
Dead fish were added to the sum of fish with symptoms
[a] Calculated using binominal probability
[b] Calculated using probit method

III. Conclusion

The acute toxicity of KWG 4168 to rainbow trout (*Oncorhynchus mykiss*) was determined in a static 96-hour test. Seven test concentrations were assessed along with a control and solvent control. Each treatment group contained 20 fish.

At test termination, mortalities of 0, 0, 0, 0, 0, 0, 0, 45 and 100% were observed in the control, solvent control and 0.78, 1.48, 3.13, 4.33, 8.80, 18.0 and 26.2 mg a.s./L test concentrations. Sub-lethal signs of intoxication were observed from the 8.80 mg a.s./L test concentration onwards, and included lying on back/side, tumbling during swimming and fish staying mainly on the bottom of the tank.

The resulting 96-hour LC_{50} was determined to be 18.5 mg a.s./L, with 95% confidence intervals of 8.80 to 26.2 mg a.s./L. The LOEC and NOEC were 8.80 and 4.33 mg a.s./L, respectively.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 203 the most up-to-date version of which is the "Fish acute toxicity test", adopted 18 June 2019.

Validity criteria according to the most up-to-date OECD 203 guideline (2019) were met:

- Control mortality to not exceed 10% at the end of the test (actual: 0.0 and 0.0% for the control and solvent control, respectively)

One of the criteria was not met:

- Dissolved oxygen concentration to be ≥60% of the air saturation value (actual: 58.93 to 93.38%)

However a dissolved oxygen concentration <60% was observed in only one individual measurement in the lowest test concentration, and later readings were above 60%. Control mortality was 0% in this test therefore this minor deviation is not considered to have had any detrimental impact on the results achieved in this study.

The study is considered acceptable.

The resulting 96-hour LC_{50} was determined to be 18.5 mg a.s./L.

Data Point:	KCA 8.2.1/02
Report Author:	[redacted]
Report Year:	1994
Report Title:	KWG 4168 techn.- Acute toxicity (96 h) to bluegill in a static test
Report No:	DOM 93063
Document No:	M-006229-01-1
Guideline(s) followed in study:	OECD 203 (1992)
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The acute toxicity of KWG 4168 to bluegill sunfish (*Lepomis macrochirus*) was determined in a static 96-hour test. Seven test concentrations were assessed, along with a control and solvent control. Each treatment group contained 20 bluegill sunfish.

At test termination, mortalities of 0, 0, 0, 0, 0, 0, 80, 100 and 100% were observed in the control, solvent control and 0.34, 1.30, 2.50, 4.22, 9.40, 15.4 and 26.2 mg a.s./L test concentrations. Sub-lethal signs of intoxication were observed from the 4.22 mg a.s./L test concentration onwards, and included lying on back/side, tumbling during swimming and fish staying mainly on the bottom or surface of the tank.

The resulting 96-hour LC_{50} was determined to be 7.13 mg a.s./L with 95% confidence intervals of 5.91 to 8.37 mg a.s./L. The LOEC and NOEC were 4.22 and 2.50 mg a.s./L, respectively.

I. Materials and Methods

A. Materials

Test Material KWG 4168

Lot/Batch #: 898114002

Purity: 97.8%

Description: Colourless liquid

Stability of test compound: Stable as shown by percent ranges for measured analytical values

Reanalysis/Expiry date: 27 January 1994

Density: Not reported

Treatments

Test rates: Nominal: 1.0, 1.78, 3.16, 5.62, 10.0, 17.8 and 31.6 mg/L
Mean measured: 0.34, 1.30, 2.50, 4.22, 9.40, 15.4 and 26.2 mg a.s./L

Solvent/vehicle: 100 µL acetone/L test water

Analysis of test concentrations: Yes, mean measured concentrations 34 – 94% of nominal

Test organisms

Species: Bluegill sunfish (*Lepomis macrochirus*)

Source:	[redacted]
Acclimatisation period:	14 days
Feeding:	Fish were not fed for 48 h before or during the study
Treatment for disease:	Not for 70 days prior to the start of the study
Test design	
Test vessel:	Glass aquaria of 32 x 36 x 38 cm containing 40 L test solution
Test medium:	Reconstituted water
Replication:	No replicates
No. of animals/vessel:	20 fish
Duration of test:	96 hours
Environmental test conditions	
Temperature:	19 – 20 °C
Dissolved oxygen:	8.6 – 9.3 mg/L (approx. 92.67 – 102.25% of saturation)
pH:	7.0 – 7.8
Photoperiod:	16 h light : 8 h dark

B. Study Design

This study was conducted in order to assess the acute toxicity of KWG 4168 to bluegill sunfish (*Lepomis macrochirus*) in a 96-hour static test. Test concentrations were determined based on the results of a preliminary range-finding test.

The mean body weight of fish at the beginning of the test was 1.0 ± 0.5 g (±SD), and the mean length was 3.8 ± 0.6 cm (±SD). Loading was 0.5 g fish/L test medium.

Nominal test concentrations were 1.0, 1.78, 3.16, 5.62, 10.0, 17.8 and 31.6 mg a.s./L. Mean measured concentrations were 34, 73, 79, 75, 94, 87 and 83% of nominal, corresponding to 0.34, 1.30, 2.50, 4.22, 9.40, 15.4 and 26.2 mg a.s./L, respectively.

Test vessels were glass aquaria of 32 x 36 x 38 cm containing 40 L test solution. To each vessel were added 20 fish, which were observed after four hours and then daily for mortalities and symptoms of intoxication.

Dissolved oxygen and pH values were determined daily in each aquarium, and the water temperature of the control aquarium was recorded hourly.

Analytical determinations of the active substance were made in the test medium at test initiation and termination.

Analytical method

Samples of water were analysed using the validated analytical method 00252 M001, report reference M-008490-02-2 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the OECD 203 guideline were met:

- Control mortality to not exceed 10% at the end of the test (actual: 0.0 and 0.0% for the control and solvent control, respectively)
- Dissolved oxygen concentration to be ≥60% of the air saturation value (actual: 92.67 to 102.25%)

Mean measured values ranged from 34 to 94% of nominal. The low recovery seen in the lowest test concentration (1.00 mg a.s./L) had no impact on test results as the subsequent two higher test levels showed no adverse effects on the fish. The results have been presented based on the mean measured concentrations.

Table CA 8.2.1/02-1 Measured concentrations of KWG 4168 in test medium

Nominal test concentration (mg a.s./L)	Measured concentration (% of nominal) at test initiation[1]	Measured concentration (% of nominal) at test termination[1]	Mean measured test concentration (% of nominal)	Mean measured test concentration (mg a.s./L)
Control	0	0	0	0
Solvent control	0	0	0	0
1.00	29	38	34	0.34
1.78	69	77	73	1.30
3.16	87	71	79	2.50
5.62	67	82	75	4.22
10.0	90	98	94	9.40
17.8	62	111	87	15.4
31.6	83	83	83	26.2

[1] Mean of two replicates, corrected for recoveries

At test termination, mortalities of 0, 0, 0, 0, 0, 80, 100 and 100% were observed in the control, solvent control and 0.34, 1.30, 2.50, 4.22, 9.40, 15.4 and 26.2 mg a.s./L test concentrations. Sub-lethal signs of intoxication were observed from the 4.22 mg a.s./L test concentration onwards, and included lying on back/side, tumbling during swimming and fish staying mainly on the bottom or surface of the tank.

Table CA 8.2.1/02-2 Mortalities and symptoms of intoxication of bluegill sunfish during exposure to KWG 4168

Mean measured concentration (mg a.s./L)	Number of mortalities / number with symptoms of intoxication[1]				
	4 h	24 h	48 h	72 h	96 h
Control	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
Solvent control	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
0.34	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
1.30	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
2.50	0 / 0	0 / 0	0 / 0	0 / 0	0 / 0
4.22	0 / 0	0 / 20	0 / 20	0 / 20	0 / 20
9.40	0 / 20	0 / 20	0 / 20	4 / 20	16 / 20
15.4	0 / 20	10 / 20	16 / 20	19 / 0	20 / 20
26.2	20 / 20	-	-	-	-
LC_{50} (mg a.s./L) (95% CI)	-	15.4[a] (9.40 – 26.2)	13.2[a] (9.40 – 15.4)	11.1[b] (9.93 – 12.5)	7.13[b] (5.91 – 8.37)

[1] 20 fish were introduced per aquarium
Dead fish were added to the sum of fish with symptoms
[a] Calculated using binominal probability
[b] Calculated using probit method

III. Conclusion

The acute toxicity of KWG 4168 to bluegill sunfish (*Lepomis macrochirus*) was determined in a static 96-hour test. Seven test concentrations were assessed, along with a control and solvent control. Each treatment group contained 20 bluegill sunfish.

At test termination, mortalities of 0, 0, 0, 0, 0, 0, 80, 100 and 100% were observed in the control, solvent control and 0.34, 1.30, 2.50, 4.22, 9.40, 15.4 and 26.2 mg a.s./L test concentrations. Sub-lethal signs of intoxication were observed from the 4.22 mg a.s./L test concentration onwards, and included lying on back/side, tumbling during swimming and fish staying mainly on the bottom or surface of the tank.

The resulting 96-hour LC_{50} was determined to be 7.13 mg a.s./L, with 95% confidence intervals of 5.91 to 8.37 mg a.s./L. The LOEC and NOEC were 4.22 and 2.50 mg a.s./L, respectively.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 203, the most up-to-date version of which is the "Fish acute toxicity test", adopted 18 June 2019.

Validity criteria according to the OECD 203 (2019) guideline were met:

- Control mortality to not exceed 10% at the end of the test (actual: 0.0 and 0.0% for the control and solvent control, respectively)
- Dissolved oxygen concentration to be ≥60% of the air saturation value (actual: 92.67 to 102.25%)

The study is therefore considered acceptable.

The resulting 96-hour LC_{50} was determined to be 7.13 mg a.s./L.

Data Point:	KCA 8.2.1/03
Report Author:	[redacted]
Report Year:	2008
Report Title:	Acute toxicity of spiroxamine to zebra fish (Danio rerio) over 96 hours
Report No:	BAY-033/4-11
Document No:	M-307809-02-1
Guideline(s) followed in study:	OECD 203, 1992, Directive 92/69/EEC, part C.1
Deviations from current test guideline:	Yes Methods: SANCO/3029/99 rev. 4. Accuracy n=1 No precision data Ecotoxicology: OECD 203 (2019) The age of the test fish is not reported, however they are of a similar length and weight Feeding schedule is not reported, nor if any mortalities were observed during the acclamation period The light intensity is not reported
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The acute toxicity of KWG 4168 to zebra fish (*Danio rerio*) was determined in a static 96-hour test. Six test concentrations were assessed, along with a control. Each treatment group contained ten fish.

Mortality of 0, 0, 0, 0, 30, 100 and 100% were observed in the control and 0.278, 0.589, 1.22, 2.22, 4.27 and 9.27 mg a.s./L test concentrations.

Sub-lethal effects of spiroxamine exposure could be observed in the 1.22 mg a.s./L test concentration and above. Such signs included uncoordinated swimming, lateral positioning, dorsal positioning, tail-heavy swimming and dark colouring.

The 96-hour LC_{50} was determined to be 2.41 mg a.s./L. The NOEC and LOEC were determined to be 0.589 and 1.22 mg a.s./L, respectively.

I. Materials and Methods

A. Materials

Test Material	KWG 4168 (spiroxamine)
Lot/Batch #:	EDTH004650
Purity:	97.0% w/w
Description:	Light brown oil
Stability of test compound:	Not reported
Reanalysis/Expiry date:	02 August 2009
Density:	No information
Treatments	
Test rates:	Nominal: 0.31, 0.63, 1.25, 2.50, 5.00 and 10.0 mg a.s./L Geomean: 0.278, 0.589, 1.22, 2.22, 4.27 and 9.27 mg a.s./L
Solvent/vehicle:	None
Analysis of test concentrations:	Yes, geometric mean measured concentrations 85 – 98 % of nominal
Test organisms	
Species:	Zebra fish (*Danio rerio*)
Source:	[redacted]
Acclimatisation period:	Fish were reared in water of the same quality as used in the test for at least three months until the start of exposure
Feeding:	Not fed during the test
Treatment for disease:	None reported
Test design	
Test vessel:	12-L glass aquaria containing approx. 10 L test solution
Test medium:	Purified drinking water
Replication:	No replicates
No. of animals/vessel:	Ten fish
Duration of test:	96 hours

Environmental test conditions

Temperature:	22.6 – 23.2°C
Dissolved oxygen:	90 – 100% saturation
pH:	7.7 – 8.6
Photoperiod:	12 h light : 12 h dark

B. Study Design

This study was conducted to assess the effects of 96-hour exposure of zebra fish (*Danio rerio*) to spiroxamine in a static test. Test concentrations were selected based on the results of a non-GLP range-finding test.

Fish were of an in-house culture, and had been reared in water of the same quality as used in the test for at least three months prior to exposure. At test initiation fish were weighed and measured, and ranged from 2.2 to 2.9 cm (mean 2.5 cm) and 0.10 to 0.22 g (mean 0.15 g).

Test vessels were 12 L glass aquaria containing approximately 10 L test solution. Test medium was drinking water purified by filtration with activated charcoal, passage through a limestone column and oxygen aeration to saturation. Aquaria were held at 22.6 to 23.2°C under a 12 hour light to 12 hour dark photoperiod.

Six test concentrations were assessed along with a control. A stock solution was prepared by mixing 500 mg spiroxamine with 5 L dilution water, which was then further diluted to obtain required test concentrations. Nominal test concentrations were 0.31, 0.63, 1.25, 2.50, 5.00 and 10.0 mg a.s./L. Analyses of fresh and aged exposure media revealed concentrations of 56 to 123% of nominal values, thus endpoints were based on geometric mean measured concentrations. Geometric mean measured concentrations were 0.278, 0.589, 1.22, 2.22, 4.27 and 9.27 mg a.s./L, corresponding to 89, 94, 98, 89, 85 and 93% of nominal respectively.

The temperature, pH and oxygen concentration of the water were measured in each vessel directly at test initiation and then daily for the remainder of the test. Fish were measured for length and weight at the beginning of the study and observed daily for mortalities and any abnormalities in appearance and behaviour.

LC_x values were determined by probit analysis using the programme ToxRat Standard v3.3.0.

Analytical method

Samples of water were analysed using the validated analytical method M-303809-02-1, report reference M-303809-02-1 **(see Doc MCA Section 4)**.

II. Results and Discussion

Validity criteria according to the OECD 203 guideline were met:

- Control mortality to not exceed 10% at the end of the test (actual: 0.0%)
- Dissolved oxygen concentration to be ≥60% of the air saturation value (actual: 90 to 100%)

Table CA 8.2.1/03-1 Measured concentrations of spiroxamine in test medium

Nominal test concentration (mg a.s./L)	0 h (mg/L)	% of nominal	48 h (mg/L)	% of nominal	96 h (mg/L)	% of nominal	Geometric mean measured concentration	
							mg/L	%
0.31	0.340	109	0.300	96	0.210	67	0.278	89

Nominal test concentration (mg a.s./L)	0 h (mg/L)	% of nominal	48 h (mg/L)	% of nominal	96 h (mg/L)	% of nominal	Geometric mean measured concentration	
							mg/L	%
0.63	0.770	123	0.530	85	0.500	80	0.589	94
1.25	1.48	118	1.49	119	0.830	66	1.22	98
2.50	2.93	117	2.65	106	1.41	56	2.22	89
5.00	6.07	121	3.55	71	3.62	72	4.27	85
10.0	11.2	112	10.2	102	6.96	70	9.27	93

LOQ Limit of quantification: 0.003 mg/L

The results have been presented based on the mean measured concentrations.

Mortality of 0, 0, 0, 0, 30, 100 and 100% were observed in the control and 0.278, 0.589, 1.22, 2.22, 4.27 and 9.27 mg a.s./L test concentrations.

Sub-lethal effects of spiroxamine exposure could be observed in the 1.22 mg a.s./L test concentration and above. Such signs included uncoordinated swimming, lateral positioning, dorsal positioning, tail-heavy swimming and dark colouring.

Table CA 8.2.1/03-2 Cumulative mortality of zebra fish during exposure to spiroxamine

Geometric mean measured concentration (mg a.s./L)	Cumulative mortality (%)				
	3 h	24 h	48 h	72 h	96 h
Control	0	0	0	0	0
0.278	0	0	0	0	0
0.589	0	0	0	0	0
1.22	0	0	0	0	0
2.22	0	0	10	30	30
4.27	0	100	100	100	100
9.27	60	100	100	100	100

10 fish were added per concentration

The 96-hour LC_{50} was determined by probit analysis using the programme ToxRat to be 2.41 mg a.s./L. The NOEC and LOEC were determined to be 0.589 and 1.22 mg a.s./L, respectively.

Table CA 8.2.1/03-3 Endpoints after 96-hour exposure of zebra fish to spiroxamine

Endpoint	mg a.s./L
NOEC	0.589
LOEC	1.22
LC_{10}	1.52
LC_{50} (95% confidence limits)	2.41 (1.97 – 2.95)

III. Conclusion

The acute toxicity of KWG 4168 to zebra fish (*Danio rerio*) was determined in a static 96-hour test. Six test concentrations were assessed, along with a control. Each treatment group contained ten fish.

Mortality of 0, 0, 0, 0, 30, 100 and 100% were observed in the control and 0.278, 0.589, 1.22, 2.22, 4.27 and 9.27 mg a.s./L test concentrations.

Sub-lethal effects of spiroxamine exposure could be observed in the 1.22 mg a.s./L test concentration and above. Such signs included uncoordinated swimming, lateral positioning, dorsal positioning, tail-heavy swimming and dark colouring.

The 96-hour LC_{50} was determined to be 2.41 mg a.s./L. The NOEC and LOEC were determined to be 0.589 and 1.22 mg a.s./L, respectively.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 203, the most up-to-date version of which is the "Fish acute toxicity test", adopted 18 June 2019.

Validity criteria according to the up-to-date OECD 203 (2019) guideline were met:

- Control mortality to not exceed 10% at the end of the test (actual: 0.0%)
- Dissolved oxygen concentration to be ≥60% of the air saturation value (actual: 90 to 100%)

The study is therefore considered acceptable.

The 96-hour LC_{50} was determined to be 2.41 mg a.s./L.

CA 8.2.2 Long-term and chronic toxicity to fish

For procedural reasons studies listed in the Table 8.2.2-1 below are included in the current dossier as available data or information previously submitted but not necessarily evaluated. However, these reports have been fully superseded by newer studies. Consequently, no summaries of the reports have been included in the dossier.

Table CA 8.2.2-1: Studies previously submitted and not relied upon for the risk assessment

Data Point	Document No.	Date	Title
KCA 8.2.2/01	M-468005-01-1	2013	SPIROXAMINE - Higher-tier effect assessment for fish based on a refined-exposure Fish Full Life Cycle study

CA 8.2.2.1 Fish early life stage toxicity test

Data Point:	KCA 8.2.2.1/01
Report Author:	[redacted]
Report Year:	1994
Report Title:	KWG 4168 techn. -Early life stage toxicity to rainbow trout (Oncorhynchus mykiss) under flow-through conditions
Report No:	DOM 94016
Document No:	M-006232-01-1
Guideline(s) followed in study:	OECD 210 (1992)
Deviations from current test guideline:	Yes OECD 210 (2013) Temperature deviated from the recommended 10 ± 1.5°C, with a max. daily temperature of 12°C frequently observed. The max. temperature on day 22 was 14°C. Mean total length of the control fish at test termination was not reported. The mean standard length of the pooled controls was 34.2 mm. Fry were first fed 14 days post-hatch rather than 19 days post-hatch, and were only fed once daily on weekends and holidays.
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The chronic toxicity of KWG 4168 to rainbow trout (*Oncorhynchus mykiss*) was determined in a flow-through early-life-stage toxicity test. The effects on 35 eggs and 15 fry were observed over a total of 93 days. Nominal test concentrations of 62.5, 125, 250, 500, 1000 and 2000 µg a.s./L were tested.

Survival at post-hatch Day 34 and Day 63 resulted in a NOEC and LOEC of 1000 and 2000 µg a.s./L, respectively, at both days. The effect threshold (EC_0) was calculated to be 1414 µg a.s./L.

Egg hatchability resulted in a NOEC at 2000 µg a.s./L and the LOEC at > 2000 µg a.s./L. An effect threshold could not be determined.

Time to hatch resulted in a NOEC at 2000 µg a.s./L and the LOEC at > 2000 µg a.s./L. An effect threshold could not be determined.

Time to 94 percent swim-up resulted in a NOEC at 500 µg a.s./L and the LOEC at 1000 µg a.s./L. The effect threshold (EC_0) was calculated to be 707 µg a.s./L.

Growth, expressed as dry weight, resulted in a NOEC at < 62.5 µg a.s./L and the LOEC at 62.5 µg a.s./L. The effect threshold (EC_0), based on a concentration-response model (linear regression model), is 14 µg a.s./L.

Growth, expressed as length, on post-hatch Day 34 resulted in a NOEC at < 62.5 µg a.s./L and the LOEC at 62.5 µg a.s./L. The effect threshold (EC_0), based on a concentration-response model (linear regression model), is 29 µg a.s./L.

Growth, expressed as length, on post-hatch Day 63 resulted in a NOEC at < 62.5 µg a.s./L and the LOEC at 62.5 µg a.s./L. The effect threshold (EC_0), based on a concentration-response model (linear regression model), is 26 µg a.s./L.

The effect threshold for KWG 4168 was based on the most sensitive endpoint (growth, expressed as dry weight), and determined to be 14 µg a.s./L.

I. Materials and Methods

A. Materials

Test Material	KWG 4168
Lot/Batch #:	898114002
Purity:	97.8%
Description:	Colourless liquid
Stability of test compound:	Certified until 27 January 1994
Reanalysis/Expiry date:	27 January 1994
Density:	Not reported
Treatments	
Test rates:	Nominal: 62.5, 125, 250, 500, 1000 and 2000 µg a.s./L Mean measured: 61.1, 126.8, 220.5, 452.8, 995.6 and 1874.5 µg a.s./L
Solvent/vehicle:	Acetone
Analysis of test concentrations:	Yes, mean measured values 88 – 101% of nominal
Test organisms	
Species:	Rainbow trout (*Oncorhynchus mykiss*)

Source:	[redacted]
Acclimatisation period:	Not applicable
Feeding:	Fry were fed with live brine shrimp (*Artemia salina*) nauplii and ground trout/salmon starter *ad libitum* starting on day 14 post-hatch. Food was added to aquaria twice daily during the week, and once daily at weekends/holidays, with each aquarium receiving an approximately equal quantity of food
Treatment for disease:	None
Test design	
Test vessel:	Egg hatching: 14 x 9 cm with a water depth of 17.5 cm (volume 2.2 L) Growth phase: 18 x 22 cm with a water depth of 19 cm (volume 7.5 L)
Test medium:	Reconstituted water
Replication:	No replicates
No. of animals/vessel:	Egg hatching: 35 eggs per vessel Growth phase: 15 alevin per vessel
Duration of test:	93 days
Environmental test conditions	
Temperature:	9 – 14°C (mean: 10.3 – 11.9°C)
Dissolved oxygen:	95 – 109% of saturation
pH:	7.1 – 7.5
Photoperiod:	16 h light : 8 h dark at 435 lux

B. Study Design

The chronic toxicity of KWG 4168 to rainbow trout (*Oncorhynchus mykiss*) was determined in a flow-through early-life-stage toxicity test over 93 days. Test concentrations were chosen based on historical toxicity results.

Nominal test concentrations of 62.5, 125, 250, 500, 1000 and 2000 µg a.s./L were tested. Prior to the initiation of the study, test solutions had been flowing through the test system for nine days.

Unfertilised eggs and milt from three female and three male adult brood fish. Eggs were fertilised in dilution water at 11 ± 1°C with the aid of gentle stirring, after which they were rinsed to clarity and randomly distributed to incubation cups.

Eggs were incubated in 1.8 mm diameter cups constructed with perforated stainless steel plate on the bottom. These incubation cups were suspended in each replicate test chamber. The glass aquaria used

for egg hatching measured approximately 14 x 9 cm with a water depth of 17.5 cm, yielding an approximate chamber volume of 2.2 L. The test solution was changed approximately 41 times per hour.

Test chambers were held under a 16 hour light to eight hour dark photoperiod at 435 lux. Developing embryos were shielded from excess UV light exposure by leaving lights off until completion of hatch.

To each of the incubation cups were added 35 impartially selected fertilised eggs. Each test concentration had four replicates. Four replicates of 50 eggs each were placed in additional exposure chambers for a viability determination. All incubation cups were observed daily, except weekends, for egg mortality, determined by a distinct change in colouration. Any dead eggs found were discarded.

The number of eggs hatched in each incubation cup was recorded daily except weekends until three days post-hatch. The post-hatch period began after 95% of living eggs in the control had hatched, on study day 30. Alevin were impartially thinned to 15 individuals per replicate on day 5 post-hatch. Observation of behaviour and mortality were made daily except weekends.

Glass aquaria used as growth chambers for the post-hatch phase measured approximately 18 x 22 cm with a water depth of 19 cm, yielding an approximate chamber volume of 7.5 L. The test solution was changed approximately 12 times per hour.

Fry were fed with live brine shrimp (*Artemia salina*) nauplii and ground trout/salmon starter *ad libitum* starting on day 14 post-hatch. Food was added to aquaria twice daily during the week and once daily at weekends/holidays, with each aquarium receiving an approximately equal quantity of food.

Growth was determined by measurement of the standard length on day 34 post-hatch using slide photography. At termination of the biological phase on day 63 post-hatch, after a total of 93 days, surviving fish were sacrificed and the standard lengths and wet weights determined. Once these measurements were completed, fish were dried at 60°C for 72 hours, and individual dry weights were determined.

Temperature was measured working daily in the control and a data logger documented hourly temperatures from a centrally-located test chamber. Dissolved oxygen and pH were measured in one replicate of the control, solvent control, and 62.5, 500 and 2000 µg a.s./L test concentrations approximately weekly.

Analytical method

Samples of water were analysed using the validated analytical method 00252 M001, report reference M-008490-02-2 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the most recent OECD 210 guideline (2013) were met:

- Dissolved oxygen concentration to be ≥60% of the air saturation value throughout the test (actual: 95 to 109%)
- Overall survival of fertilised eggs (*i.e.* hatching success) and post-hatch survival in the controls should both be ≥75% for rainbow trout (100 and 90.5% achieved, respectively, in the pooled controls)

One of the criteria was not met:

- Water temperature to not differ by more than ±1.5°C between test chambers or between successive days at any time during the test. The temperature was only measured in one test chamber, with one deviation of +2°C to preceding and successive days recorded on study day 22. The mean temperature on day 22 was 11.7 °C ± 0.9 (SD).

Mean measured values ranged from 88 to 101% of nominal. At days 42 and 49 in the 2000 µg a.s./L test group, measured concentrations of 43258 and <0.1 µg a.s./L, respectively, were found. These two values were most likely outliers resulting from sample handling errors, and as a result were excluded from further statistical analysis. On several sample days, KWG 4168 was detected in control and solvent

control samples at amounts in the same range as in the blank samples, due to cross-contamination during the analytical phase.

Table CA 8.2.2.1/01-1 Measured concentrations of KWG 4168 in test medium

Nominal test concentration (µg a.s./L)	Mean measured concentration (µg a.s./L)	Standard deviation (µg a.s./L)	Mean measured concentration (%)
Control	-	-	-
Solvent control	-	-	-
62.5	61.1	13.0	97.8
125	126.8	32.3	101.4
250	220.5	59.2	88.2
500	452.8	82.8	90.6
1000	995.6	375.3	99.6
2000	1874.5	677.2	93.7

Results corrected for recoveries

The results have been based on nominal test concentrations.

Percent egg hatchability was not significantly affected by the treatment at any concentration when compared to the pooled controls. Mean fry survival on day 64 (post-hatch day 34) was 93, 95, 98, 88, 97, 93, 87 and 82%, with survival in the highest test concentration, 2000 µg a.s./L, being significantly reduced compared to the pooled controls. On day 93 (post-hatch day 63, mean fry survival was 87, 94, 98, 88, 93, 92, 87 and 73%, with fry survival in the 2000 µg a.s./L again significantly reduced compared to the pooled controls.

Table CA 8.2.2.1/01-2 Mean egg hatchability and fry survival with exposure to KWG 4168

Nominal test concentration (µg a.s./L)	Mean egg hatch (%)[1]	Mean post-hatch day 34 survival (%)	Mean post-hatch day 63 survival (%)
Control	100	93	87
Solvent control	100	95	94
62.5	100	98	98
125	100	88	88
250	100	97	93
500	100	93	92
1000	100	87	87
2000	100	82*	73*

Results are means of four replicates

* Statistically significantly different from the pooled controls ($\alpha = 0.05$)

[1] Post-hatch day 3

Egg hatching began on study day 28 and continued until study day 33. Percent hatch on study day 33 was 100% for all test levels, and there was no statistically significant different in percent hatchability at any treatment level. As ≥95% on the eggs in the control had hatched on day 30, this was taken as day 0 of the post-hatch period.

Table CA 8.2.2.1/01-3 Time to hatch of rainbow trout fry with exposure to KWG 4168

Nominal test concentration (µg a.s./L)	Percent hatch (%)						
	Day 27	Day 28	Day 29	Day 30	Day 31	Day 32	Day 33
Control	0	2	74	96	99	100	100
Solvent control	0	9	87	99	100	100	100
62.5	0	13	73	97	100	100	100
125	0	16	73	100	100	100	100

Nominal test concentration (µg a.s./L)	Percent hatch (%)						
	Day 27	Day 28	Day 29	Day 30	Day 31	Day 32	Day 33
250	0	15	77	96	99	100	100
500	0	25	68	97	98	100	100
1000	0	24	69	98	99	100	100
2000	0	17	64	98	100	100	100

Results are means of four replicates

Newly hatched fry began swimming up from the bottom of test chambers on study day 47. A 94% swim-up was achieved on day 49 in the pooled controls, during which time there was a statistically significant difference in the 1000 and 2000 mg a.s./L test concentrations.

Table CA 8.2.2.1/01-4 Percent swim-up of rainbow trout fry with exposure to KWG 4168

Nominal test conc. (µg a.s./L)	Percent swim up by study day (%)													
	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Control	0	57	80	93	80	65	73	83	90	95	92	90	95	95	95
Solvent control	0	67	88	95	97	90	93	95	97	98	98	98	98	98	98
62.5	0	64	57	88	85	90	95	95	95	95	90	97	100	100	100
125	0	18	36	81	76	71	69	66	79	79	91	98	100	100	100
250	0	48	34	78	73	75	52	53	85	63	73	44	91	91	91
500	0	32	77	93	86	68	40	95	21	14	61	33	55	53	51
1000	0	2	14	41*	54	52	42	42	24	22	20	12	7	5	5
2000	0	0	0	11*	10	7	7	7	8	4	2	2	4	4	8

Results are means of four replicates

* Statistically significant different to the pooled control (α=0.05)

Fry growth expressed as standard length on post-hatch day 34 was significantly reduced at all test concentrations when compared to the pooled controls. A concentration-response model (linear regression) demonstrated an effect threshold (EC_0) at 29 µg a.s./L.

Fry growth expressed as standard length was also significantly reduced on post-hatch day 63 in all test concentrations compared to the pooled controls. A concentration-response model (linear regression) demonstrated an effect threshold (EC_0) at 26 µg a.s./L.

Fry growth expressed as dry weight was significantly reduced on post-hatch day 63 in all test concentrations compared to the pooled controls. A concentration-response model (linear regression) demonstrated an effect threshold (EC_0) at 14 µg a.s./L.

Table CA 8.2.2.1/01-5 Mean length and weight of rainbow trout with exposure to KWG 4168

Nominal test concentration (µg a.s./L)	Post-hatch day 34 length (mm)	Post-hatch day 63		
		Length (mm)	Wet weight (mg)	Dry weight (mg)
Control	30.2	34.5	552	91.9
Solvent control	30.9	33.9	491	86.8
Pooled control	30.5	34.2	522	89.4
62.5	28.4*	31.9*	-	77.3*
125	26.6*	29.4*	-	71.3*
250	24.7*	26.5*	-	69.5*
500	21.9*	23.2*	-	59.1*
1000	20.4*	21.9*	-	55.5*
2000	19.1*	21.2*	-	50.8*

Results are means of four replicates

Nominal test concentration (µg a.s./L)	Post-hatch day 34 length (mm)	Post-hatch day 63		
		Length (mm)	Wet weight (mg)	Dry weight (mg)

* Statistically significant different to the pooled control (α=0.05)

During the post-hatch period, the following morphological and behavioural effects were observed: darkened colouration, swollen belly, oversized yolk sac, exophthalmia, kyphosis, scoliosis, fish lying on side or back, loss of equilibrium and vertical orientation. The lack of a dose-response coupled with the level of random mortality observed in the controls, indicated no compound-related effects in the case of swollen belly, oversized yolk sac, exophthalmia, kyphosis, scoliosis and loss of equilibrium.

In the case of darkened colouration, there was a dose-response relationship observed between post-hatch day 33 and the end of the study in the 250, 500, 1000 and 2000 µg a.s./L test concentrations. In the case of vertical orientation there was a dose-response between post-hatch day 33 and the end of the study in the 500, 1000 and 2000 µg a.s./L test concentrations. In the case of fish lying on side or back there was a transient dose-response between post-hatch day 18 and post-hatch day 22 at all test levels. This symptom was observed at the end of the study only in the 500, 1000 and 2000 µg a.s./L test concentrations.

The endpoints resulting from exposure of rainbow trout to KWG 4168 are summarised in the table below:

Table CA 8.2.2.1/01-6 Endpoints (µg a.s./L) after exposure to KWG 4168

Endpoint	NOEC	LOEC	Effect threshold (EC_0)
Survival at post-hatch day 34	1000	2000	1414
Survival at post-hatch day 63	1000	2000	
Egg hatchability	2000	>2000	>2000
Time to hatch	2000	>2000	>2000
Time to 94% swim-up	500	1000	707
Growth, expressed as dry weight	<62.5	62.5	14
Growth (standard length) on post-hatch day 34	<62.5	62.5	29
Growth (standard length) on post-hatch day 63	<62.5	62.5	26

III. Conclusion

Based on the statistical analysis of survival, egg hatchability, time to hatch, time to swim-up and growth (expressed as weight and length), the NOEC and LOECs were determined. In addition, effect thresholds (EC_0) were estimated with a good curve fitting for growth parameters based on a concentration-response model (linear regression model).

Survival at post-hatch Day 34 and Day 63 resulted in a NOEC and LOEC of 1000 and 2000 µg a.s./L, respectively, at both days. The effect threshold (EC_0) was calculated to be 1414 µg a.s./L.

Egg hatchability resulted in a NOEC at 2000 µg a.s./L and the LOEC at >2000 µg a.s./L. An effect threshold could not be determined.

Time to hatch resulted in a NOEC at 2000 µg a.s./L and the LOEC at > 2000 µg a.s./L. An effect threshold could not be determined.

Time to 94 percent swim-up resulted in a NOEC at 500 µg a.s./L and the LOEC at 1000 µg a.s./L. The effect threshold (EC_0) was calculated to be 707 µg a.s./L.

Growth, expressed as dry weight, resulted in a NOEC at < 62.5 µg a.s./L and the LOEC at 62.5 µg a.s./L. The effect threshold (EC_0), based on a concentration-response model (linear regression model), is 14 µg a.s./L.

Growth, expressed as length, on post-hatch Day 34 resulted in a NOEC at < 62.5 µg a.s./L and the LOEC at 62.5 µg a.s./L. The effect threshold (EC_0), based on a concentration-response model (linear regression model), is 29 µg a.s./L.

Growth, expressed as length, on post-hatch Day 63 resulted in a NOEC at < 62.5 µg a.s./L and the LOEC at 62.5 µg a.s./L. The effect threshold (EC_0), based on a concentration-response model (linear regression model), is 26 µg a.s./L.

The effect threshold for KWG 4168 was based on the most sensitive endpoint (growth, expressed as dry weight), and determined to be 14 µg a.s./L.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 210 (1992), the most up-to-date version of which is the "Fish, early-life stage toxicity test", adopted 26 July 2013.

Validity criteria according to the most recent OECD 210 guideline (2013) were met:

- Dissolved oxygen concentration to be ≥60% of the air saturation value throughout the test (actual: 95 to 109%)
- Overall survival of fertilised eggs (*i.e.* hatching success) and post-hatch survival in the controls should both be ≥75% for rainbow trout (100 and 90.5% achieved, respectively, in the pooled controls)

One of the criteria was not met:

- Water temperature to not differ by more than ±1.5°C between test chambers or between successive days at any time during the test. The temperature was only measured in one test chamber, with one deviation of +2°C to preceding and successive days recorded on study day 22. The mean temperature on day 22 was 11.7 °C ± 0.9 (SD).

The water temperature deviation was transient and only slight, and thought to have no impact on the integrity of the study. The study is therefore considered acceptable.

Statistically significant effects were determined at the lowest concentration tested which was 62.5 µg a.s./L. As a result an EC_0 was calculated and determined to be 14 µg a.s./L. Whilst the study is considered to be valid the EC_0 endpoint is not conventionally used in risk assessment and should therefore be treated with caution.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

The NOEC was determined to be <62.5 µg a.s./L. The EC_0 for KWG 4168 was based on the most sensitive endpoint (growth, expressed as dry weight), and determined to be 14 µg a.s./L. A more conservative chronic fish endpoint is available from a fish full life cycle study and has been used in the risk assessment.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.2.2.1/04
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10 and EC20 values for Oncorhynchus mykiss with spiroxamine TG in an early life stage study
Report No:	0471836-ECO1
Document No:	M-760407-01-1
Guideline(s) followed in study:	none
Deviations from current test guideline:	None
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 M-006232-01-1 on the effects of Spiroxamine TG in the rainbow trout (Oncorhynchus mykiss) early life stage study did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013. Effect Concentrations with a 10% and 20% effect on length when compared to the pooled controls were re-calculated. As effects were less than 50% an EC_{50} could not be determined. As effects were >10%, no EC_{10} calculations were conducted for dry weight. Although ECx values and 95% confidence intervals were calculated for % swim up, as the EC_{10} is was above the test rate that showed an effect around 10% the results are deemed unfit for use within a risk assessment. As effects of 5% and 9% were seen in the controls, which were not corrected for in the test item concentration survival, actual effects would therefore be minimal and so reliable ECx calculations could not be determined. As there were no effects on hatchability and time to hatch, with a lack of dose response, no ECx values could be calculated.

The resulting EC_{10}, and EC_{20} values for length at 34 days post-hatch (dph) of 67.87 (95%CL: 23.52 – 123.02) and 288.97 (95%CL: 173.78 – 408.69) µg a.s./L, respectively, are considered reliable as the criteria for goodness of fit were met. The resulting EC_{10} and EC_{20} values for length at 63 dph of 58.21 (95%CL: 27.16 – 93.81) and 327.40 (95%CL: 232.84 – 459.19) µg a.s./L, respectively, meet the goodness of fit criteria and are therefore considered reliable. However, from a visual interpretation of the data an EC_{10} > 62.5 µg a.s./L should still be considered more appropriate for use in the risk assessment.

I. Methods

The statistical evaluation was performed with statistical software ToxRat Standard v3.3.0.

Effect concentrations with 10 and 20% from the test item treatment when compared to the pooled controls were calculated but due to lack of effects and the non-existence of a dose response these could not be determined for some parameters. EC_{10} and EC_{20} values were determined for length and % swim up. For the determination of the EC_{10} and EC_{20} values for length at 34 and 63dph a Probit function using linear maximum likelihood regression and linear weighted regression, respectively was used along with 95% ECx confidence limits. For the determination of the EC_{10} and EC_{20} values for % swim up at 49 dph a Weibull function using linear maximum likelihood regression was used along with 95% ECx confidence limits.

II. Results and Discussion

A more detailed explanation is given for regression analysis endpoints for dry weight, total length and % swim up. These details can be found below. For the parameters hatchability and time to hatch, no statistical calculations were possible to perform as no effects were observed.

Total length at 34 days post hatch (dph)

Regarding the calculation of EC_{10} and EC_{20} values for total length at 34 dph, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.00, showing no significant deviation between fit and data, and a statistically significant concentration/response was found ($p(F) < 0.001$) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table below.

Table CA 8.2.2.1/04-1 Results of the Probit analysis (max. likelihood regression) with total length at 34 dph: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (according to Fieller`s theorem)

Parameter	Total length	
	EC_{10} (95 % confidence interval) [µg a.s./L]	EC_{20} (95 % confidence interval) [µg a.s./L]
Effect on total length at 34 dph	67.87 (23.52-123.02)	288.97 (173.78-408.69)

The resulting EC_{10} and EC_{20} values of 67.87 (95% CL: 23.52 – 123.02) and 288.97 (95% CL: 173.78 – 408.69) µg a.s./L, respectively, meet the goodness of fit criteria and therefore the estimated EC_{10} value is considered reliable for use in the risk assessment.

Total length at 63 days post hatch (dph)

Regarding the calculation of EC_{10} and EC_{20} values for total length at 63 dph, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.00, showing no significant deviation between fit and data, and a statistically significant concentration/response was found ($p(F) < 0.001$) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table.

Table CA 8.2.2.1/04-2 Results of the Probit analysis (linear weighted regression) with total length at 63 dph: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (according to Fieller`s theorem)

Parameter	Total length	
	EC_{10} (95 % confidence interval) [µg a.s./L]	EC_{20} (95 % confidence interval) [µg a.s./L]
Effect on total length at 63 dph	58.21 (27.16 - 93.81)	327.40 (232.84 - 459.19)

The resulting EC_{10} and EC_{20} values of 58.21 (95%CL: 27.16 – 93.81) and 327.40 (95%CL: 232.84 – 459.19) µg a.s./L, respectively, meet the goodness of fit criteria and therefore the estimated EC_{10} value is considered reliable. However, from a visual interpretation of the data an $EC_{10} > 62.5$ µg a.s./L should still be considered more appropriate for use in the risk assessment.

% swim up

Regarding the calculation of EC_{10} and EC_{20} values for % swim up at 49 dph, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.00, showing no significant deviation between fit and data, and a statistically significant concentration/response was found ($p(F) < 0.001$) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table and figure below.

Table CA 8.2.2.1/04-3 Results of the Weibull analysis with % swim up at 49 dph: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (by bootstrapping (1000 resamplings); bias-corrected)

Parameter	% swim up	
	EC_{10} (95 % confidence interval) [µg a.s./L]	EC_{20} (95 % confidence interval) [µg a.s./L]
Effect on % swim up at 49 dph	321.75 (176.14 – 519.90)	502.71 (327.41 – 703.62)

The resulting EC_{10} and EC_{20} values of 321.75 (95%CL: 176.14 – 519.90) and 502.71 (95%CL 327.41 – 703.62) µg a.s./L, respectively, meet the goodness of fit criteria and therefore the estimated EC_{10} value is considered reliable. However, as the endpoint is way above the test rate that showed an effect around 10% the results are deemed unfit for use within a risk assessment.

A summary of the obtained endpoints is presented in the following table.

Table CA 8.2.2.1/04-4 Overall endpoints of the statistical re-calculation of the *Oncorhynchus mykiss* study with spiroxamine

Parameter	Endpoint (µg a.s./L)
	EC_{10} (95% confidence intervals)
Survival 34 dph	n.d.
Survival 63 dph	n.d.
Dry weight	n.d.
Length 34 dph	67.87 (23.52 – 123.02)
Length 63 dph	>62.5
% swim up	n.d.
Hatchability	n.d.
Time to hatch	n.d.

Due to the lack of a concentration dose response when compared to the controls, the calculation of EC_{10} and EC_{20} values for hatchability and time to hatch was not possible and therefore no EC_{10} or EC_{20} values were determined. Due to effects at all the tested rates being more than 10% below the controls values, and as recommended by OECD (OECD 54), the EC_{10} could not be calculated for dry weight. Due to a mortality of 5 and 9% in the pooled controls at 34 and 63 dph respectively, which were not used to correct test item survival, EC_{10} values could not be reliably calculated for survival as the real effects would be even smaller than those presented.

III. Conclusion

The resulting EC_{10}, and EC_{20} values for length at 34 dph of 67.87 (95%CL: 23.52 – 123.02) and 288.97 (95%CL: 173.78 – 408.69) µg a.s./L, respectively, are considered reliable as the criteria for goodness of fit were met. The resulting EC_{10} and EC_{20} values for length at 63 dph of 58.21 (95%CL: 27.16 – 93.81) and 327.40 (95%CL: 232.84 – 459.19) µg a.s./L, respectively, meet the goodness of fit criteria and are therefore considered reliable. However, from a visual interpretation of the data an EC_{10} >62.5 µg a.s./L should still be considered more appropriate for use in the risk assessment.

<u>Assessment and conclusion by applicant:</u>

The statistical re-evaluation of the data has determined EC_{10} and EC_{20} values for some of the parameters but many of these values, although reliable according to the statistical software, were considered unsuitable for use in the risk assessment. Expert judgement has therefore been used for some of these parameters. The most critical endpoint determined remains to be a NOEC of <62.5 µg a.s./L based on effects on dry weight. However, due to the limited effects seen at this test concentration there is argument to use expert judgement to set the NOEC at 62.5 µg a.s./L.

An EC_0 of 14 µg a.s./L was determined in the original study report and has been taken as the most critical endpoint for this study in order to remain consistent with the original study report. The values determined in the re-evaluation work are considered to be fully valid.

Data Point:	KCA 8.2.2.1/02
Report Author:	[redacted]
Report Year:	2004
Report Title:	14C-KWG 4168 - Early life stage toxicity to rainbow trout (Oncorhynchus mykiss) under flow-through conditions (supplemental raw data)
Report No:	DOM 95017
Document No:	M-006449-02-1
Guideline(s) followed in study:	OECD Guideline 210; "Fish, Early-life Stage Toxicity Test"
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A flow-through early-life stage toxicity test was conducted to determine the toxicity of ^{14}C-KWG 4168 to the early-life stages of the rainbow trout (*Oncorhynchus mykiss*).

Six test concentrations (3.75, 7.5, 15, 30, 60 and 120 µg a.s./L) were investigated, each with four replicates of initially 100 fertilised eggs. The effects of exposure on different life stages of the fish, including early life stages, juvenile growth, reproduction and early life stages of the filial generation were assessed under continuous exposure.

Based on the statistical analysis of survival, egg hatchability, time to hatch, time to swim-up, and growth (expressed as weight and length), the no-observed-effect-concentrations (NOECs) and the lowest-observed-effect-concentrations (LOECs) were determined as follows. All test levels listed are based on mean measured concentrations (3.65, 7.41, 14.2, 28.9, 61.8 and 119 µg a.s./L) of the test substance:

Survival at day 71 (post-hatch day 36) resulted in a NOEC at ≥119 µg a.s./L and the LOEC at >119 µg a.s./L.

Survival at 96 (post-hatch day 61) resulted in a NOEC at ≥119 µg a.s./L and the LOEC at >119 µg a.s./L.

Egg hatchability at day 38 resulted in a NOEC at ≥119 µg a.s./L and the LOEC at >119 µg a.s./L.

Time to hatch at day 38 resulted in a NOEC at ≥119 µg a.s./L and the LOEC at >119 µg a.s./L.

Time to swim-up at day 55 resulted in a NOEC at ≥119 µg a.s./L and the LOEC at >119 µg a.s./L.

Growth at day 71 (post-hatch day 36), expressed as standard length, resulted in a NOEC at 28.9 µg a.s./L and the LOEC at 61.8 µg a.s./L.

Growth at day 96 (post-hatch day 61), expressed as standard length, resulted in a NOEC at 14.2 µg a.s./L and the LOEC at 28.9 µg a.s./L.

Growth at day 96 (post-hatch day 61), expressed as dry weight, resulted in a NOEC at 28.9 µg a.s./L and the LOEC at 61.8 µg a.s./L.

The lowest effect threshold (geometric mean of NOEC and LOEC) for ^{14}C-KWG 4168 was based on the most sensitive endpoint, the fry growth, expressed as standard length, on study day 96 (post hatch day 61). So, the lowest effect threshold in this study was calculated to be 20.3 µg test substance/L.

I. Materials and Methods

A. Materials

	Non-radiolabelled	Radiolabelled
Test Material	KWG 4168 technical	THS 4429
Lot/Batch #:	898114002	Not reported
Purity:	96.7%	> 98%
Description:	Colourless liquid	Not reported
Stability of test compound:	Certified until 25 January 1995	Not reported
Reanalysis/Expiry date:	25 January 1995	Not reported
Density:	Not reported	Not reported
Treatments		
Test rates:	3.75, 7.5, 15, 30, 60 and 120 µg a.s./L	
Solvent/vehicle:	Acetone	
Analysis of test concentrations:	Yes on days -1, 0, 7, 14, 21, 28, 36, 37, 43, 50, 56, 64, 70, 77, 86, 91 and 96 (mean measured values 94 – 103% of nominal)	
Test organisms		
Species:	Rainbow trout (*Oncorhynchus mykiss*)	
Source:	[redacted]	
Acclimatisation period:	Not applicable	
Feeding:	Live brine shrimp (*Artemia salina*) and trout/salmon starter pellets	
Treatment for disease:	Eggs obtained from certified disease free hatchery	
Test design		
Test vessel:	18 cm x 22 cm with a water depth of 19 cm = 7.5 L volume	
Test medium:	Reconstituted water	
Replication:	Four replicates per control and test group	
No. of animals/vessel:	35	
Duration of test:	96 days	
Environmental test conditions		

Temperature:	9.1 – 10.7°C (mean: 9.8°C)
Dissolved oxygen:	93 – 100% of saturation
pH:	7.0 – 7.6
Photoperiod:	16 h light : 8 h dark at 435 lux

Study Design

A flow-through early-life stage toxicity test was conducted to determine the toxicity of ^{14}C-KWG 4168 to the early-life stages of the rainbow trout (*Oncorhynchus mykiss*).

Nominal test concentrations of 3.75, 7.5, 15, 30, 60 and 120 µg a.s./L were tested. Prior to the initiation of the study, test solutions had been flowing through the test system.

Eggs transported in plastic bags, were transferred to a dry stainless steel bowl and the milt was mixed with the eggs (with an autoclaved goose feather). Milt, transported in a separate plastic bag, was examinated under the microscope. There were no negative findings. Dilution water (10 ± 1°C) was added until the eggs were covered.

A total amount of 135 L/h dilution water was used in this study. This dilution water was split into 9 different water streams of 15 L/h, controlled by flow meters. A diluter system with two programmed Microlab Hamilton MT 4-fold syringe pumps was used for the intermittent introduction of 1.5 mL/h (volumes of 25 µL each 60 seconds) of stock solutions with different concentrations of ^{14}C-KWG 4168. Stock solutions of ^{14}C-KWG 4168 in acetone were added to each of these streams of dilution water (resulting in a concentration of 100 µL acetone per litre of dilution water), except for the dilution water control group and the fertilisation control group. The solvent control group was also prepared with acetone. The maximum concentration of acetone in the test system, was 100 µL/L.

All stock solutions were continuously agitated during the test by magnetic stirrers. Flow splitting cells divided the water streams after the introduction of stock solutions and after passing mixing chambers into four aliquots per test concentration before being delivered to replicate test chambers.

The accuracy of the test solution splits was checked prior to the test initiation and after test termination for both the egg hatching chamber system (for embryos from fertilisation to hatching) and the growth chamber system (for alevins). The accuracy of splits was within 10% of nominal. The diluter system and syringe pump function were checked and documented working daily.

Eggs were incubated in incubation cups, constructed from 8 cm diameter teflon pipes with stainless steel plates perforated (hole diameter: 1.8 mm) on the bottom. These incubation cups were suspended in each replicate test chamber. To facilitate circulation of water and keep eggs clean, incubation cups were oscillated vertically with a lifting movement of approximately 4 cm in the test chamber by means of a rocker arm apparatus driven by a low rpm electric motor.

The glass aquaria used for egg hatching measured approximately 14 cm x 9 cm with a water depth of 17.5 cm, yielding an approximate chamber volume of 2.2 liters and resulting in approximately 41 changes of test solution per day. The glass aquaria used as growth chambers for the post hatch phase measured approximately 18 cm x 22 cm with a water depth of 19 cm, yielding an approximate chamber volume of 7.5 liters and resulting in approximate 12 changes of test solution per day. All glass aquaria used as growth chambers for the post hatch phase were covered with stainless steel screens to prevent escaping of alevins.

The number of eggs hatched in each incubation cup was recorded until 3 days post-hatch. The post-hatch period began after 95% of all living eggs in the control had hatched (study day 35, post hatch day 0). Alevin were impartially thinned to 15 individuals per replicate on study day 38 (post-hatch day 3). This was accomplished by transferring 15 impartially selected fry from each replicate and by releasing in the corresponding replicate aquarium on the same day. Observations of abnormal behaviour, normal

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

Feeding began on day 47 (post-hatch day 12). Fry were fed *ad libitum,* taking care that each aquaria received an equal quantity of food. Food was added to the aquaria twice daily, except on weekends/holidays when food was added once daily. Live brine shrimp *(Artemia salina)* nauplii were fed to the fry until one day before study termination. In addition, the fry were also fed from post-hatch day 14 with ground trout/salmon starter until one day before study termination.

Growth was determined by measurement of standard length (mm) on study day 71 (post hatch day 36) using slide photography. The water depth in each test chamber was lowered to approximately 2 cm in order to take the photo. A millimeter scale and tank identification plate with study number, test concentration and replicate number were positioned on the bottom of the chamber. The developed photographic slides were projected parallax-free on a wall and standard length of each fish was measured using the photographed metric scale as the standard.

At the termination of the biological phase (post hatch day 61) after a total of 96 days the surviving fish were sacrificed. The standard length (mm) was determined and recorded for each individual fish. Standard lengths were determined by measuring from the tip of the snout to the tip of the caudal peduncle using a millimeter scale. Wet weight of control and solvent control fish was recorded for evaluation of test system loading. Fish were blotted on paper towels to remove excess moisture prior to weighing. Once length and wet weight measurements were completed each single fish were then placed into labelled open pans and placed in a 60°C drying oven for 60 hours. The dry weights of the individual fish were measured to ± 1 mg using an analytical balance.

II. Results and Discussion

Validity criteria were not assessed as part of the study report.

The mean measured concentrations of ^{14}C-KWG 4168 during the test were 3.65, 7.41, 14.2, 28.9, 61.8, and 119 µg a.s./L. These mean values ranged from 94 - 103 % of nominal during the total test period and for all test levels. All reported results are related to the mean measured concentrations of test substance.

The stability of ^{14}C-KWG 4168 in the prime stock solution, used for preparing of all 5 series of stock solutions with acetone, was >99% during the study. The stability of ^{14}C-KWG 4168 in the fifth series of stock solutions with acetone was >98.8%. The stability of ^{14}C-KWG 4168 in water samples from the aquaria had an overall mean of 95% during the study. Thus, ^{14}C-KWG 4168 was stable under test conditions.

Table CA 8.2.2.1/02-1 Analytical measurements of ^{14}C-KWG 4168 in test water

Nominal test concentration (µg a.s./L)	Mean measured concentration (µg a.s./L)	Mean	SD	Measured (X) concentration in %	Measured (X) concentration in µg/L
3.75	5.237 5.226 5.312	5.258	0.038	98	3.66
7.5	5.33 5.091 5.38	5.267	0.126	98	7.34
15	4.632 5.198 5.364	5.065	0.313	94	14.11
30	5.167 4.989 4.952	5.036	0.094	94	28.07
120	5.243 5.072 5.213	5.176	0.075	96	115.39

Nominal test concentration (µg a.s./L)	Mean measured concentration (µg a.s./L)	Mean	SD	Measured (X) concentration in %	Measured (X) concentration in µg/L
Lab recovery spikes (before test series)	564.665 529.227 535.91	538.301	12.119	-	-
Lab recovery spikes (after test series)	537.329 531.566 561.107				

Fry survival was analysed on study day 71 (post-hatch day 36). On post-hatch day 36, fry survival ranged from 95 percent to 100 percent: Control (95 %), Solvent Control (98 %), 3.65 µg/L (100 %), 7.41 µg/L (100 %), 14.2 µg/L (100 %), 28.9 µg/L (100 %), 61.8 µg/L (97 %), and 119 µg/L (98 %). There was no statistical difference in percent hatchability in any test treatment compared to the pooled control data.

Fry survival was again analysed on study day 96 (post-hatch day 61; study termination). On post-hatch day 61, fry survival ranged from 95 percent to 100 percent: Control (95 %), Solvent Control (98 %), 3.65 µg/L (100%), 7.41 µg/L (100%), 14.2 µg/L (98%), 28.9 µg/L (100 %), 61.8 µg/L (97 %), and 119 µg/L (98 %). There was no statistical difference in percent hatchability in any test treatment compared to the pooled control data.

Percent egg hatchability was evaluated on study day 38 (post-hatch day 3). Hatch data, corrected for viability, ranged from 93 percent to 100 percent: Control (98 %), Solvent Control (97 %), 3.65 µg/L (98 %), 7.41 µg/L (93 %), 14.2 µg/L (93 %), 28.9 µg/L (96 %), 61.8 µg/L (100 %), and 119 µg/L (95 %). There was no statistical difference in percent hatchability in any test treatment compared to the pooled control data.

Table CA 8.2.2.1/02-2 Mean egg hatchability and fry survival with exposure to ^{14}C-KWG 4168

Mean measured test concentration (µg a.s./L)	Post-hatch day 3 (study day 38)		Post-hatch day 36 survival (%)	Post-hatch day 61 (study termination)
	Mean egg hatch (%)[1]	Mean post-hatch day 34 survival (%)	Survival (%)	Survival (%)
Control	89	98	95	95
Solvent control	89	97	98	98
3.65	91	98	100	100
7.41	84	93	100	100
14.2	84	93	100	98
28.9	86	96	100	100
61.8	93	100	97	97
119	86	95	98	98

Fry growth, expressed as standard length, was measured on study day 71 (post-hatch day 36). Analysis of data showed a significant difference from the pooled controls in the 61.8, and 119 µg/L test levels. Fry growth, expressed as standard length, was again measured on study day 96 (post-hatch day 61; study termination). Analysis of data showed a significant difference from the pooled controls in the 28.9, 61.8, and 119 µg/L test levels.

Fry growth, expressed as dry weight, was measured on study day 96 (post-hatch day 61; study termination). Analysis of data showed a significant difference from the pooled controls in the 61.8, and 119 µg/L test levels.

The biomass loading factor for the study was determined using the wet weights of the control and solvent control fish at study termination. The mean wet weight was 346 mg/fish in the pooled controls. The biomass loading factor based upon the 7.5 litre volume of a single growth chambers was 692 mg fish per litre. The biomass loading factor based upon a flow of 90 litres per day (12 changes of test solution

per day) through each single test chamber was 58 mg fish per litre and day. These loads were well within the requirements to ensure adequate dissolved oxygen levels and to avoid crowding of fish.

Table CA 8.2.2.1/02-3 Mean standard length, wet and dry weight of Rainbow trout (*Oncorhynchus mykiss*) exposed to 14C-KWG 4168 in an early life-stage toxicity study

Mean measured test concentration (µg a.s./L)	Post-hatch day 36 mean length (mm)	Post-hatch day 61		
		Mean length (mm)	Mean wet weight (mg)	Mean dry weight (mg)
Control	26.4	30.9	331	55.4
Solvent control	25.9	31.6	361	60.3
Pooled control	26.1	31.2	346	57.8
3.65	26.6	31.5	-	55.0
7.41	27.0	31.7	-	56.2
14.2	27.4	31.6	-	57.2
28.9	26.3	29.9*	-	50.7
61.8	25.0*	29.0*	-	50.0
119	22.2*	27.3*	-	46.1*

*denotes statistically significant difference (Williams test) from pooled controls

Time to hatch was evaluated for all test levels. Egg hatching began on study day 33 and continued until day 38. The mean percent hatch on study day 38 ranged from 98 percent to 100 percent. Control (98 %), Solvent Control (100 %), 3.65 ug/L (100 %), 7.41 ug/L (100 %), 14.2 ug/L (100%), 28.9 ug/L (100%), 61.8 ug/L (100%), and 119 ug/L (100%). There was no statistical difference in time to hatch in any test treatment compared to the pooled control data.

Table CA 8.2.2.1/02-4 Mean time to hatch for Rainbow trout (*Oncorhynchus mykiss*) eggs during the ^{14}C-KWG early life-stage toxicity study

Mean measured test concentration (µg a.s./L)	Percent hatched[1)] study day					
	33	34	35	36	37	38
Control	0	11	96	98	98	98
Solvent control	0	10	93	100	100	100
3.65	0	2	90	100	100	100
7.41	1	37	96	99	100	100
14.2	0	14	90	99	100	100
28.9	0	21	90	99	100	100
61.8	0	5	92	99	100	100
119	0	2	93	97	100	100

[1] Percent hatch = [# of alevin] /(# of alevin + # of eggs) on Day 38, post-hatch day 3] * 100

Newly hatched fry began swimming up from the bottom of the test chambers on study day 50 (post-hatch day 15). Swim-up was observed for a 7 day period between study day 50 and 57. A >95% swim-up was achieved on day 55 in the pooled controls (control: 96 percent; solvent control: 95 percent). For all other treatment levels on day 55 a swim-up between 91 and 100 percent was reached. There was no statistical difference in time to swim up in any test treatment compared to the pooled control data.

Table CA 8.2.2.1/02-5 Mean percent swim-up of newly hatched rainbow trout (*Oncorhynchus mykiss*) fry during the 14C-KWG 4168 early-life stage toxicity study

Mean measured test concentration (µg a.s./L)	Percent hatched[1)] study day							
	50	51	52	53	54	55	56	57
Control	3	5	20	20	56	96	100	97
Solvent control	3	15	32	42	92	95	98	98
3.65	0	15	30	35	73	98	98	98
7.41	3	20	45	40	87	100	100	100
14.2	3	5	23	35	68	100	100	100
28.9	2	12	33	45	90	98	98	98
61.8	3	8	17	32	73	100	100	100
119	0	7	17	27	59	91	100	100

Egg viability was checked 12 days after fertilisation with the 200 additional eggs (50 per incubation cup) that were placed in separate egg incubation cups at test initiation. The viability determination (fertilisation success) in the four replicates ranged from 86 to 94 percent with a mean of 89.5 percent.

Table CA 8.2.2.1/02-6 Rainbow trout (*Oncorhynchus mykiss*) embryo viability during the 14C-KWG 4168 early life-stage toxicity study

Total eggs in egg cup	Number of viable eggs	Number of nonviable eggs	Percent viability
50	47	3	94
50	43	7	86
50	43	7	86
50	46	4	92
Mean:			89.5

III. Conclusion

Based on the statistical analysis of survival, egg hatchability, time to hatch, time to swim-up, and growth (expressed as weight and length), the no-observed-effect-concentrations (NOECs) and the lowest-observed-effect-concentrations (LOECs) were determined as follows. All test levels listed are based on mean measured concentrations (3.65, 7.41, 14.2, 28.9, 61.8 and 119 µg a.s./L) of the test substance:

Survival at day 71 (post-hatch day 36) resulted in a NOEC at ≥119 µg a.s./L and the LOEC at >119 µg a.s./L.

Survival at 96 (post-hatch day 61) resulted in a NOEC at ≥119 µg a.s./L and the LOEC at >119 µg a.s./L.

Egg hatchability at day 38 resulted in a NOEC at ≥119 µg a.s./L and the LOEC at >119 µg a.s./L.

Time to hatch at day 38 resulted in a NOEC at ≥119 µg a.s./L and the LOEC at >119 µg a.s./L.

Time to swim-up at day 55 resulted in a NOEC at ≥119 µg a.s./L and the LOEC at >119 µg a.s./L.

Growth at day 71 (post-hatch day 36), expressed as standard length, resulted in a NOEC at 28.9 µg a.s./L and the LOEC at 61.8 µg a.s./L.

Growth at day 96 (post-hatch day 61), expressed as standard length, resulted in a NOEC at 14.2 µg a.s./L and the LOEC at 28.9 µg a.s./L.

Growth at day 96 (post-hatch day 61), expressed as dry weight, resulted in a NOEC at 28.9 µg a.s./L and the LOEC at 61.8 µg a.s./L.

The lowest effect threshold (geometric mean of NOEC and LOEC) for ^{14}C-KWG 4168 was based on the most sensitive endpoint, the fry growth, expressed as standard length, on study day 96 (post hatch day 61). So, the lowest effect threshold in this study was calculated to be 20.3 µg test substance/L.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 210 (1992), the most up-to-date version of which is the "Fish, early-life stage toxicity test", adopted 26 July 2013.

Validity criteria according to the most recent OECD 210 guideline (2016) were met:

- Dissolved oxygen concentration to be ≥60% of the air saturation value throughout the test (actual: 95 to 98%)
- Overall survival of fertilised eggs and post-hatch success in the controls to be ≥75% (95 and 98%, in the control ad solvent control, respectively)
- Water temperature to not differ by more than ±1.5°C between test chambers or between successive days at any time during the test. The temperature range for the duration of the test was 1.6°C, daily temperatures were not reported, however, it can be assumed that the criteria was met based on the temperature range for the entire test period.

The validity criteria according to the current test guideline have been met therefore this study is considered to be valid.

Growth at day 96 (post-hatch day 61), expressed as standard length, resulted in the lowest NOEC at 14.2 µg a.s./L.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.2.2.1/05
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10 and EC20 values for Oncorhynchus mykiss with 14C spiroxamine TG in an early life stage study
Report No:	0471836-ECO3
Document No:	M-760403-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	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 report (M-006449-02-1) on the effects of ^{14}C Spiroxamine TG in the rainbow trout (*Oncorhynchus mykiss*) early life stage study did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013. Effect Concentrations with a 10% and 20% effect on length when compared to the pooled controls were re-calculated. As there were no effects (<10%) on survival, hatchability, % swim up and time to hatch, with a lack of dose response ECx values were estimated to be >119.00 µg a.s./L. Although ECx values and 95% confidence intervals were calculated for the parameter dry weight, as confidence intervals were large, spanning more than 2 concentrations and the dose response curve is not covered by the data, it was not possible to determine reliable ECx values.

The resulting EC_{10}, and EC_{20} values for length at 36dph were 96.73 (95%CL: 91.75 – 100.84) and 138.15 (95%CL: 131.86 – 146.92) µg a.s./L, respectively. The resulting EC_{10}, and EC_{20} values for length at 61dph were 91.46 (95%CL: 72.83 – 114.65) and 195.28 (95%CL: 146.45 – 369.35) µg a.s./L, respectively. Although the EC_{10} values are considered reliable as the criteria for goodness of fit were met, as effects were <20%, the EC_{20} cannot be considered reliable as it was calculated based on extrapolation.

I. Methods

The statistical evaluation was performed with statistical software ToxRat Standard v3.3.0.

Effect concentrations with 10 and 20% from the test item treatment when compared to the pooled controls were calculated but due to lack of effects and the non-existence of a dose response these could not be determined for some parameters. EC_{10} and EC_{20} values could only be determined for dry weight at 61dph and length at 36 and 61 dph. For the dry weight at 61 dph a Probit function using linear maximum likelihood regression was used along with 95% EC_x confidence limits to calculate EC_{10} and EC_{20} values. For the length at 36 and 61 dph, a Logit function using linear maximum likelihood regression was used along with 95% EC_X confidence limits to calculate EC_{10} and EC_{20} values.

II. Results and Discussion

A complete list of the obtained endpoints is presented below. A more detailed explanation is given for regression analysis endpoints for total length and dry weight. These details can be found below. For the parameters survival, hatchability, time to hatch and % swim up, no statistical calculations were possible as no effects were observed.

Dry weight at 61 days post hatch (dph)

Regarding the calculation of EC_{10} and EC_{20} values for dry weight at 61 dph, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.00, showing no significant deviation between fit and data, and a statistically significant concentration/response was found ($p(F) = 0.019$) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table.

Table CA 8.2.2.1/05-1 Results of the Probit analysis (max. likelihood regression) with dry weight at 61 dph: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (according to Fieller's theorem)

Parameter	Dry weight	
	EC_{10} (95% confidence interval) [µg a.s./L]	EC_{20} (95 % confidence interval) [µg a.s./L]
Effect on dry weight at 61 dph	33.94 (4.84 – 61.93)	115.92 (63.47 – 979.11)

The resulting EC_{10} and EC_{20} values of 33.94 (95% CL: 4.84 – 61.93) and 115.92 (95% CL: 63.47 – 979.11) µg a.s./L, respectively, meet the goodness of fit criteria. However, as the EC_x confidence intervals span more than 2 concentrations and the dose response curve is not covered by the data (as effects were between 1.0% and 20.3%), the EC_{10} value is not considered reliable for use within a risk assessment.

Total length at 36 dph

Regarding the calculation of EC_{10} and EC_{20} values for total length at 36 dph, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.00, showing no significant deviation between fit and data, and a statistically significant concentration/response was found ($p(F) < 0.001$) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table and figure below.

Table CA 8.2.2.1/05-2 Results of the Logit analysis with total length at 36 dph: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (according to Fieller's theorem)

Parameter	Total length	
	EC_{10} (95 % confidence interval) [µg a.s./L]	**EC_{20} (95 % confidence interval) [µg a.s./L]**
Effect on total length at 36 dph	96.73 (91.75-100.84)	138.15 (131.86-146.92)

The resulting EC_{10} and EC_{20} values of 96.73 (95% CL: 91.75 – 100.84) and 138.15 (95% CL: 131.86 – 146.92) µg a.s./L, respectively, meet the goodness of fit criteria and therefore the estimated EC_{10} value is considered reliable for use in the risk assessment.

Total length at 61 dph

Regarding the calculation of EC_{10} and EC_{20} values for total length at 61 dph, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.00, showing no significant deviation between fit and data and a statistically significant concentration response was found ($p(F) = 0.003$) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table below.

Table CA 8.2.2.1/05-3 Results of the Logit analysis (max. likelihood regression) with total length at 61 dph: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (according to Fieller's theorem)

Parameter	Total length	
	EC_{10} (95 % confidence interval) [µg a.s./L]	**EC_{20} (95 % confidence interval) [µg a.s./L]**
Effect on total length at 61 dph	91.46 (72.83-114.65)	195.28 (146.45-369.35)

The resulting EC_{10} and EC_{20} values of 91.46 (95% CL: 72.83 – 114.65) and 195.28 (95% CL: 146.45 – 369.35) µg a.s./L, respectively, meet the goodness of fit criteria and therefore the estimated EC_{10} value is considered reliable.

A summary of the obtained endpoints is presented in the following table.

Table CA 8.2.2.1/05-4 Overall endpoints of the statistical re-calculation of the *Oncorhynchus mykiss* study with spiroxamine

Parameter	Endpoint (µg a.s./L)
	EC_{10} (95% confidence intervals)
Survival 36 dph	>119.00
Survival 61 dph	>119.00
Dry weight 61 dph	n.d.
Length 34 dph	96.73 (91.75-100.84)
Length 61 dph	91.46 (72.83-114.65)
% swim up	>119.00
Hatchability	>119.00
Time to hatch	>119.00

The calculation of EC_{10} and EC_{20} values for dry weight at 61 dph did not generate reliable values, therefore no EC_{10} and EC_{20} values are presented. Due to the lack of effects above 10% when compared to the controls, the calculation of EC_{10} and EC_{20} values for hatchability, % swim up, hatchability and

time to hatch was not possible. The EC_{10} and EC_{20} values for hatchability, % swim up, hatchability and time to hatch are therefore estimated to be above the highest tested rate of 119.00 µg a.s./L.

III. Conclusion

The resulting EC_{10}, and EC_{20} values for length at 36 dph were 96.73 (95% CL: 91.75 – 100.84) and 138.15 (95% CL: 131.86 – 146.92) µg a.s./L, respectively. The resulting EC_{10}, and EC_{20} values for length at 61 dph were 91.46 (95% CL: 72.83 – 114.65) and 195.28 (95% CL: 146.45 – 369.35) µg a.s./L, respectively. Although the EC_{10} values are considered reliable as the criteria for goodness of fit were met, as effects were <20%, the EC_{20} cannot be considered reliable as it was calculated based on extrapolation. The calculation of EC_{10} and EC_{20} values for dry weight at 61 dph did not generate reliable values, therefore no EC_{10} and EC_{20} values were determined. The EC_{10} and EC_{20} values for hatchability, % swim up, hatchability and time to hatch were estimated to be above the highest tested rate of 119.00 µg a.s./L.

Assessment and conclusion by applicant:

The statistical re-evaluation of the data has determined EC_{10} and EC_{20} values for some of the parameters assessed but the EC_{20} values are largely based on extrapolation and are therefore not considered suitable for use in the risk assessment.

The lowest EC_{10} value of 33.9 µg a.s./L was determined for dry weight but has been considered to be unsuitable for risk assessment due to the wide confidence intervals has not been considered suitable for use in the risk assessment.

The NOEC of 14.2 µg a.s./L from the original study report shall remain the critical endpoint determined for this study.

The values determined in the re-evaluation work are considered to be fully valid.

Data Point:	(KCA 8.2.2.1/03)
Report Author:	[redacted]
Report Year:	2008
Report Title:	Effects of spiroxamine technical on selected early life stages of rainbow trout (Oncorhynchus mykiss) in a static water/sediment system
Report No:	EBKWX092
Document No:	M-304369-01-1
Guideline(s) followed in study:	FIFRA Guideline 72-4; OPPTS Guideline 850.1400 (draft); OECD Guideline 210
Deviations from current test guideline:	Yes Methods: SANCO/3029/99 rev. 4 Accuracy n<5 Some fortification levels have no precision The calibration curve only has 4 data points Ecotoxicology: OECD 210 (2013) The test follows a non-standard exposure regime There were only two replicates per concentration rather than the minimum four specified
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

This study was designed to evaluate the chronic toxicity on the most sensitive early life stages of rainbow trout (*Oncorhynchus mykiss*) (swim-up phase, growth phase) exposed to spiroxamine under

consideration of more realistic exposure conditions. This was achieved by using a static water/sediment system that used three pulsed application times. For this purpose fish were exposed in a static water/artificial sediment system using three pulsed application times to a control, solvent control and initial nominal pulse concentrations of 60.0, 180, 540, 1620 and 4860 µg a.s./L.

Each test concentration had two replicates of 20 eyed eggs, which were thinned to 15 alevin after the hatching phase. The test duration was 56 days.

Sub-lethal effects were observed in the two highest test concentrations, 1620 and 4860 µg a.s./L. Fry survival was significantly reduced compared to the controls in the 4860 µg a.s./L test concentration.

At test termination, fish length and weight were significantly reduced in the 180, 540 and 1620 µg a.s./L test concentrations. No measurements were made in the 4680 µg a.s./L test concentration due to mortality.

The overall chronic NOEC and LOEC for the most sensitive early life stage of rainbow trout (*Oncorhynchus mykiss*) exposed to spiroxamine in a static water/artificial sediment system were 60.0 and 180 µg a.s./L, based on the effects observed on fish length and weight.

I. Materials and Methods

A. Materials

Test Material	Spiroxamine technical
Lot/Batch #:	EDTH004650
Purity:	97.0%
Description:	Light brown oil
Stability of test compound:	Stable at 25 ± 5°C until the expiry date
Reanalysis/Expiry date:	02 August 2009
Density:	Not reported
Treatments	
Test rates:	60.0, 180, 540, 1620 and 4860 µg a.s./L
Solvent/vehicle:	100 µL/L acetone
Analysis of test concentrations:	Yes, measured test concentrations 69 – 126% of initial nominal in the nominally 60.0, 180 and 540 µg a.s./L concentrations, and 122 – 408% in the nominally 1620 and 4860 µg a.s./L concentrations
Test organisms	
Species:	Rainbow trout (*Oncorhynchus mykiss*)
Source:	[redacted]
Acclimatisation period:	Eggs acclimated from 0.5°C to 9.9°C over approx. 4 hours
Feeding:	Brine shrimp (*Artemia salina*) starting on study day 22.
Treatment for disease:	None reported
Test design	

Test vessel:	34.3 x 21.6 x 30.5 cm 22-L glass aquaria with a 3 cm sediment layer and a 21.3 cm deep water column containing 15.8 L test medium
Test medium:	Soft blended water (spring water blended with RO water)
Replication:	Two replicates
No. of animals/vessel:	20 eyed eggs at experimental start, thinned to 15 alevin after hatching phase
Duration of test:	56 days
Environmental test conditions	
Temperature:	10.7 – 11.5°C
Dissolved oxygen:	9.4 – 11.0 mg/L (85 – 100% saturation)
pH:	7.6 – 8.0
Photoperiod:	16 h light, 8 h dark with 30 minute transition period at 627 to 860 lux (mean 747 lux) Developing embryos/larvae were shielded from light exposure until one week post hatch.

B. Study Design

This study was designed to evaluate the chronic toxicity on the most sensitive early life stages of rainbow trout (*Oncorhynchus mykiss*) (swim-up phase, growth phase) exposed to spiroxamine under consideration of more realistic exposure conditions.

Due to the fast dissipation of spiroxamine from the water phase and ELS study under flow-through conditions would be unrealistic, therefore a special test design was followed.

For this purpose fish were exposed in a static water/artificial sediment system using three pulsed application times to a control, solvent control and initial nominal pulse concentrations of 60, 180, 540, 1620 and 4860 µg a.s./L.

Test sediment was prepared based on OECD 219 (draft) and constituted 4% finely ground air-dried sphagnum peat moss, 76% white quartz (SiO_2) sand and 20% kaolinite clay. Calcium carbonate was added to the mixture to obtain a pH value of the 6.5 to 8.0. Approximately 2.5 kg dry sediment was added to each test vessel to give an approximate sediment layer of 3 cm. Test vessels were filled to a depth of 24.3 cm, a water volume of 15.8 L. Formulated sediment was conditioned for eight days under test conditions prior to use in the study.

Serially diluted stock solutions were added to the aquaria in 10-day intervals on study days 22, 32 and 42.

Test solutions were prepared by adding 1.58 mL of the relevant stock solution into the upper water phase of the corresponding aquaria and gently stirring. Test solutions had a solvent load of 0.1 mL/L. Solvent controls were prepared by adding 1.58 mL to the upper water phase, and the control was prepared by gently stirring.

On study day 0, 20 eggs were impartially placed in each egg cup and transferred to test vessels. Eggs were observed daily for mortality and hatching, with any non-viable eggs removed.

On post-hatch day 3, alevin were impartially thinned to 15 per replicate, and were observed daily for hatchability, abnormal behaviour, physical changes, swim-up behaviour and mortality. Growth expressed as standard length and dry weight was measured at test termination on day 56.

Fish were fed brine shrimp (*Artemia sakina*) starting on study day 22 at least once daily on weekends and holidays and at least two times daily on weekdays until approximately 24 hours prior to study termination.

Analytical method

Samples of water were analysed using the validated analytical method M-304369-01-1, report reference M-304369-01-1 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the most up-to-date OECD 210 guideline (2013) were met:

- Dissolved oxygen concentrations to be greater than 60% of the air saturation value throughout the test (actual: 85 to 100%)
- Water temperature to be 10 ± 1.5°C (actual: 10.7 to 11.5°C)
- Overall hatching success in the controls to be at least 75% (actual: 95 to 100% across all groups)
- Overall post-hatch survival in the controls to be at least 75% (actual: 97 and 97%)

Initial measured concentrations on application days ranged from 69 to 126% of nominal for the test concentrations 60, 180 and 540 µg a.s./L. In the two highest test concentrations, 1620 and 4860 µg a.s./L, unexpectedly high spiroxamine concentrations of 122 and 408% of nominal were found.

It appeared that spiroxamine bound quickly to the suspended solids in water and was extracted out of the suspended solids together with spiroxamine from the water. As a result, spiroxamine was found higher than the nominal concentrations. This was not noted at the lower treatment levels. Stock solution results of all test levels ranged from 87 to 105% of nominal values and were therefore within acceptable ranges. As expected from the physicochemical properties, spiroxamine disappeared rapidly from the water body of the test system. The calculated DT_{50} was 3.5 days. The toxicity values were calculated based on initial nominal concentrations.

Table CA 8.2.2.1/03-1 Measured concentrations of spiroxamine in stock solution

Study day	Nominal concentration (µg/L)				
	60	180	540	1620	4860
22	94	98	100	103	97
32	96	93	100	91	105
42	95	92	90	98	87

Table CA 8.2.2.1/03-2 Measured concentrations of spiroxamine in test medium

Study day	Nominal concentration (µg/L)						
	Control	Solvent control	60	180	540	1620	4860
22	<LOQ <LOQ	<LOQ <LOQ	60.9 59.4	151 146	370 438	2662 3320	11613 10310
32	<LOQ <LOQ	<LOQ <LOQ	67.6 66.2	159 151	486 560	1982 2473	7769 8173
42	<LOQ <LOQ	<LOQ <LOQ	59.4 64.5	191 176	600 681	3785 4689	12822 19831
Mean % of nominal [1]	-	-	105	90.2	96.8	194.6	241.8
Range % of nominal	-	-	99 - 113	81 - 106	69 - 126	122 – 290[a]	160 – 408[a]

Calculations were made using unrounded data

[1] Calculated using rounded data

[a] In the two highest test levels unexpectedly high spiroxamine concentrations were found

The results have been presented based on nominal test concentrations.

Time to hatch was recorded for all test levels from study day 7 until study day 13 (post hatch day 3). The post-hatch period began after 90% of all living eggs in the controls had hatched. Percent hatch was not statistically analysed as all test levels were untreated at the time of hatch. Hatching success ranged from 95 to 100%.

From study day 14 onwards observations of fish were made with reservations due to the turbidity of the water caused by the fish stirring up the sediment. In the highest test concentration, 4860 µg a.s./L fish were on the bottom, and showed a loss of equilibrium and dark colouration. Fish in the 1620 µg a.s./L showed a dark colouration.

Time to swim up could not be quantified due to the high turbidity of the water.

Fry survival on day 56 in the control, solvent control and 60, 180, 540, 1620 and 4860 µg a.s./L test concentrations was 97, 97, 97, 100, 97, 93 and 0% respectively. A statistically significant reduction in fry survival compared to the control was found in the 4860 µg a.s./L test concentration.

Table CA 8.2.2.1/03-3 Fry survival of rainbow trout after 56 days exposure to spiroxamine

Initial nominal concentration (µg a.s./L)	Fry survival (%)
Control	97
Solvent control	97
60	97
180	100
540	97
1620	93
4860	0*

* Significantly different ($p \leq 0.05$) from the controls

At test termination (study day 56) fish were sacrificed and measured for standard length and dry weight. No measurements were made in the highest test concentration, 4860 µg a.s./L, as all fish had died by test termination. The mean lengths of surviving fish ranged from 25.3 to 38.1 mm. Length was significantly reduced from the pooled controls at test concentrations 180, 540 and 1620 µg a.s./L. The mean dry weights of fish ranged from 49.6 to 114.8 mg. Dry weight was significantly reduced from the pooled controls at test concentrations 180, 540 and 1620 µg a.s./L.

Table CA 8.2.2.1/03-4 Effects on length and weight of rainbow trout after 56 days exposure to spiroxamine

Initial nominal concentration (µg a.s./L)	Standard length (mm)	Dry weight (mg)
Control	38.1	113.6
Solvent control	37.5	114.0
60	36.6	114.8
180	31.9*	92.1*
540	28.4*	90.7*
1620	25.3*	49.6*
4860	[a]	-[a]

[a] All fish had died prior to test termination

* Significantly different ($p \leq 0.05$) from the controls

III. Conclusion

This study was designed to evaluate the chronic toxicity on the most sensitive early life stages of rainbow trout (*Oncorhynchus mykiss*) (swim-up phase, growth phase) exposed to spiroxamine under consideration of more realistic exposure conditions. This was achieved by using a static water/sediment system that used three pulsed application times. For this purpose fish were exposed in a static water/artificial sediment system using three pulsed application times to a control, solvent control and initial nominal pulse concentrations of 60, 180, 540, 1620 and 4860 µg a.s./L.

Each test concentration had two replicates of 20 eyed eggs, which were thinned to 15 alevin after the hatching phase. The test duration was 56 days.

Sub-lethal effects were observed in the two highest test concentrations, 1620 and 4860 µg a.s./L. Fry survival was significantly reduced compared to the controls in the 4860 µg a.s./L test concentration.

At test termination, fish length and weight were significantly reduced in the 180, 540 and 1620 µg a.s./L test concentrations. No measurements were made in the 4680 µg a.s./L test concentration due to mortality.

The overall chronic NOEC and LOEC for the most sensitive early life stage of rainbow trout (*Oncorhynchus mykiss*) exposed to spiroxamine in a static water/artificial sediment system were 60 and 180 µg a.s./L, based on the effects observed on fish length and weight.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 210, the most up-to-date version of which is the "Fish, early-life stage toxicity test", adopted 26 July 2013.

Validity criteria according to the most up-to-date OECD 210 guideline (2013) were met:

- Dissolved oxygen concentrations to be greater than 60% of the air saturation value throughout the test (actual: 85 to 100%)
- Water temperature to be 10 ± 1.5°C (actual: 10.7 to 11.5°C)
- Overall hatching success in the controls to be at least 75% (actual: 95 to 100% across all groups)
- Overall post-hatch survival in the controls to be at least 75% (actual: 97 and 97%)

The study is considered acceptable on the basis that the validity criteria for OECD 210 were achieved. It is noted that this study used a non-standard test design by adopting a pulsed exposure regime and by incorporating sediment. The endpoint achieved in this study is not considered suitable for use directly in the risk assessment but is considered to provide supporting information for the risk assessment.

The overall chronic NOEC for the most sensitive early life stage of rainbow trout (*Oncorhynchus mykiss*) exposed to spiroxamine in a static water/artificial sediment system was 60 µg a.s./L.

CA 8.2.2.2 Fish full life cycle test

Data Point:	KCA 8.2.2.2/01
Report Author:	[redacted]
Report Year:	2009
Report Title:	Zebra fish (Danio rerio), life cycle test, flow through conditions
Report No:	47758001
Document No:	M-304458-02-1
Guideline(s) followed in study:	-OECD Guideline for Testing of Chemicals, 210 Fish, Early Life Stage Toxicity Test, 1992 -OECD Guideline for Testing of Chemicals, 215 Fish, Juvenile Growth Test, 2000 -OECD "Draft Proposal for a new Guideline: Fish Two-generation Test", 2002. .-EPA-FIFRA § 72-5/SEP-EPA-540/9-86-137 "Standard Evaluation Procedure: Fish Life-Cycle Toxicity Tests"(OPPTS 850.1400), 1986 - Nagel, R. (1998): Der vollständige Life Cycle Test (Complete Life Cycle Test, CLC Test) mit dem Zebrabärbling (Danio rerio, vormals Brachydanio rerio), Entwuf. UBA-Texte 58/98
Deviations from current test guideline:	Yes Methods: SANCO/3029/99 rev. 4 Accuracy n=4 Ecotoxicology: OECD 210 (2013) and OECD 215 (2000) It was not reported if flow rates were checked to not vary by >10% Water was not held at 26 ± 1.5°C, instead at 23.7 – 27.8°C Juvenile fish were not weighed to determine detection of a minimum variation of significant growth rate Frequency of feeding was not reported Dilution water had exceedances in iron, copper and zinc concentrations Amended by additional endocrine test parameters and endpoints Mean measured concentrations ranged in week 4 between 31 – 77 %
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017) The overall NOEC for the FFLC test is the EC10 for the survival observed in the F1-ELS of 2.0 µg as/L and should be compared to the PECSW max.
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

This was a fish full life cycle study conducted to examine the potential for long-term adverse effects of spiroxamine exposure to populations of zebrafish (*Danio rerio*).

Five test concentrations were investigated, each with four replicates of initially 100 fertilised eggs. The effects of exposure on different life stages of the zebrafish, including early life stages, juvenile growth, reproduction and early life stages of the filial generation were assessed under continuous exposure.

At the early life stage, no direct effects on hatching could be found, however survival and growth had a NOEC of 6.4 µg a.s./L.

At the juvenile growth stage, the NOEC values for survival, length and 'pseudo' specific growth rate were 16, 6.4 and 6.4 µg a.s./L, respectively.

At the adult stage, the NOEC values for eggs/female/day, cumulative egg number, fertilisation rate, survival and sex ratio were 16, 16, 16, 40 and 6.4 µg a.s./L, respectively. NOEC values for male length, female length, male weight and female weight were 6.4, 16, 40 and 16 µg a.s./L, respectively. Biomarker NOEC values for male and female vitellogenin content were 40 and 2.6 µg a.s./L, respectively, and for histology were 16 and 6.4 µg a.s./L in males and females, respectively.

In the early life stage of the filial generation, population NOEC values for hatching and survival were 16 and 2.6 µg a.s./L, and growth NOEC values for length and weight were both 16 µg a.s./L.

Based on the most sensitive endpoint, survival of the F_1 generation, the overall NOEC was found to be 2.6 µg a.s./L.

I. Materials and Methods

A. Materials

Test Material	Spiroxamine
Lot/Batch #:	AE 1344293-01-01
Purity:	97%
Description:	Light brown oil
Stability of test compound:	Stable at pH 7 and 9 and 25°C with half-life >1 year
Reanalysis/Expiry date:	02 August 2009
Density:	No information
Treatments	
Test rates:	2.6, 6.4, 16, 40 and 100 µg spiroxamine/L
Solvent/vehicle:	None
Analysis of test concentrations:	Yes, 92 – 101% of nominal
Test organisms	
Species:	Zebrafish (*Danio rerio*). Fertilised eggs, four cells used for the study were collected from a glass spawning tray placed at the bottom of the adult holding vessels
Source:	[redacted]
Acclimatisation period:	n/a
Feeding:	Larvae were fed daily *ad libitum* from day 6 onwards with finely ground breeding food (TetraMin Baby). From day 9 onwards brine shrimp nauplii (*Artemia salina*) were added *ad libitum*, and from day 16 onwards ground TetraMin flake food were added *ad libitum*.
Treatment for disease:	None reported
Test design	
Test vessel:	Glass 40 x 28 x 28 cm 28-L aquaria containing approx. 25 L solution
Test medium:	Purified drinking water
Replication:	Four replicates
No. of animals/vessel:	100 fertilised eggs per replicate, later reduced to 50 fish and then 30
Duration of test:	56 days

Environmental test conditions	
Temperature:	24.0 – 27.8°C (four measurements recorded temperatures <24.0°C, with a minimum recorded of 23.7°C)
Dissolved oxygen:	68 – 102%
pH:	7.4 – 8.6
Photoperiod:	12 h light : 12 h darkness

B. Study Design

This was a fish full life cycle study conducted to examine the potential for long-term adverse effects of spiroxamine exposure to populations of zebrafish (*Danio rerio*). The effects on different life stages of the zebrafish, including early life stages, juvenile growth, reproduction and early life stages of the filial generation were assessed under continuous exposure.

Test vessels were glass 28-litre aquaria of 40 x 28 x 28 cm containing approximately 25 litres test solution. Test solution was prepared in a mixing chamber by the addition of relevant amounts of stock solution to purified drinking water, with a daily water exchange rate of approximately five volumes. The flow-through system was served by test solutions at least 24 hours prior to the additions of the eggs.

The study was conducted using nominal concentrations of 2.6, 6.4, 16, 40 and 100 µg spiroxamine/L and a control, each with four replicates under flow-through conditions. Exposure was started with 100 fertilised eggs per vessel and replicate. Larvae were fed daily *ad libitum* from day 6 onwards with finely ground breeding food (TetraMin Baby). From day 9 onwards brine shrimp nauplii (*Artemia salina*) were added *ad libitum*, and from day 16 onwards ground TetraMin flake food were added *ad libitum*.

After 28 days, the fish number was randomly reduced to 50 per replicate for investigation of juvenile growth. Fish were photographed on days 28 and 56 to determine survival and growth.

After 56 days, fish numbers were reduced to 30 per replicate for the investigation of reproduction. After the last reduction to 30 individuals, glass spawning trays were introduced and monitored daily for spawned eggs. The time until first findings of eggs was recorded. Egg production per female per day and fertilisation rate was measured.

All fish were measured for length and weight. A blood sample was taken and the biomarker vitellogenin was measured for a subsample of five males and five females per replicate, if possible. In all replicates of the control and 2.6 µg a.s./L test concentration all male fish were measured for vitellogenin content.

To confirm the macroscopic sex determination, all gonads were histologically inspected, with a detailed examination performed on five male and five female fish per replicate, if possible. Blood samples of the same fish were analysed for vitellogenin.

To start the F_1 generation, 100 fertilised eggs per test vessel were placed in stainless steel fry chambers. Larvae were fed daily *ad libitum* with finely ground breeding food (TetraMin Baby). From day 9 onwards brine shrimp nauplii (*Artemia salina*) were added *ad libitum*, and from day 16 onwards ground TetraMin flake food were added *ad libitum*. After 35 days, F_1 fish were sacrificed and measured for length and weight.

Chemical analysis of the test solutions was performed weekly in all test concentrations and replicates. Samples were analysed by HPLC-MS/MS, with a LOQ of 0.8 µg/L. The temperature, pH and oxygen concentration of the water was measured in each aquarium was measured directly before adding the fish and afterwards twice weekly.

Data were analysed for statistical differences as compared with the untreated control by performing ANOVA followed by Williams' or Dunnett's test or respective non-parametric approaches. All statistical tests and probit analysis were conducted using the software ToxRat Professional 2.09.

Analytical method

Samples of water were analysed using the validated analytical method M-304458-02-1, report reference M-304458-02-1 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the study report were achieved:

- Total survival in the control replicates was >70%
- Hatching rate in the control replicates to be ≥70% (actual: 91.1%)
- Dissolved oxygen concentration to be ≥60% in all test vessels throughout the test (actual: 68 102%)
- Water temperatures were kept within 26 ± 2 °C.

The overall mean measured concentrations, determined for each replicate vessel, were between 92 and 101% of the nominal concentrations. Thus, the evaluation of the effect concentrations was based on nominal concentrations of the test item.

The single test media concentrations ranged between 31 and 167% of nominal values. Due to a malfunction of the dosing system, the analytical results for week 4 showed reduced recovery rates of 31 to 82% over all treatment groups. In week 20, a pump malfunction resulted in low test item concentration in two replicates of the 16 µg a.s./L test concentration. A decrease in stock solution and test vessel concentrations could be observed in week 20, 24 and 30, resulting in recoveries of 46 to 77%. All other measurements were between 68 and 167%.

Table CA 8.2.2.2/01-1 Measured concentrations of spiroxamine during the test

	Nominal concentration (µg a.s./L)					
	Control	**2.6**	**6.4**	**16**	**40**	**100**
Mean measured test concentration ±SD						
µg a.s./L	**<LOQ**	2.6 ± 0.1	6.1 ± 0.2	14.9 ± 0.1	38.8 ± 1.4	96.8 ± 3.2
%	**-**	99 ± 2.3	95 ± 2.9	93 ± 0.4	97 ± 3.5	97 ± 3.2

SD Standard deviation
LOQ Limit of quantification: 0.8 µg/L

F_0 generation, early life phase

A slight delay and reduction in hatching could be observed in the 6.4, 16, 40 and 100 µg a.s./L and above compared to the control. This is thought to be due to the suppression on microbiological growth of the egg surface by the active substance, resulting in an increased stability of the egg shell. No negative impact on related endpoints was observed, therefore the finding is regarded as an indirect effect and is not considered to be relevant for the toxicity assessment.

Survival of the hatched larvae as well as the length of the larval fish on day 28 post fertilisation was significantly reduced in the 16, 40 and 100 µg a.s./L test concentrations.

Table CA 8.2.2.2/01-2 Effects of spiroxamine exposure on the early life phase of the F_0 generation

Parameter	**Nominal concentration (µg a.s./L)**					
	Control	**2.6**	**6.4**	**16**	**40**	**100**
Hatching, day 4 (%)	26.8 ± 16.8	16.4 ± 11.2	13.1 ± 7.2	13.9 ± 7.2	10.6 ± 4.8*	6.3 ± 6.4*
Hatching, day 5 (%)	91.1 ± 5.5	90.5 ± 4.4	81.7 ± 5.8*	73.6 ± 12.3*	80.6 ± 10.2*	75.6 ± 6.4*
Survivors, day 14 (%)	92.4 ± 6.2	93.9 ± 3.0	93.4 ± 7.1	88.5 ± 3.8	81.0 ± 8.0*	81.7 ± 2.5*
Survivors, day 21 (%)	86.0 ± 4.8	88.5 ± 5.2	80.1 ± 10.1	69.4 ± 14.4*	24.5 ± 13.5*	7.6 ± 6.8*

Parameter	Nominal concentration (µg a.s./L)					
	Control	2.6	6.4	16	40	100
Survivors, day 28 (%)	79.3 ± 3.4	83.1 ± 6.3	77.6 ± 7.8	65.0 ± 12.2*	14.2 ± 4.4*	2.0 ± 2.4*
Length, day 28 (cm)	0.84 ± 0.04	0.80 ± 0.02	0.78 ± 0.03	0.76 ± 0.01*	0.75 ± 0.09*	0.74 ± 0.02*

Results are means of four replicates ± standard deviation
* Significantly reduced compared to the control (William's test, $p<0.05$)

F_0 generation, juvenile growth phase

Survival of the juvenile fish on day 56 was significantly reduced in the 40 and 100 µg a.s./L test concentrations.

Fish length on day 56 and 'pseudo' specific growth rate, based on the length measurements on days 28 and 56, were significantly reduced in the 16 and 40 µg a.s./L test concentrations.

Table CA 8.2.2.2/01-3 Effects of spiroxamine exposure on the juvenile growth phase of the F_0 generation

Parameter	Nominal concentration (µg a.s./L)				
	Control	2.6	6.4	16	40
Survivors, day 56 (%)	98.5 ± 1.9	98.5 ± 1.9	98.0 ± 1.6	91.0 ± 10.9	25.9*
Length, day 56 (cm)	2.26 ± 0.05	2.25 ± 0.03	2.20 ± 0.01	1.99 ± 0.06†	1.73†
Pseudo specific growth rate	3.583 ± 0.10	3.722 ± 0.05	3.720 ± 0.14	3.409 ± 0.09*	2.864*

Results are means of four replicates ± standard deviation
* Significantly reduced compared to the control (William's test, $p<0.05$)
† Significantly reduced compared to the control (Dunnett's test, $p<0.05$)

F_0 generation, adult phase

Reproduction

No dose-dependent effects of spiroxamine exposure on reproduction could be detected. Due to the low number of remaining fish at the highest test concentration, the 40 µg a.s./L level was excluded from the statistical evaluation. However, the fertilisation rate as well as the cumulative number of fertilised eggs was significantly reduced at the lowest test concentration.

Since no clear dose-response could be detected, the NOEC with regard to reproduction was determined to be 16 µg a.s./L, the highest level tested during this phase.

Table CA 8.2.2.2/01-4 Effects of spiroxamine exposure on the reproduction of the adult phase of the F_0 generation

Parameter	Nominal concentration (µg a.s./L)			
	Control	2.6	6.4	16
Time to first spawning (days)	104 ± 9	124 ± 20	114 ± 14	101 ± 6
Eggs/female/day	6 ± 0.6	6 ± 4.6	4 ± 1.3	8 ± 1.7
Cumulative number of fertilised eggs	7723 ± 584	4065 ± 1334†	5331 ± 2439	6710 ± 1.0
Fertilisation rate (%)	87 ± 5.5	75 ± 9.8*	82 ± 1.0	82 ± 1.0

Results are means of four replicates ± standard deviation
* Significantly reduced compared to the control (William's test, $p<0.05$)
† Significantly reduced compared to the control (Dunnett's test, $p<0.05$)

Survival and growth

No exposure-related effects on survival of adult fish could be observed in the study. Length and weight of fish was found to be increased for female fish in the 40 µg a.s./L test concentration. A significant decrease was found in male fish at the 16 and 40 µg a.s./L test concentration. Male weight was not affected.

Table CA 8.2.2.2/01-5 Effects of spiroxamine exposure on the survival and growth of the adult phase of the F_0 generation

Parameter	Nominal concentration (µg a.s./L)				
	Control	**2.6**	**6.4**	**16**	**40**
Survival at termination (%)	87 ± 9.4	90 ± 17.8	98 ± 5.0	78 ± 16.2	80
Length, males (cm)	3.7 ± 0.07	3.7 ± 0.08	3.7 ± 0.03	3.6 ± 0.03*	3.6*
Length, females (cm)	3.6 ± 0.06	3.7 ± 0.07	3.6 ± 0.07	3.6 ± 0.08	3.9†
Weight, males (g)	0.484 ± 0.03	0.490 ± 0.03	0.478 ± 0.02	0.466 ± 0.02	0.493
Weight, females (g)	0.493 ± 0.03	0.546 ± 0.03	0.528 ± 0.04	0.521 ± 0.03	0.763†

Results are means of four replicates ± standard deviation
* Significantly reduced compared to the control (William's test, p<0.05)
† Significantly reduced compared to the control (Dunnett's test, p<0.05)

Sex ratio

Regarding sex ratio, a shift towards an increased number of males could be detected in the 16 and 40 µg a.s./L test concentrations. It must, however, be noted that the number of males per group was quite small in the control and low test concentrations.

In the 16 µg a.s./L test concentration the number of males significantly increased, however values remained within the historical range. In the 40 µg a.s./L test concentration, the percentage of males was over 80%. The resulting NOEC for sexual development was determined to be 6.4 µg a.s./L.

Vitellogenin

A decrease of vitellogenin concentrations in female blood plasma could be observed in the 6.4, 16 and 40 µg a.s./L test concentrations compared to the control. Vitellogenin concentrations in male blood plasma were not significantly affected.

Table CA 8.2.2.2/01-6 Effects of spiroxamine exposure on the sex ratio and vitellogenin concentration of the adult phase of the F_0 generation

Parameter	Nominal concentration (µg a.s./L)				
	Control	**2.6**	**6.4**	**16**	**40**
Sex ratio					
Males (%)	33.4 ± 6.5	20.6 ± 4.4	28.9 ± 2.8	56.7 ± 6.3*	91.7*
Females (%)	66.6 ± 6.5	79.4 ± 6.5	71.1 ± 2.8	43.3 ± 6.3*	8.3*
Vitellogenin					
Males (ng/µg)	0.154 ± 0.05	0.348 ± 0.28	0.139 ± 0.01	0.131 ± 0.02	0.095
Females (ng/µg)	198.8 ± 68.0	162.1 ± 51.8	90.0 ± 13.9*	114.1 ± 4.3*	77.7*

Results are means of four replicates ± standard deviation
* Significantly reduced compared to the control (William's test, p<0.05)

Histopathology

No specific findings indicative of substance-related toxic effects could be seen in the test fish. Histopathological investigation of the gonads and gonadal ducts revealed in females a higher grade of inflammatory changes in the ovary and oviduct in the 16 µg a.s./L test concentration.

Concomitantly, the incidence of egg debris in the oviduct was slightly elevated. In a few fish, the body cavity also showed inflammation/granulomas. A relation to the treatment could not be fully excluded, although inflammatory changes were seen at a higher test rate in only one of the replicates.

F_1 generation, early life stage phase

Hatching rate of the F_1 generation was greater than 80% in the control and treatment groups. Survival of fish was significantly reduced at test concentrations 6.4 µg a.s./L and above, resulting in an F_1 generation survival NOEC of 2.6 µg a.s./L.

Table CA 8.2.2.2/01-7 Effects of spiroxamine exposure on the early life phase of the F_1 generation

Parameter	Nominal concentration (µg a.s./L)			
	Control	2.6	6.4	16
Hatching, day 4 (%)	91.2 ± 3.0	93.2 ± 8.7	84.3 ± 9.4	82.8 ± 9.1
Hatching, day 5 (%)	92.2 ± 2.3	90.4 ± 0.4	90.9 ± 2.4	90.2 ± 0.8
Hatching, day 6 (%)	92.2 ± 2.3	90.4 ± 0.4	90.9 ± 2.4	90.2 ± 0.8
Survivors, day 14 (%)	82.2 ± 9.7	82.6 ± 4.4	78.1 ± 8.5	71.0 ± 8.1*
Survivors, day 21 (%)	79.7 ± 7.4	70.8 ± 5.8	61.5 ± 3.5*	58.3 ± 14.8*
Survivors, day 28 (%)	77.0 ± 6.3	66.3 ± 6.7	59.5 ± 3.6†	50.9 ± 18.3†
Survivors, day 35 (%)	75.0 ± 6.0	65.1 ± 5.9	55.3 ± 3.5†	41.1 ± 17.4†
Length, day 35 (cm)	1.01 ± 0.03	1.05 ± 0.005	1.03 ± 0.02	0.94 ± 0.10
Weight, day 35 (g)	0.013 ± 0.001	0.014 ± 0.001	0.013 ± 0.001	0.011 ± 0.003

Results are means of four replicates ± standard deviation
* Significantly reduced compared to the control (William's test, $p = 0.05$)
† Significantly reduced compared to the control (Welch t-test, $p = 0.05$)

A summary of the relevant NOEC and LOEC endpoints is presented below.

Table CA 8.2.2.2/01-8 Summary of endpoints after exposure of zebrafish to spiroxamine

Population	Endpoint		NOEC (µg a.s./L)	LOEC (µg a.s./L)
F_0 generation, early life stage	Population	Hatching	2.6[a]	6.4
		Survival	6.4	16
	Growth	Length	6.4	16
F_0 generation, juvenile growth	Population	Survival	16	40
	Growth	Length	6.4	16
		'Pseudo' specific growth rate	6.4	16
F_0 generation, adult	Population	Eggs/female/day	16	>16
		Cumulative egg number	16	>16
		Fertilisation rate	16	>16
		Survival	40	>40
		Sex ratio	6.4	16
	Growth	Male length	6.4	16
		Female length	16	40
		Male weight	40	>40
		Female weight	16	40
	Vitellogenin	Males	40	>40
		Females	2.6	6.4

Population	Endpoint		NOEC (µg a.s./L)	LOEC (µg a.s./L)
	Histology	Males: no change	16	40
		Females: inflammatory changes in the ovaries, egg debris	6.4	16
F_1 generation, early life stage	Population	Hatching	16	>16
		Survival	2.6	6.4
	Growth	Length	16	16
		Weight	16	16

[a] Endpoint not relevant as based on indirect effects

III. Conclusion

This was a fish full life cycle study conducted to examine the potential for long-term adverse effects of spiroxamine exposure to populations of zebrafish (*Danio rerio*).

Five test concentrations were investigated, each with four replicates of initially 100 fertilised eggs. The effects of exposure on different life stages of the zebrafish, including early life stages, juvenile growth, reproduction and early life stages of the filial generation were assessed under continuous exposure.

At the early life stage, no direct effects on hatching could be found, however survival and growth had a NOEC of 6.4 µg a.s./L.

At the juvenile growth stage, the NOEC values for survival, length and 'pseudo' specific growth rate were 16, 6.4 and 6.4 µg a.s./L, respectively.

At the adult stage, the NOEC values for eggs/female/day, cumulative egg number, fertilisation rate, survival and sex ratio were 16, 16, 16, 40 and 6.4 µg a.s./L, respectively. NOEC values for male length, female length, male weight and female weight were 6.4, 16, 40 and 16 µg a.s./L, respectively. Biomarker NOEC values for male and female vitellogenin content were 40 and 2.6 µg a.s./L, respectively, and for histology were 16 and 6.4 µg a.s./L in males and females, respectively.

In the early life stage of the filial generation, population NOEC values for hatching and survival were 16 and 2.6 µg a.s./L, and growth NOEC values for length and weight were both 16 µg a.s./L.

Based on the most sensitive endpoint, survival of the F_1 generation, the overall NOEC was found to be 2.6 µg a.s./L.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guidelines 210, the most up-to-date version of which is the "Fish, early-life stage toxicity test", adopted 26 July 2013 and 215 "Fish, juvenile growth test", adopted 21 January 2000. There is no formal OECD test guideline for a fish full-life cycle test therefore the study validity has been based on the OECD 210 guidelines. The OECD 215 test guideline is no longer used and therefore the validity criteria of this guideline have not been assessed here.

The study report listed it's own validity criteria (see above) and these were considered to have been achieved. However, validity criteria according to the most up-to-date OECD 210 (2013) test guideline have also been assessed and the following criteria were met:

- Hatching rate in the control replicates to be ≥70% (actual: 91.1%)
- Post-hatch survival in the control replicates to be ≥75% (actual: 79.3%)
- Dissolved oxygen concentration to be ≥60% in all test vessels throughout the test (actual: 68 – 102%)

One of the criteria were not met:

- Water temperature to be at 26 ± 1.5°C during the test (actual: 23.7 – 27.8°C). Four measurements recorded temperatures <24.0°C, with a minimum recorded of 23.7°C.

The deviations noted above are not considered to have adversely affected the integrity of the test therefore the study is considered to be acceptable.

Based on the most sensitive endpoint, survival of the F_1 generation, the overall NOEC was found to be 2.6 µg a.s./L.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.2.2.2/04
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10 and EC20 values for Danio rerio with spiroxamine TG in a full life cycle test
Report No:	0471836-ECO23
Document No:	M-760413-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
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 M-304458-02-1 on the effects of Spiroxamine TG in the zebrafish (*Danio rerio*) full life cycle test did not provide estimates of EC_{10} or EC_{20}. Therefore these values have been calculated in accordance with the Annex to Com. Reg. 283/2013. Effect concentrations with a 10 or 20% effect on F_0 length at 28 dpf and 56 dpf, F_0 pseudo specific growth rate, F_0 sex ratio based on females, F_1 post-hatch survival at 28 and 35 dpf and F1 survival at 35 dpf ($EC_{10,20}$) were re-calculated.

I. Methods

The statistical evaluation was performed with statistical software ToxRat Professional v3.3.0.

F0 generation (early life stage)

The calculation of an EC_{10} value for F_0 length after 28 days was performed with a Probit function using linear maximum likelihood regression and with the confidence limits determined by Normal approximation. The determination of reliable EC_{10} and EC_{20} values for F_0 hatching after 4 and 5 days was not possible due to the lack of a dose response or effects with a magnitude of 10% among the tested treatment rates. The determination of reliable EC_{10} and EC_{20} values for F_0 survival after 14, 21 and 28 days was not possible due to a poor goodness of fit in the data and the data scattering around the dose response.

F0 generation (juvenile growth stage)

The calculation of EC_{10} and EC_{20} values for F_0 length after 56 days and pseudo specific growth rate after 56 days post fertilisations (dpf) was performed with a Probit function using linear maximum likelihood regression and with the confidence limits determined by Fieller's theorem. Due to a lack of data for survival at 56 dpf within the study report, EC_{10} and EC_{20} calculations could not be performed.

F_0 generation (adult life phase)

The calculation of EC_{10} and EC_{20} values for F_0 sex ratio based on females was performed with a Probit function using linear maximum likelihood regression and with the confidence limits determined by Fieller's theorem.

The determination of reliable EC_{10} and EC_{20} values for F_0 egg number per female per day, cumulative egg number, fertilisation rate, vitellogenin concentration in male and female plasma and sex ratio based on males, was not possible due to the lack of a dose response. Due to the lack of effects above 10% when compared to the control on F_0 length at termination in males and females, and weight at termination in males and females, the EC_{10} and EC_{20} values are estimated to be >40 µg a.s./L.

Due to a lack of data for survival at termination and time to spawn within the study report, EC_{10} and EC_{20} calculations could not be performed.

F1 generation (early life stage)

The determination of reliable EC_{10} and EC_{20} values for F_1 survival at 14 and 21 dpf was not possible due to the lack of a dose response. The EC_{10} and EC_{20} values for F_1 post-hatch survival at 28 and 35 dpf were performed with a Probit function using linear maximum likelihood regression and with the confidence limits determined by Fieller's theorem. The EC_{10} and EC_{20} values for F_1 survival (based on number of eggs introduced) at 35 dpf were performed with a logit function using linear maximum likelihood regression and with the confidence limits determined by Fieller's theorem.

Due to the lack of effects above 10% when compared to the control on F_1 hatching day 4 and 5, length at 35 dpf and weight at 35 dpf the EC_{10} and EC_{20} values are estimated to be >16 µg a.s./L.

II. Results and Discussion

An explanation is given for regression analysis endpoints and endpoints where the statistical software could not produce a reliable results and where expert judgment was needed. These details can be found below.

F_0 generation (early life stage)

Length at 28 days post fertilisation (dpf)

Regarding the calculation of the EC_{10} value for length at 28 dpf, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.000, showing no significant deviation between fit and data, and a statistically significant concentration/response was found (p(F) = 0.006) for this parameter.

The resulting EC_{10} value and the respective confidence intervals are represented in the following table.

Table CA 8.2.2.2/04-1 Results of the Probit analysis (max. likelihood regression) with length at 28 dpf: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (by Normal approximation)

Parameter	Length	
	EC_{10} (95 % confidence interval) [µg a.s./L]	EC_{20} (95 % confidence interval) [µg a.s./L]
Effect on length at 28 dpf	42.896 (24.536 – 74.994)	n.d.

n.d.: not determinable

The resulting EC_{10} value of 42.896 (95%CL: 24.536 – 74.994) µg a.s./L, meet the goodness of fit criteria and therefore the calculated EC_{10} value is considered reliable.

F_0 generation (juvenile growth stage)

Length at 56 days post fertilisation (dpf)

Regarding the calculation of EC_{10} and EC_{20} values for length at 56 dpf, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 0.999, showing no significant deviation between fit and data, and a statistically significant concentration/response was found (p(F) = 0.015) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table.

Table CA 8.2.2.2/04-2 Results of the Probit analysis (max. likelihood regression) with length at 56 dpf: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (by Fieller's theorem)

Parameter	Length	
	EC_{10} (95 % confidence interval) [µg a.s./L]	EC_{20} (95 % confidence interval) [µg a.s./L]
Effect on length at 56 dpf	14.915 (6.341 – 21.039)	32.974 (23.982 – 51.492)

The resulting EC_{10} value of 14.915 (95%CL: 6.341 – 21.039) and 32.974 (95%CL: 23.982 – 51.492) µg a.s./L, meets the goodness of fit criteria and therefore the estimated EC_{10} value is considered reliable.

Pseudo specific growth rate at 56 days post fertilisation (dpf)

Regarding the calculation of EC_{10} and EC_{20} values for pseudo specific growth rate at 56 dpf, the criteria for goodness of fit were met as the P(Chi²) value was 1.000, showing no significant deviation between fit and data, and a statistically significant concentration/response was found (p(F) = 0.003) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table.

Table CA 8.2.2.2/04-3 Results of the Probit analysis (max. likelihood regression) with pseudo specific growth rate at 56 dpf: Selected effective concentrations (EC_x) of the test item and their 95% confidence limits (by Fieller's theorem)

Parameter	Pseudo specific growth rate	
	EC_{10} (95 % confidence interval) [µg a.s./L]	EC_{20} (95 % confidence interval) [µg a.s./L]
Effect on pseudo specific growth rate at 56 dpf	24.665 (21.482 – 27.133)	39.833 (37.154 – 43.187)

The resulting EC_{10} value of 24.665 (95%CL: 21.482 – 27.133) and 39.833 (95%CL: 37.154 – 43.187) µg a.s./L, meets the goodness of fit criteria and therefore the estimated EC_{10} value is considered reliable.

F_0 generation (adult life phase)

Sex ratio (based on females)

Regarding the calculation of EC_{10} and EC_{20} values for sex ratio (based on females) at 56 dpf, the criteria for goodness of fit were met as the P(Chi²) value was 0.999, showing no significant deviation between fit and data, and a statistically significant concentration/response was found (p(F) = 0.003) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table.

Table CA 8.2.2.2/04-4 Results of the Probit analysis (max. likelihood regression) with sex ratio (based on females) at 56 dpf: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (by Fieller's theorem)

Parameter	Sex ratio (based on females)	
	EC_{10} (95 % confidence interval) [µg a.s./L]	EC_{20} (95 % confidence interval) [µg a.s./L]
Effect on sex ratio (based on females) at 56 dpf	9.767 (8.043-11.038)	12.084 (10.598-13.187)

The resulting EC_{10} value of 9.767 (95%CL: 8.043 – 11.038) and 12.084 (95%CL: 10.598 – 13.187) µg a.s./L, meets the goodness of fit criteria and therefore the estimated EC_{10} value is considered reliable.

F_1 generation (early life stage)

Post-hatch survival at 28 days post fertilisation (dpf)

Regarding the calculation of EC_{10} and EC_{20} values for post-hatch survival at 28 dpf, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 0.739, showing no significant deviation between fit and data, and a statistically significant concentration/response was found (p(F) = 0.032) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table below.

Table CA 8.2.2.2/04-5 Results of the Probit analysis (max. likelihood regression) with pseudo specific growth rate at 56 dpf: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (by Fieller's theorem)

Parameter	Post-hatch survival	
	EC_{10} (95 % confidence interval) [µg a.s./L]	EC_{20} (95 % confidence interval) [µg a.s./L]
Effect on post-hatch survival at 28 dpf	1.915 (1.038 – 2.742)	5.868 (4.545 – 7.269)

The resulting EC_{10} value of 1.915 (95%CL: 1.038 – 2.742) and 5.868 (95%CL: 4.545 – 7.269) µg a.s./L, meets the goodness of fit criteria however as it falls below the lowest dose of 2.6 µg a.s./L and in accordance with the OECD Series on Testing and Assessment Number 54, the estimated EC_{10} value should not be considered reliable.

Post-hatch survival at 35 days post fertilisation (dpf)

Regarding the calculation of EC_{10} and EC_{20} values for post-hatch survival at 28 dpf, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 0.880, showing no significant deviation between fit and data, and a statistically significant concentration/response was found (p(F) = 0.010) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table.

Table CA 8.2.2.2/04-6 Results of the Probit analysis (max. likelihood regression) with post-hatch survival at 35 dpf: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (by Fieller's theorem)

Parameter	Post-hatch survival	
	EC_{10} (95 % confidence interval) [µg a.s./L]	EC_{20} (95 % confidence interval) [µg a.s./L]
Effect on post-hatch survival at 35 dpf	2.263 (1.619-2.868)	4.828 (3.998-5.625)

The resulting EC_{10} value of 2.263 (95%CL: 1.619 – 2.868) and 4.828 (95%CL: 3.998 – 5.625) µg a.s./L, meets the goodness of fit criteria however as it falls below the lowest dose of 2.6 µg a.s./L and in accordance with the OECD Series on Testing and Assessment Number 54, the estimated EC_{10} value should not be considered reliable.

Survival at 35 days post fertilisation (dpf)

In line with previous EU reviews of the data, survival based on eggs introduced has also been calculated at 35 dpf.

Regarding the calculation of EC_{10} and EC_{20} values for survival at 35 dpf, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 0.905, showing no significant deviation between fit and data, and a statistically significant concentration/response was found (p(F) = 0.008) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table.

Table CA 8.2.2.2/04-7 Results of the Probit analysis (max. likelihood regression) with post-hatch survival at 35 dpf: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (by Fieller's theorem)

Parameter	Survival	
	EC_{10} (95 % confidence interval) [µg a.s./L]	**EC_{20} (95 % confidence interval) [µg a.s./L]**
Effect on survival at 35 dpf	1.878 (1.271-2.465)	4.456 (3.610-5.243)

The resulting EC_{10} value of 1.878 (95%CL: 1.271 – 2.465) and 4.456 (95%CL: 3.610 – 5.243) µg a.s./L, meets the goodness of fit criteria however as it falls below the lowest dose of 2.6 µg a.s./L and in accordance with the OECD Series on Testing and Assessment Number 54, the estimated EC_{10} value should not be considered reliable.

A summary of the obtained endpoints is presented in the following table.

Table CA 8.2.2.2/04-8 Overall endpoints of the statistical re-calculation of the *Danio rerio* study with spiroxamine

Period	Endpoint (µg a.s./L)		
	Development stage	**Parameter**	**EC_{10} (95% confidence intervals)**
F0 generation	Early life stage	Hatching d4	n.d.
		Hatching d5	n.d.
		Survival at 14 dpf	n.d.#
		21 dpf	n.d.#
		28 dpf	n.d.#
		Length at 28 dpf	42.896 (24.536-74.994)
	Juvenile growth	Survival at 56 dpf	-*
		Length at 56 dpf	14.915 (6.341-21.039)
		Pseudo specific growth rate	24.665 (21.482-27.133)
	Adult	Time to first spawn	-*
		Egg number per female per day	n.d.
		Cumulative egg number	n.d.
		Fertilisation rate	n.d.
		Survival	-*
		Sex ratio males	n.d.
		Sex ratio females	9.767 (8.043-11.038)
		Length males	>40
		Length females	>40
		Weight males	>40
		Weight females	n.d.
		Vitellogenin males	n.a.
		Vitellogenin females	n.a.
F1 generation	Early life stage	Hatching d4	>16
		Hatching d5	>16
		[+]PH survival 14 dpf	n.d.
		PH survival 21 dpf	n.d.
		PH survival 28 dpf	1.915 (1.038-2.742)**
		PH survival 35 dpf	2.263 (1.619-2.868)**
		[+]Survival 35 dpf	1.878 (1.271-2.465)**

Period	Endpoint (µg a.s./L)		
	Development stage	Parameter	EC_{10} (95% confidence intervals)
		Length	≥16
		Weight	≥16

Due to the lack of a concentration dose response or when the test rate with 10% when compared to the controls are below the tested range of concentrations, the calculation of EC_{10} and EC_{20} values for the parameters marked in the above table as "n.d." was not possible and therefore no EC_{10} or EC_{20} values were determined. As data was not available within the study report, statistical analyses of parameters marked with a * was not possible.

Where the calculated EC_{10} values were below the lowest dose, as per the OECD series on testing and assessment number 54 (Current approaches in the statistical analysis of ecotoxicity data: A guidance to application), these values are not considered reliable enough and are marked with a **.

[+]Post-hatch survival was used to calculate the ECx values for the F1 generation, resulting in survival being independent of hatch. However, as the number of eggs hatched at day 5 was less than the number surviving on day 14 (eggs assumed to have continued to hatch after the measured time point of day 5 however raw data not available to confirm), this was not possible for the F_0 generation and therefore number of eggs introduced was used to determine survival at 14 dpf, 21 dpf and 28 dpf. Survival was therefore, not independent of hatch for this stage.

The determination of reliable EC_{10} and ECx values for parametets marked with a # was not possible due to a poor goodness of fit in the data and the data scattering around the dose response.

As vitellogenin concentration is a non-apical endpoint, this parameter was not deemed relevant for further analysis and therefore ECx values were not determined for both male and female fish.

III. Conclusion

Effect concentrations with a 10 or 20% effect on F_0 length at 28 dpf and 56 dpf, F_0 pseudo specific growth rate, F0 sex ratio based on females, F1 post-hatch survival at 28 and 35 dpf and F_1 survival at 35dpf ($EC_{10,20}$) were re-calculated.

The lowest determined EC_{10} value was 1.878 µg a.s./L for survival of the F_1 generation but it is noted that this is an extrapolated value and should be used with caution.

Assessment and conclusion by applicant:

The statistical re-evaluation of the data has determined EC_{10} and EC_{20} values for many of the parameters assessed. It must be noted that EC_{10} values for survival are extrapolated values below the concentration range tested and therefore should be treated with caution.

In the RAR (2010), RAR (2017) an EC_{10} was determined by the RMS and used in preference to the NOEC in the original study report.

The lowest EC_{10} value of 1.88 µg a.s./L, based on overall survival, is lower than the overall NOEC of 2.6 µg a.s./L and therefore has been taken as the critical endpoint determined for this study. It must be noted that the EC_{10} value of 1.88 µg a.s./L is below the lowest test concentration and therefore may be potentially unreliable.

The values determined in the re-evaluation work are considered to be fully valid.

Data Point:	KCA 8.2.2.2/02
Report Author:	[redacted]
Report Year:	2009
Report Title:	Statement concerning questions related to the fish full life cycle test listed in the draft re-assessment report on Spiroxamine
Report No:	M-347595-01-1
Document No:	M-347595-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

In the study M-304458-02-1 [BVL Doc ID 1798135; Zebra fish, life cycle test, flow through conditions.] the following aspect needs further clarification.

During the critical phase for sexual development of zebra fish, in the fourth week, the mean test concentrations dropped down to 71 % of nominal at the NOEC-level (4.5 µg/L instead of 6.4 µg/L). At the LOEC-level the measured concentration was 6.1 µg as/L (38 % of nominal). At the lowest test concentration the measured value was 1.85 µg as/L compared to 2.6 µg as/L nominal (71.2 %). The decrease of the concentrations was due to malfunctions of the pumps. Probably, the concentrations were measured a second time for control purposes in the 4th week. This information may be helpful to define the time period of low exposure. Such data are not shown in the study report, probably they are available from the raw data and can be provided.

Since effects from exposure to endocrine disruptors may result from relatively short exposure periods of critical developmental stages, it can not be excluded that the effects on sexual development found in this study are underestimated, if related to the nominal concentrations.

During the evaluation of the Fish Full Life cycle report questions concerning the exposure situation especially at week 4 arose. The respective questions were discussed with the study director ([redacted]) and the following answer can be given:

The stock solutions for the flow through device were prepared freshly each day. Thus, the weekly chemical analysis is only a representative one. Additionally, the consumption of the stock solution was checked at least daily, assuring that malfunctions of the dosing pumps, if occurring, could be detected within 24 hours. On working days, the correct function of pumps was checked twice daily, therefore it can be stated that a severe malfunction would have been detected within 15 hours.

The problems observed within week 4 of the experiment were caused by two different incidents.

1) Until week 3 the test item concentrations in the aquaria were representatively measured to be between 75 and 102 % of the nominal concentrations. The first measurement in week 4 (Feb 27, day 1 of week 4) revealed a drop of concentrations down to values ranging between 18 and 81 %. To overcome the low exposure situation, the amount of test item weighed in was increased from that time point onwards. Due to daily preparation of the stock solution and the at least 5-fold water exchange rate of the flow through device, it can be assumed that the measured concentrations in week 4 (first measurement) represent the lowest values of the development of test concentrations between the weeks 3 and 4 and a recovery of test concentrations took place immediately, as confirmed for most of the replicates by the second measurement of week 4 (Feb 29).

2) Independently from this, malfunctions of the dosing pumps were observed on Friday of week 4 (Feb 29). Two replicates each of test level 1 (replicate 1 and 2) and test level 4 (replicate 3 and 4) were affected. The pumps were immediately repaired and samples were taken for analytical measurements (as second sampling point during week 4). No further analytical samples were taken at the following day (Saturday) due to the weekend situation. The visual control of the stock solution consumption from Friday onwards was in the expected range again. The results of the accompanying chemical analysis for week 5 (80-123 % recovery) demonstrate the correct function of the flow through device. Due to the at least 5 fold exchange of the water volume per day it can be assumed that the aimed concentration were achieved rapidly again after repairing of the flow through device on Friday.

The first analytical measurement of week 4 (Feb 27) was on day 21 after test initiation and day 16 post hatch. The pump malfunction occurred on Feb 29, which is day 23 after test initiation (post fertilisation) and day 18 post hatch. Therefore, this low exposure event took place during week 3 post hatch and was terminated at the beginning of week 4. The results for week 5 (representing week 4 post hatch) and the following weeks are within the acceptable range (mean values above 80% of nominal). Based on the available literature concerning gonad development of zebrafish, the time period of low exposure is just before the early beginning of the critical phase of gonad development of zebrafish. This is confirmed by the homogeneity of the study results of the different replicates: the two replicates not negatively influenced by pump malfunctions did not show different effects compared to the ones affected by the pump malfunctions. In conclusion, there is clear evidence that the existing problems of the flow through system revealed on Feb 27 and 29 did not negatively affect the general outcome of the study. Therefore the use of the nominal concentrations which was based on mean measured values ranging between 92 and 101 % of the nominal values seems reasonable.

In order to be conservative, a worst case assumption for the calculation of the exposure concentrations in week 4 in the study report by applying a weighted approach (see Table 2). The lower values measured at the first sampling were weighted 6 fold. The second measurement in week 4, revealing by far higher recovery values than the first measurement in most cases (except the 4 replicates negatively impacted by pump malfunctions), was weighted only one fold.

The evaluator of the Fish Full Life Cycle used the LC_{10} instead of NOEC for the endpoint F_1 survival (day 35). This seems to be questionable. The Fish Full Life Cycle study was performed using a test design appropriate to generate a NOEC ("NOEC-approach"). Five concentrations tested in four replicates each were investigated. This is in line with existing guidelines for Fish Full Life Cycle Tests and other chronic fish studies (e.g. ELS –tests according OECD 210) and represents the state of the art.

The use of EC_{10} values is a possibility to create risk assessment relevant results for a chronic study in cases where a NOEC determination is not possible. In the presented study, a NOEC confirmed by statistical analysis was achieved. The evaluator re-evaluated this endpoint by calculating an LC_{10} and additionally excluded the replicate with the lowest survival rate. It was stated that the respective replicate was not fulfilling the validity criteria according to OECD TG 210. However, the validity criteria are defined for the mean value rather than for the single replicates. Especially for the second generation early life stages of a Fish Full Life Cycle test the validity criteria of the OECD 210 should be discussed anyway. Nevertheless BCS evaluated the data for the endpoint under discussion again excluding the respective replicate as well. This evaluation revealed the same NOEC as the originally presented data evaluation. Additionally the ECx calculations were carried out. The originally presented NOEC of 2.6 µg/L ranged in all cases within the 95% confidence limits of the LC_{10} calculations. Based on the above given arguments we recommend the use of the NOEC instead of the LC_{10} for the endpoint F1 survival (day 35).

On page 212 of the spiroxamine Draft Re-assessment Report (yellow draft) it is stated that:

"Since it can not be excluded that also short term exposure of females may impair the egg quality permanently and hence the fish population, the use of PEC_{twa} values for the risk assessment is not appropriate and the PEC max values have to be preferred, as discussed in the guidance document on aquatic Ecotoxicology". In the Aquatic guidance document the following can be found on page 27:

"The use of PEC_{twa} may not be appropriate for use with endocrine disrupting compounds since these effects may result from relatively short periods of exposure at critical developmental periods."

Zebrafish is known to be a sensitive fish species concerning sex reversal. The critical developmental period for this is lasting for about 3 to 4 weeks. Thus, it should be possible to use a PEC_{twa} of 7 days, especially when the single peaks originating from exposure modelling are not higher than the NOEC generated in a Fish full life Cycle study covering the most sensitive developmental period. The aquatic guidance document states that it may not be appropriate to use the PEC_{twa}, therefore the definitive decision in each case should be depending on the available data package. In the case of zebrafish and Spiroxamine the possibility to use the PEC_{twa}, should be discussed on the basis of the respective exposure patterns.

Assessment and conclusion by applicant:

The supporting statement summarised above has been submitted here for completeness and to present the arguments regarding the concerns over the analytical measurements in week 4 of the test. On this basis it is considered to be acceptable.

Some of the other issues discussed here (such as use of PEC_{twa} values) are no longer considered relevant for the current submission. Please refer to Document M-CP Section 10 for the discussion on the selection of toxicity endpoints and the exposure values for the risk assessment.

Data Point:	KCA 8.2.2.2/03
Report Author:	[redacted]
Report Year:	2014
Report Title:	Zebrafisch (Danio rerio), full life cycle test under static conditions in a water sediment system - Test item: Spiroxamine
Report No:	M-467979-03-1
Document No:	M-467979-03-1
Guideline(s) followed in study:	Special study, considers to OECD 210; US EPA OCSPP 850.1500
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The 56-day chronic toxicity of spiroxamine to different life stages of the zebrafish (*Danio rerio*) was studied under static renewal conditions. Three groups consisting of different life stages of zebrafish (fertilised eggs, juveniles and mature spawning adults) were concurrently exposed to four spiroxamine concentrations under static conditions in 260 L subdivided glass aquaria with a water column of 48 cm depth and a 3 cm layer of artificial sediment. The nominal initial spiroxamine concentrations were 12, 24, 48 and 192 µg a.s./L. Sediment was included in the test vessels to simulate a realistic dissipation of the test substance, mimicking exposure and effects following two applications at a 14-day interval.

Organisms were exposed to mean initial measured test concentrations of 15.8, 30.4, 63.9 and 255 µg a.s./L.

The survival of F_1 fish larvae was interpreted as the most sensitive population relevant endpoint.

The corresponding effect concentration (EC_{10}) was calculated to be 23.3 µg spiroxamine/L.

Beside larval survival, the sex ratio after juvenile exposure as well as the growth of adult females showed similar sensitivity (NOEC: 30.4 µg spiroxamine/L). A decrease of the concentration of the biomarker vitellogenin could be observed at ≥30.4 µg a.s./L thereby giving a NOEC for this parameter of 15.8 µg a.s./L. The histopathological evaluation of the fish gonads revealed no substance related effect.

I. Materials and Methods

A. Materials

Test Material	Spiroxamine
Lot/Batch #:	AE 1344293-01-05 (origin batch: EDTH08883)
Purity:	98.2%
Description:	Light yellow liquid
Stability of test compound:	Not reported
Reanalysis/Expiry date:	05 July 2014
Density:	Not reported
Treatments	
Test rates:	Initial nominal: 12, 24, 48 and 192 µg a.s./L Initial measured: 15.8, 30.4, 63.9 and 255 µg a.s./L
Solvent/vehicle:	None
Analysis of test concentrations:	Yes, mean measured initial concentrations 127 – 133% of nominal
Test organisms	
Species:	Zebrafish (*Danio rerio*)
Source:	[redacted]
Acclimatisation period:	Fish were raised in the test facility, and holding water was of the same quality as that used in the test
Feeding:	Fed daily *ad libitum* suitable diet depending on fish age
Treatment for disease:	None
Test design	
Test vessel:	260-L glass aquaria subdivided into four, with a 48 cm deep water column and 3 cm deep layer of artificial sediment
Test medium:	Purified drinking water according to OECD 215
Replication:	Four control replicates, three per test concentration
No. of animals/vessel:	A: 50 fertilised eggs B: 30 four-week old juveniles C: 30 adult fish
Duration of test:	56 days

Environmental test conditions

Temperature:	24.0 – 26.9°C
Dissolved oxygen:	82 - 125% saturation
pH:	5.6 – 8.9
Photoperiod:	12 h light : 12 h dark

B. Study Design

This study was conducted in order to assess the effects of spiroxamine exposure on different life stages of the zebrafish, *Danio rerio*, including early life stages, juvenile growth, reproduction and early life stages of the filial generation. Test concentrations were selected based on available data on the chronic toxicity of spiroxamine to fish.

Three groups consisting of different life stages of zebrafish (fertilised eggs, juveniles, and mature spawning adults) were concurrently exposed to four spiroxamine concentrations under static conditions in 260 L subdivided glass aquaria with a water column of 48 cm depth and a 3 cm layer of artificial sediment. The nominal initial spiroxamine concentrations were 12, 24, 48 and 192 µg spiroxamine/L. Three aquaria replicates were used per test concentration, and four untreated aquaria served as controls.

Sediment was included in the test vessels to simulate a realistic dissipation of the test substance, mimicking exposure and effects following two applications at a 14-day interval. Dissipation in artificial sediment still represents a worst case exposure as it shows lower biological activity compared to natural systems.

Fertilised eggs were collected from a healthy parental stock, and the time from spawning to exposure in the test vessels did not exceed two hours.

Test medium was drinking water purified according to OECD 215, *via* filtration with activated charcoal, passage through a limestone column and aeration until oxygen saturation. The sediment was prepared according to OECD 219, and was composed by dry weight as 4 to 5% peat, 20% kaolin clay (kaolinite content >30%) and 75 to 76% quartz sand. The final organic carbon content was determined to be 1.2%.

Application solutions of the test substance were prepared by weighing adequate amounts and dissolving it in dilution water. Vessels were treated twice with a 14-day interval by replacing 10 L test media with 10 L application solution, with 3333 mL being introduced into each of the compartments with different fish life stages.

The in-life phase began with the simultaneous application of the test item to 50 fertilised eggs, 30 4-week old juveniles and 30 adult fish. Each group was carefully segregated in separate compartments within each test aquaria. When the fish from fertilised eggs reached the age of 28 days (juveniles) their numbers were reduced to 30.

Measurements of mortality and abnormal behaviour were made daily.

At 28 and 56 days of age, fish were digitally photographed. Survival rates and fish lengths as well as the pseudo-specific growth rate (based on the length measurements) were determined by evaluating photographs using electronically supported counting and analysis. In the pre-adult life stage, *i.e.* day 56 and later, spawning trays were introduced and monitored daily for spawned eggs. The time until first findings of eggs was recorded. The spawning success was monitored for at least 20 days beginning from when which sufficient egg numbers (at least 15 eggs per replicate vessel) and a fertilisation of ≥80% was achieved on three successive days. Egg production per female per day and fertilisation rate was measured daily. After termination of the parental generation, a blood plasma sample was taken from each fish and the concentration of vitellogenin was measured in blood plasma of five males and five females. Finally, histopathology of the fish gonads was conducted for five males and five females. Primary evaluation criteria were increased proportion of spermatogonia, presence of testis-ova,

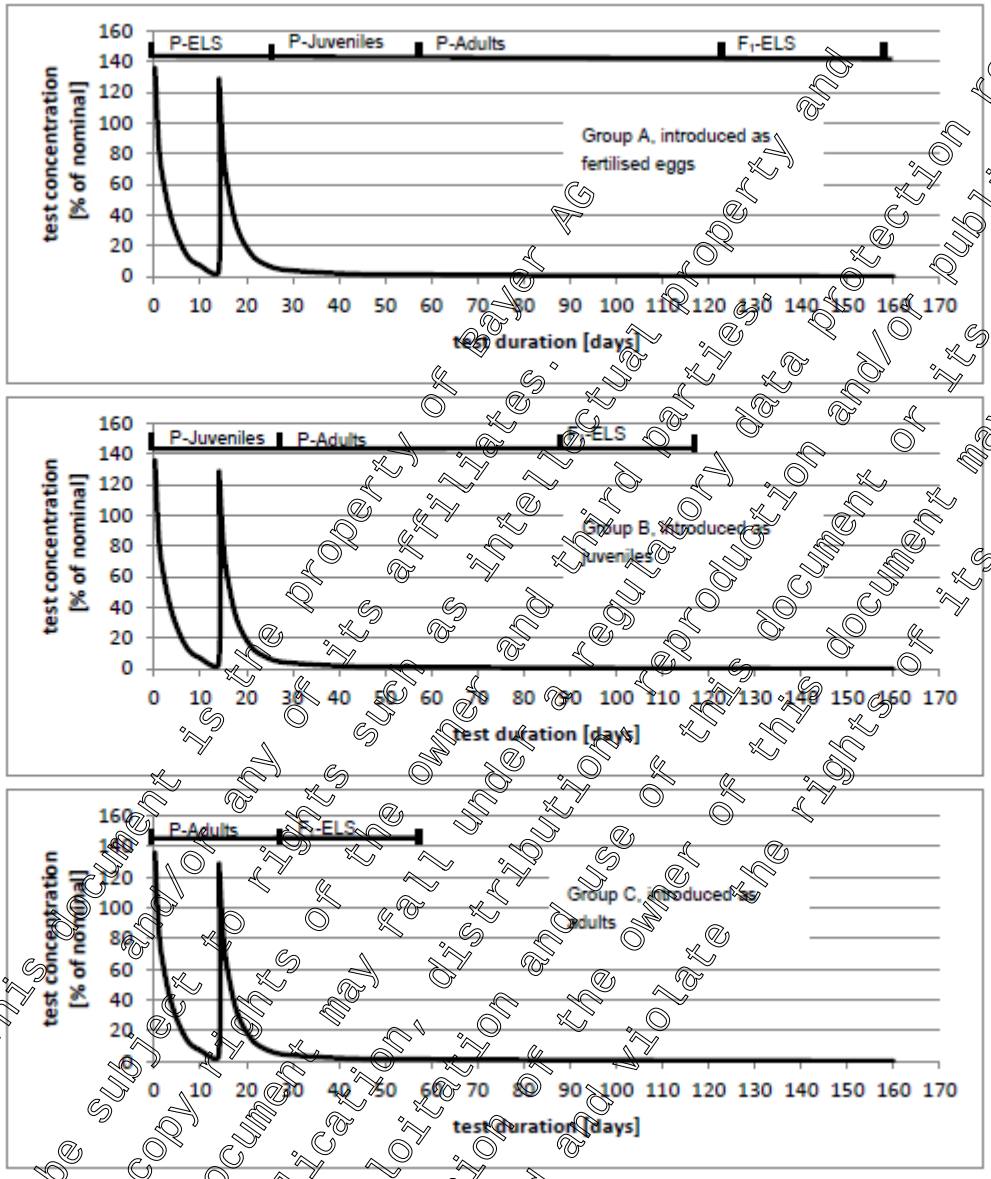
increased testicular degeneration and leydig cell hyperplasia/hypertrophy for males, and increased oocyte atresia, perifollicular hyperplasia/hypertrophy, decreased yolk formation and change in gonadal staging for females. Secondary evaluation criteria were decreased proportion of spermatogonia, increased proteinaceous fluid, asynchronous gonad development, altered proportions of spermatocytes and spermatids, altered gonadal staging and granulomatomous inflammation in males, and interstitial fibrosis, egg debris in oviduct, granulomatomous inflammation and altered number of post-ovulatory follicles in females.

For the filial generation (F_1) of each group, survival rates during the early life stage period (up to 28 days of age) were observed. After finishing the early life stage period, the fish were measured for total length. Furthermore, the group dry weight was measured and the single dry weight per fish was calculated.

Measurements of water temperature were made continuously in all test vessels. The oxygen concentration and pH of the water was measured in each vessel directly before adding the fish and afterwards at least twice weekly. Nitrate, nitrite and ammonium were measured once per week.

The different life stages tested were marked as group A (fish introduced as fertilised eggs, embryos), group B (fish introduced as juveniles) and group C (fish introduced as spawning adults). The figure presented below summarises the three exposure groups tested.

Figure CA 8.2.2.2/03-1
Exposure of Groups A, B and C during the modified exposure fish full life cycle study



Analytical method

Samples of water were analysed using the validated analytical method M-467979-03-1, report reference M-467979-03-1 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the study report were met:

- Survival rate in the controls was greater than 70%
- Dissolved oxygen concentration to be >60% saturation throughout the test (actual: 82 to 125%)
- Water temperature was within 25 ± 2°C throughout the study

1st application (day 0)

The measured concentrations of the application solutions corresponded to 104 to 137% of nominal. One hour after treatment, the measurements of the water samples revealed concentrations between 118 and

143% of nominal (mean 134%). After 4 hours, the results were between 117 and 151% of nominal (mean 136%). After 1 day the analysed values were between 63 and 104% of nominal (mean 86.0%).

The dissipation characteristic was similar in all test aquaria. On day 2 of the study, between 36 and 80% of nominal were found in the water column (mean value of 62.1%). On day 4, 7 and 10, mean concentrations of 35.1, 15.2 and 8.3% of nominal, respectively, were measured. On day 14, before the second application, the mean concentration was measured to be 4.8% of nominal.

2nd application (day 14)

The measured concentrations of the application solutions corresponded to 107 to 144% of nominal. One hour after treatment, the measurements of the water samples revealed concentrations between 118 and 140% of nominal (mean 128%). After 4 hours, the results were between 114 and 131% of nominal (mean 121%). After 1 day the analysed values were between 68 and 92% of nominal (mean 80.1%).

The dissipation characteristic was similar in all aquaria. On day 2 after the 2nd application, between 43 and 69% of nominal were found in the water column (mean value of 57.6%). On day 4, 7 and 10 after the 2nd application, mean concentrations of 31.3, 14.6 and 9.1% of nominal, respectively, were measured. On day 14 after the 2nd application, the mean concentration was determined to be 5.4% of nominal. For the following sampling dates on day 28, 42, 70 and at test end, only low amounts or amounts below the LOQ of 0.6 µg spiroxamine were detected.

The mean measured initial concentrations were calculated to be 15.8, 30.4, 63.9 and 255 µg a.s./L. Results have been presented based on these mean measured initial concentrations.

Table CA 8.2.2.2/03-1 Mean measured initial concentrations of spiroxamine (maximum peak) during the test

Nominal concentration (µg a.s./L)	Replicate	Measured concentration				Mean measured concentration			
		1 h after 1st application		1 h after 2nd application		By replicate		Total	
		µg/L	%	µg/L	%	µg/L	%	µg/L	%
Control	A	<LOQ*	-	<LOQ*		-	-	-	-
	B	<LOQ*	-	<LOQ*					
	C	<LOQ*		<LOQ*	-				
	D	<LOQ*	-	<LOQ*	-				
12.0	A	17.2	143	16.2	135	16.7	139	15.8	131
	B	14.5	121	16.8	140	15.7	131		
	C	14.2	118	15.7	131	15.0	125		
24.0	A	31.1	130	28.2	118	29.7	124	30.4	127
	B	30.7	128	29.6	123	30.2	126		
	C	30.6	127	32.1	134	31.3	130		
48.0	A	68.6	143	59.0	123	63.8	133	63.9	133
	B	68.8	143	61.1	127	64.9	135		
	C	66.6	139	59.0	123	62.8	131		
192	A	261	134	251	131	256	133	255	133
	B	273	142	248	129	260	136		
	C	263	137	233	121	248	129		

* Limit of quantification (LOQ) = 0.6 µg/L

Survival and growth

The survival of the F_1 fish larvae was found to be the most sensitive population relevant endpoint after peak exposure to spiroxamine with statistically significant effects observed at test concentrations of 30.4 µg/L and higher in all test groups.

For the F_1-generation of group C, a statistically significant difference compared to the control was detected for survival at ≥30.4 µg spiroxamine/L (NOEC: 15.8 µg/L). An EC_{10} of 23.3 µg spiroxamine/L (based on mean measured initial concentrations) was calculated.

The survival of parental fish larvae (group A) was affected at the same concentration level as the F_1 fish larvae of groups A and B (NOEC: 30.4 µg/L). In contrast, survival of juvenile and adult fish was not affected in any group.

Growth retardation could be observed at all parental life stages at 63.9 µg/L and higher. No impact on growth could be observed for the filial fish. The parental larvae (Group A) and juvenile fish (Group A and B) showed an impact on growth in terms of reduced lengths (NOEC: 63.9 µg spiroxamine/L). The adult females of group B showed significant decrease of weight at the two highest test concentrations (NOEC: 30.4 µg spiroxamine/L). Group C fish were not affected.

Reproduction

Fecundity, represented by the egg number per female and day, was not affected after peak exposure to the test item in any of the tested concentrations. There was also no impact on the fertilisation rate (NOEC: ≥255 µg spiroxamine/L).

Sexual development, biomarkers and histopathology of gonads

A significant shift in sex ratio towards an increased number of male fish could be observed in group B (fish exposed as juveniles) at test concentrations ≥63.9 µg/L (NOEC: 30.4 µg spiroxamine/L). Also for group A (fish exposed as fertilised eggs) an effect on sex ratio could be detected. An increased number of males was found at the highest test level (NOEC: 63.9 µg spiroxamine/L).

Beside the apical endpoints, the biomarker vitellogenin (VTG) was analysed. This parameter was analysed to provide additional information on physiological processes in the exposed fish and to assist the interpretation of the effects observed for the population relevant endpoints.

No effect on VTG concentrations in blood plasma of both female and male fish could be detected in the parental fish groups exposed as adults and juveniles (group B and C). However, for the parental group A (fish exposed as fertilised eggs), a significant decrease of VTG concentrations was detected at ≥30.4 µg spiroxamine/L.

The decrease of VTG amount was quite pronounced, but a clear dose response relationship was missing.

As a further parameter, which encourages the interpretation of apical effects, a histopathological analysis of fish gonads was performed. The histopathological evaluation of the adult animals of all groups (A, B and C) revealed no substance related effects on fish gonads. There were single findings of egg debris, fibrosis, atresia in females and testis-ova in males, but these were not treatment related.

Table CA 8.2.2.2/03-2 Group A, P-generation: Early life stage: hatch, survival and growth, day 28 pf

Endpoint	Mean measured initial concentration (µg a.s./L)				
	Control	15.8	30.4	63.9	255
Number of replicates	4	3	3	3	3
Number of eggs introduced	200	150	150	150	150
Survival, day 21 pf (%)	92.0 ± 4.3	89.3 ± 3.1	90.0 ± 2.0	75.3 ± 4.6*	46.0 ± 15.9*
Survival, day 28 pf (%)	91.5 ± 3.4	89.3 ± 3.1	90.0 ± 2.0	74.7 ± 4.2†	41.3 ± 16.8†
Length, day 28 pf (%)	1.27 ± 0.04	1.31 ± 0.05	1.29 ± 0.02	1.30 ± 0.07	0.89 ± 0.09*

Mean values ± standard deviation are presented
pf Post fertilisation
* Statistically significantly different to the control, $p<0.05$, Williams test, one-sided smaller
† Statistically significantly different to the control, $p<0.05$, Welch t- test, one-sided smaller

Table CA 8.2.2.2/03-3 Group A, P-generation: Juvenile stage: hatch, survival and growth, day 56 pf

Endpoint	Mean measured initial concentration (µg a.s./L)				
	Control	15.8	30.4	63.9	255
Number of replicates	4	3	3	3	3
Number of eggs introduced	121	90	90	89	62
Survival between day 28 and 56 pf (%)	99.2 ± 1.6	98.9 ± 1.9	98.9 ± 1.9	100 ± 0.0	94.9 ± 8.9
Length, day 56 pf (cm)	2.76 ± 0.09	2.81 ± 0.03	2.84 ± 0.05	2.73 ± 0.07	2.48 ± 0.06*
Pseudo specific growth rate (based on length)	2.779 ± 0.16	2.735 ± 0.14	2.842 ± 0.11	2.667 ± 0.23	3.307 ± 0.32#

Mean values ± standard deviation are presented
pf Post fertilisation
* Statistically significantly different to the control, $p<0.05$, Williams test, one-sided smaller
# Statistically significantly different to the control, $p<0.05$, Williams test, one-sided greater

Table CA 8.2.2.2/03-4 Group A, P-generation: Reproduction

Endpoint	Mean measured initial concentration (µg a.s./L)				
	Control	15.8	30.4	63.9	255
Number of replicates	4	3	3	3	3
Time to regular spawning (days)	71 ± 1	70 ± 2	70 ± 1	69 ± 1	71 ± 2
Egg number per day and female	6 ± 3	6 ± 0	7 ± 2	5 ± 2	11 ± 1
Fertilisation rate (%)	97.4 ± 0.7	95.7 ± 2.1	97.8 ± 0.5	98.1 ± 0.2	97.0 ± 0.7

Mean values ± standard deviation are presented

Table CA 8.2.2.2/03-5 Group A, P-generation: Termination – survival, growth, sex ratio and biomarker

Endpoint	Mean measured initial concentration (µg a.s./L)				
	Control	15.8	30.4	63.9	255
Number of replicates	4	3	3	3	3
Survival (%)	99.2 ± 1.7	100 ± 0.0	97.7 ± 2.0	96.6 ± 3.3	100 ± 0.0
Length males (cm)	3.6 ± 0.1	3.6 ± 0.1	3.6 ± 0.1	3.5 ± 0.1	3.6 ± 0.1
Weight males (g)	0.410 ± 0.019	0.448 ± 0.042	0.414 ± 0.045	0.390 ± 0.009	0.439 ± 0.025
Length females (cm)	3.5 ± 0.1	3.5 ± 0.0	3.5 ± 0.0	3.4 ± 0.0	3.5 ± 0.2
Weight females (g)	0.424 ± 0.017	0.435 ± 0.009	0.420 ± 0.012	0.430 ± 0.023	0.485 ± 0.105
Sex ratio (% females)	60.4 ± 8.0	68.7 ± 9.8	54.2 ± 8.8	63.7 ± 1.0	23.3 ± 16.2*
Sex ratio (% males)	39.6 ± 8.0	31.3 ± 9.8	45.8 ± 8.8	36.3 ± 1.0	76.7 ± 16.2#
Vitellogenin males (ng/µg)	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.08 ± 0.12	0.20 ± 0.34
Vitellogenin females (ng/µg)	309.4 ± 142.1	277.3 ± 40.6	143.3 ± 101.2*	126.8 ± 33.4*	165.5 ± 3.3*

Mean values ± standard deviation are presented
* Statistically significantly different to the control, $p<0.05$, Williams test, one-sided smaller
# Statistically significantly different to the control, $p<0.05$, Williams test, one-sided greater

Table CA 8.2.2.2/03-6 Group A, F1-generation: Early life stage – hatch, survival and growth, day 28 pf

Endpoint	Mean measured initial concentration (µg a.s./L)				
	Control	15.8	30.4	63.9	255
Number of replicates	4	3	3	3	3
Number of eggs introduced	202	152	152	153	151
Survival, day 21 pf (%)	85.2 ± 9.0	75.0 ± 1.7	75.7 ± 8.9	63.4 ± 12.5*	61.5 ± 12.3*
Survival, day 28 pf (%)	82.7 ± 6.4	72.4 ± 2.1	75.1 ± 7.8	62.1 ± 11.4*	60.2 ± 13.4*
Length, day 28 pf (cm)	0.99 ± 0.04	0.94 ± 0.06	0.99 ± 0.01	1.03 ± 0.06	1.07 ± 0.09

Endpoint	Mean measured initial concentration (µg a.s./L)				
	Control	15.8	30.4	63.9	255
Group dry weight, day 28 pf (mg)	70.0 ± 4.2	50.7 ± 12.5	48.1 ± 7.0	59.7 ± 20.2	61.9 ± 19.8
Single dry weight, day 28 pf (mg)	1.7 ± 0.2	1.4 ± 0.3	1.3 ± 0.1	1.9 ± 0.4	2.1 ± 0.6

Mean values ± standard deviation are presented
* Statistically significantly different to the control, $p<0.05$, Williams test, one-sided smaller

Table CA 8.2.2.2/03-7 Group B, P-generation: Juvenile growth: survival and growth, day 56 pf

Endpoint	Mean measured initial concentration (µg a.s./L)				
	Control	15.8	30.4	63.9	255
Number of replicates	4	3	3	3	3
Number of eggs introduced	120	91	90	91	90
Survival between day 28 and 56 pf (%)	99.2 ± 1.7	96.8 ± 5.6	95.6 ± 1.9	98.9 ± 1.9	98.9 ± 1.9
Length, day 56 pf (cm)	2.49 ± 0.10	2.30 ± 0.10*	2.25 ± 0.09*	2.39 ± 0.05*	2.00 ± 0.14*
Pseudo specific growth rate (based on length)	4.793 ± 0.22	4.540 ± 0.08	4.515 ± 0.17	4.746 ± 0.11	3.900 ± 0.27*

Mean values ± standard deviation are presented
pf Post fertilisation
* Statistically significantly different to the control, $p<0.05$, Williams test, one-sided smaller

Table CA 8.2.2.2/03-8 Group B, P-generation: Reproduction

Endpoint	Mean measured initial concentration (µg a.s./L)				
	Control	15.8	30.4	63.9	255
Number of replicates	4	3	3	3	3
Time to regular spawning (days)	68 ± 1	71 ± 1	73 ± 2	69 ± 1	71 ± 0
Egg number per day and female	14 ± 3	15 ± 2	15 ± 3	13 ± 3	17 ± 4
Fertilisation rate (%)	94.1 ± 2.3	96.2 ± 0.5	94.6 ± 0.6	95.2 ± 0.3	96.3 ± 1.6

Mean values ± standard deviation are presented

Table CA 8.2.2.2/03-9 Group B, P-generation: Termination – survival, growth, sex ratio and biomarker

Endpoint	Mean measured initial concentration (µg a.s./L)				
	Control	15.8	30.4	63.9	255
Number of replicates	4	3	3	3	3
Survival (%)	97.5 ± 1.7	98.9 ± 1.9	96.4 ± 6.2	100 ± 0.0	93.3 ± 6.7
Length males (cm)	3.7 ± 0.04	3.7 ± 0.08	3.7 ± 0.09	3.6 ± 0.09	3.5 ± 0.06*
Weight males (g)	0.426 ± 0.03	0.430 ± 0.03	0.425 ± 0.03	0.396 ± 0.03	0.408 ± 0.02
Length females (cm)	3.6 ± 0.03	3.5 ± 0.05	3.5 ± 0.05	3.4 ± 0.04*	3.4 ± 0.15
Weight females (g)	0.458 ± 0.03	0.418 ± 0.01	0.425 ± 0.04	0.383 ± 0.01*	0.398 ± 0.05*
Sex ratio (% females)	53.6 ± 10.4	41.3 ± 8.3	45.1 ± 10.8	37.4 ± 2.3*	19.2 ± 8.1*
Sex ratio (% males)	46.5 ± 10.4	58.7 ± 8.3	54.9 ± 10.8	62.6 ± 2.3#	80.8 ± 8.1#
Vitellogenin males (ng/µg)	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
Vitellogenin females (ng/µg)	221.7 ± 67.0	180.7 ± 47.5	213.0 ± 6.3	171.0 ± 81.3	281.7 ± 35.0

Mean values ± standard deviation are presented
* Statistically significantly different to the control, $p<0.05$, Williams test, one-sided smaller
\# Statistically significantly different to the control, $p<0.05$, Williams test, one-sided greater

Table CA 8.2.2.2/03-10 Group B, F_1-generation: Early life stage – hatch, survival and growth, day 28 pf

Endpoint	Mean measured initial concentration (µg a.s./L)				
	Control	**15.8**	**30.4**	**63.9**	**255**
Number of replicates	4	3	3	3	3
Number of eggs introduced	200	150	152	153	152
Survival, day 21 pf (%)	87.5 ± 1.0	80.7 ± 7.0	84.9 ± 6.0	77.1 ± 5.2*	75.7 ± 2.7*
Survival, day 28 pf (%)	86.5 ± 1.9	80.7 ± 7.0	84.2 ± 7.0	74.5 ± 3.8*	75.7 ± 2.7*
Length, day 28 pf (cm)	0.99 ± 0.04	0.94 ± 0.06	0.99 ± 0.01	1.03 ± 0.06	1.07 ± 0.09
Group dry weight, day 28 pf (mg)	63.3 ± 22.6	53.3 ± 10.9	50.6 ± 15.1	51.7 ± 20.0	68.6 ± 8.9
Single dry weight, day 28 pf (mg)	1.5 ± 0.5	1.3 ± 0.2	1.2 ± 0.4	1.4 ± 0.6	1.8 ± 0.3

Mean values ± standard deviation are presented

* Statistically significantly different to the control, *p*<0.05, Williams test, one-sided smaller

Table CA 8.2.2.2/03-11 Group C, P-generation: Reproduction

Endpoint	Mean measured initial concentration (µg a.s./L)				
	Control	**15.8**	**30.4**	**63.9**	**255**
Number of replicates	4	3	3	3	3
Egg number per day and female	11 ± 2	12 ± 3	13 ± 1	13 ± 0	15 ± 5
Fertilisation rate (%)	95.1 ± 2.2	94.0 ± 3.6	95.6 ± 0.8	96.0 ± 0.9	96.5 ± 0.5

Mean values ± standard deviation are presented

Table CA 8.2.2.2/03-12 Group C, P-generation: Termination – survival, growth, sex ratio and biomarker

Endpoint	Mean measured initial concentration (µg a.s./L)				
	Control	**15.8**	**30.4**	**63.9**	**255**
Number of replicates	4	3	3	3	3
Survival (%)	98.4 ± 1.9	95.6 ± 7.7	95.5 ± 3.9	95.6 ± 5.1	93.2 ± 6.9
Length males (cm)	4.0 ± 0.09	4.0 ± 0.04	4.1 ± 0.06	4.1 ± 0.05	4.1 ± 0.10
Weight males (g)	0.499 ± 0.03	0.517 ± 0.01	0.520 ± 0.02	0.539 ± 0.01	0.514 ± 0.02
Length females (cm)	4.0 ± 0.08	4.1 ± 0.04	4.0 ± 0.05	4.0 ± 0.01	4.1 ± 0.05
Weight females (g)	0.623 ± 0.06	0.659 ± 0.10	0.569 ± 0.03	0.608 ± 0.02	0.639 ± 0.05
Sex ratio (% females)	49.7 ± 15.4	37.4 ± 6.5	38.9 ± 6.2	38.2 ± 9.4	41.2 ± 8.1
Sex ratio (% males)	50.3 ± 15.4	62.6 ± 6.5	61.1 ± 6.2	61.8 ± 9.4	58.8 ± 8.1
Vitellogenin males (ng/µg)	0.02 ± 0.0	0.05 ± 0.01	0.04 ± 0.03	0.03 ± 0.02	0.03 ± 0.01
Vitellogenin females (ng/µg)	333.3 ± 65.9	245.0 ± 23.7	343.7 ± 96.8	217.5 ± 15.9	311.4 ± 96.3

Mean values ± standard deviation are presented

Table CA 8.2.2.2/03-13 Group C, F_1-generation: Early life stage – hatch, survival and growth, day 28 pf

Endpoint	Mean measured initial concentration (µg a.s./L)				
	Control	**15.8**	**30.4**	**63.9**	**255**
Number of replicates	4	3	3	3	3
Number of eggs introduced	199	152	151	151	152
Survival, day 21 pf (%)	92.9 ± 4.9	88.8 ± 4.6	82.8 ± 3.9	45.6 ± 9.7*	5.2 ± 9.1*
Survival, day 28 pf (%)	92.9 ± 4.9	88.8 ± 4.6	82.8 ± 3.9*	37.8 ± 0.4*	5.2 ± 9.1*
Length, day 28 pf (cm)	1.18 ± 0.08	1.17 ± 0.06	1.16 ± 0.08	1.23 ± 0.17	1.31[1]
Group dry weight, day 28 pf (mg)	147 ± 30.7	139 ± 28.6	123 ± 32.2	75.0 ± 34.6*	40.0* [1]

Endpoint	Mean measured initial concentration (µg a.s./L)				
	Control	15.8	30.4	63.9	255
Single dry weight, day 28 pf (mg)	3.2 ± 0.9	3.1 ± 0.5	2.9 ± 0.7	3.9 ± 1.8	5.0[1]

Mean values ± standard deviation are presented
* Statistically significantly different to the control, $p<0.05$
1 Surviving fish larvae could only be observed at replicate B at 255 µg/L. Thus, only one replicate value is presented.

A summary of the resulting endpoints, based on mean measured concentrations, is presented in the table below:

Table CA 8.2.2.2/0-14 Summary of endpoints for each group

Group A (introduced as fertilised eggs)		Group B (introduced as juveniles)		Group C (introduced as adults)	
***Life phase,* Parameter**	**NOEC (µg/L)**	***Life phase,* Parameter**	**NOEC (µg/L)**	***Life phase,* Parameter**	**NOEC (µg/L)**
Parental, early life stage		*Parental, juvenile growth*		*Reproduction*	
Hatching	-[1]	Survival	≥255	Egg number/ female/day	≥255
Survival	30.4	Length [2] growth	63.9	Fertilisation rate	≥255
Length	63.9	*Reproduction*		*Filial, early life stage*	
Juvenile growth		Time to regular spawning	≥255	Survival [4]	EC_{10}: 23.3
Survival	≥255	Egg number/ female/day	≥255	Length, weight	≥255
Length, growth	63.9	Fertilisation rate	≥255	*Termination, parental adults*	
Reproduction		*Filial, early life stage*		Survival	≥255
Time to regular spawning	≥255	Survival	30.4	Sex ratio	≥255
Egg number/ female/day	≥255	Length, weight	≥255	Length, weight	≥255
Fertilisation rate	≥255	*Termination, parental adults*		Biomarker VTG	≥255
Filial, early life stage		Survival	≥255	-	-
Survival	30.4	Sex ratio	30.4		
Length, weight	≥255	Length, male	≥255		
Termination, parental adults		Length, female	≥255		
Survival	≥255	Weight, male [3]	≥255		
Sex ratio	63.9	Weight, female	30.4		
Length, weight	≥255	Biomarker VTG	≥255		
Biomarker VTG	15.8	-			

1 Due to the presence of sediment in the fry chambers, it was not possible to monitor hatching success.
2 The statistical evaluation of length at day 56 revealed a significant difference to the control at ≥15.8 µg spiroxamine/L. However, the calculated difference of the single treatment levels were found to be <10% compared to control at 15.8, 30.4 and 63.9 µg/L, with 7.3%, 9.3% and 3.9% effect, respectively. Since variation between the single replicates was very low, the statistical test was able to detect a significant difference already for a small decrease in length. Moreover, no clear dose response relationship of the fish length could be observed within the tested concentration range. At 63.9 µg/L, only a minor deviation from the control was found (3.9%).
3 A slight but significant decrease of male lengths could be observed at 255 µg/L. However, this effect was less than 5%, compared to the control males, and thus can be considered to be not biologically relevant.

Group A (introduced as fertilised eggs)		Group B (introduced as juveniles)		Group C (introduced as adults)	
Life phase, Parameter	NOEC (µg/L)	*Life phase,* Parameter	NOEC (µg/L)	*Life phase,* Parameter	NOEC (µg/L)

[4] In the filial generation of the parental fish, introduced as adults, the decrease of survival was >50% (related to the number of introduced eggs) compared to control and the effects showed a clear dose-related response. An EC_{10} was therefore able to be calculated.

III. Conclusion

The survival of F_1 fish larvae was interpreted as the most sensitive population relevant endpoint.

The corresponding effect concentration (EC_{10}) was calculated to be 23.3 µg a.s./L.

Beside larval survival, the sex ratio after juvenile exposure as well as the growth of adult females showed similar sensitivity (NOEC: 30.4 µg a.s./L). A decrease of the concentration of the biomarker vitellogenin could be observed at ≥30.4 µg a.s./L thereby giving a NOEC for this parameter of 15.8 µg a.s./L. The histopathological evaluation of the fish gonads revealed no substance-related effect.

Assessment and conclusion by applicant:

Validity criteria according to the study report were met:

- Survival rate in the controls was greater than 70%
- Dissolved oxygen concentration to be >60% saturation throughout the test (actual: 82 to 125%)
- Water temperature was within 25 ± 2°C throughout the study

The study report listed it's own validity criteria (see above) and these were considered to have been achieved. However, validity criteria according to the most up-to-date OECD 210 (2013) test guideline have also been assessed and the following criteria were met:

- Hatching rate in the control replicates was >70%
- Post-hatch survival in the control replicates was ≥75%
- Dissolved oxygen concentration to be ≥60% in all test vessels throughout the test (actual: 82 – 125%)

One of the criteria were not met but this was a very minor deviation:

- Water temperature to be at 26 ± 1.5°C during the test (actual: 24.0 – 26.9°C).

The study is therefore considered acceptable. It is acknowledged that this was a non-standard test design but the study was considered to have been successfully conducted in accordance with its aims. The use of this study in a refined risk assessment has been discussed in Document M-CP Section 10.

The lowest NOEC determined in the study was 15.8 µg a.s./L.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.2.2.2/05
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10 and EC20 values for Danio rerio with spiroxamine TG in a full life cycle test
Report No:	0471836-ECO18
Document No:	M-760412-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
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 M-467979-03-1 on the effects of spiroxamine TG in the zebrafish (*Danio rerio*) full life cycle test did not provide estimates of EC_{10} or EC_{20}. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013. Due to the lack of effects above 10% when compared to the control, the EC_{10} and EC_{20} values for Group A F_0 survival at 56 days post fertilization (dpf), length at 56 dpf, fertility, survival at termination, length at termination (males and females), weight at termination (males and females), F_1 length at 28 dpf, Group B F_0 length at 28 dpf, survival at 56 dpf, egg number per day and female, fertility, survival at termination, length at termination (males and females), weight at termination (males), F_1 length at 28 dpf, Group C F_0 egg number per day and female, fertility, survival at termination, length at termination (males), weight at termination (males and females) and F_1 weigh at 28 dpf, were estimated to be > 255 µg a.s./L. For the remaining parameters, no reliable EC_{10} or EC_{20} values were possible to calculate.

I. Methods

The statistical evaluation was performed with statistical software ToxRat Professional v3.3.0.

Group A

The determination of reliable EC_{10} and EC_{20} values for F_0 length after 28 days, pseudo specific growth rate, eggs per female per day, sex ratio and F_1 survival at day 21 and day 28 and F_1 weight at day 28 was not possible as the obtained results do not fit with the raw data due to the lack of a significant monotone dose response. Due to the lack of effects above 10% when compared to the control on F_0 survival after 56 days, fertility, survival at test termination, length at termination (males and females), weight (males and females), length after 56 dpf and F_1 total length after 28 days, the EC_{10} and EC_{20} values are estimated to be > 255 µg a.s./L. The determination of reliable EC_{10} and EC_{20} values for F_0 survival after 21 and 28 days was not possible due to a poor goodness of fit in the data.

Group B

Due to the lack of effects above 10% when compared to the control on F_0 length after 28 days, survival at 56 days, eggs per female per day, fertility, survival at termination, length of males and females at termination, weight of males at termination and F_1 total length at 28d, the EC_{10} and EC_{20} values are estimated to be > 255 µg a.s./L. The determination of reliable EC_{10} and EC_{20} values for F_0 total length at 56 days, pseudo specific growth rate, sex ratio based on male, weight of females at termination, F_1 survival after 21 and 28 days and weight at 28 days was not possible due to the lack of a significant monotone dose response. Due to the lack of effects below 10% on F_0 sex ratio based on female on all treatments, no reliable EC_{10} value could be calculated inside the test concentration range. Therefore, no EC_{10} is presented for F_0 sex ratio based on female.

Group C

Due to the lack of effects above 10% when compared to the control on F_0 eggs per female per day, fertility, survival at termination, male length at termination, male and female weight at termination, F_1 weight at 28dpf, EC_{10} and EC_{20} values are estimated to be >255 µg a.s./L.

The determination of reliable EC_{10} and EC_{20} values for F0 sex ratio based on females and males, female length at termination and F_1 total length at 28dpf was not possible due to the lack of a significant monotone dose response.

The determination of reliable EC_{10} and EC_{20} values for F_1 survival after 21 and 28 days was not possible due to a poor goodness of fit in the data and the data scattering around the dose response.

II. Results

Due to the lack of effects above 10% when compared to the control, the EC_{10} and EC_{20} values for Group A F_0 survival at 56 dpf, length at 56 dpf, fertility, survival at termination, length at termination (males and females), weight at termination (males and females), F_1 length at 28 dpf, Group B F_0 length at 28 dpf, survival at 56 dpf, egg number per day and female, fertility, survival at termination, length at termination (males and females), weight at termination (males), F_1 length at 28 dpf, Group C F_0 egg number per day and female, fertility, survival at termination, length at termination (males), weight at termination (males and females) and F_1 weight at 28 dpf, were estimated to be >255 µg a.s./L. For the remaining parameters, no reliable EC_{10} or EC_{20} values were possible to calculate.

A summary of the obtained endpoints is presented in the following table.

Table CA 8.2.2.2/05-1 Overall endpoints of the statistical re-calculation of the *D.rerio* study with spiroxamine

Parameter	Endpoint (µg a.s./L) EC_{10} (95% confidence intervals)
Group A	
Parental larvae (F_0)	
Survival at 21 dpf	n.d.*
Survival at 28 dpf	n.d.*
Length at 28 dpf	n.d.
Survival at 56 dpf	>255
Length at 56 dpf	>255
Pseudo specific growth rate	n.d.
Egg number per day and female	n.d.
Fertility (hatching success)	>255
Survival at termination	>255
Sex ratio (males)	n.d.
Sex ratio (females)	n.d.
Length at termination (males)	>255
Length at termination (females)	>255
Weight at termination (males)	>255
Weight at termination (females)	>255
Vitellogenin in males	n.a.
Vitellogenin in females	n.a.
F1 generation	
Survival at 21dpf	n.d.
Survival at 28dpf	n.d.
Length at 28 dpf	>255
Weight at 28 dpf	n.d.
Group B	
Parental larvae (F0)	
Length at 28 dpf	>255
Survival at 56 dpf	>255

Parameter	Endpoint (µg a.s./L)
	EC_{10} (95% confidence intervals)
Length at 56dpf	n.d.
Pseudo specific growth rate	n.d.
Egg number per day and female	>255
Fertility (fertilisation rate)	>255
Survival at termination	>255
Sex ratio (males)	n.d.
Sex ratio (females)	n.d.
Length at termination (males)	>255
Length at termination (females)	>255
Weight at termination (males)	>255
Weight at termination (females)	n.d.
Vitellogenin in males	n.a.
Vitellogenin in females	n.a.
F1 generation	
Survival at 21 dpf	n.d.
Survival at 28 dpf	n.d.
Length at 28 dpf	>255
Weight at 28 dpf	n.d.
Group C	
Parental larvae (F_0)	
Egg number per day and female	>255
Fertility (fertilisation rate)	>255
Survival at termination	>255
Sex ratio (males)	n.d.
Sex ratio (females)	n.d.
Length at termination (males)	>255
Length at termination (females)	n.d.
Weight at termination (males)	>255
Weight at termination (females)	>255
Vitellogenin in males	n.a.
Vitellogenin in females	n.a.
F1 generation	
Survival at 21 dpf	n.d.*
Survival at 28 dpf	n.d.*
Length at 28 dpf	n.d.
Weight at 28 dpf	>255

Due to the lack of a concentration dose response when compared to the control, the calculation of EC_{10} and EC_{20} values for the parameters marked in the above table as "n.d." was not possible and therefore no EC_{10} or EC_{20} values were determined. The determination of reliable EC_{10} and EC_{20} values for parameters marked with a * was not possible due to a poor goodness of fit in the data and the data scattering around the dose response. ** Due to variability within the data set and the observation of effects in all concentrations, there is uncertainty in the NOEC determined by the statistical software, ToxRat Professional.

As vitellogenin concentration is a non-apical endpoint, this parameter was not deemed relevant for further analysis and therefore EC_x values were not determined for both male and female fish.

III. Conclusion

Due to the lack of effects above 10% when compared to the control, the EC_{10} and EC_{20} values for Group A F_0 survival at 56 dpf, length at 56 dpf, fertility, survival at termination, length at termination (males and females), weight at termination (males and females), F_1 length at 28 dpf, Group B F_0 length at 28 dpf, survival at 56 dpf, egg number per day and female, fertility, survival at termination, length at termination (males and females), weight at termination (males), F_1 length at 28 dpf, Group C F_0 egg number per day and female, fertility, survival at termination, length at termination (males), weight at

termination (males and females) and F_1 weigh at 28 dpf, were estimated to be > 255 µg a.s./L. For the remaining parameters, no reliable EC_{10} or EC_{20} values were possible to calculate.

Assessment and conclusion by applicant:

The statistical re-evaluation of the data did not allow for the determination of reliable EC_{10} and EC_{20} values for many of the parameters assessed. This was largely due to the absence of an adequate dose-response or due to poor fit of the data.

The lowest endpoint determined remains the NOEC of 15.8 µg a.s./L based on effects on VTG in female fish therefore this continues to be the critical endpoint determined for this study.

The values determined in the re-evaluation work are considered to be fully valid.

CA 8.2.2.3 Bioconcentration in fish

Data Point:	KCA 8.2.2.3/01
Report Author:	[redacted]
Report Year:	1995
Report Title:	KWG 4168: Bioconcentration in bluegill sunfish
Report No:	BF-011
Document No:	M-006479-01-1
Guideline(s) followed in study:	HAMELINK, J.L., "Current Bioconcentration Test Methods and Theory" in: Aquatic Toxicology and Hazard Evaluation, ASTM publication STP 634, 149 - 161, 1977
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Spiroxamine has a Log P_{ow} of 2.79 and 2.98 at pH 7 for diastomers A and B, respectively but at pH 9 these value are 4.88 and 5.08, respectively. A fish bioconcentration study with spiroxamine has therefore been conducted and has been summarised below.

The Log P_{ow} of spiroxamine-desethyl (M01) is 2.41, 1.97 and >3.64 at pH 4, 7 and 9, respectively. The Log P_{ow} of spiroxamine-despropyl (M02) is 1.95, 1.41 and >3.44 at pH 4, 7 and 9, respectively. The Log P_{ow} of spiroxamine-N-oxide (M03) is 2.82, 2.59 and 2.63 at pH 4, 7 and 9, respectively. The Log P_{ow} of spiroxamine-carboxylic acid (M06) is 0.45, -0.25 and 0.10 at pH 4, 7 and 9, respectively. Thus, an assessment of the potential risk from bioconcentration of spiroxamine-desethyl (M01) and spiroxamine-despropyl (M02) also needs to be addressed in the risk assessment.

Executive Summary

The objective of the bioconcentration study was to measure uptake and depuration of [^{14}C]-KWG 4168 in bluegill sunfish by determining, if possible, its uptake rate constant (K_1), depuration rate constant (K_2) and steady-state bioconcentration factor (BCF).

KWG 4168 was accumulated by bluegill sunfish with a maximum total residue bioconcentration factor of 87 for whole fish. Kinetic modelling yielded BCF values of 31 and 24 (edible parts) for the 20 µg [^{14}C]-KWG 4168/L and 200 µg [^{14}C]-KWG 4168/L groups, respectively and BCF values of 87 and 71 (whole fish) for the 20 µg [^{14}C]-KWG 4168/L and 200 µg [^{14}C]-KWG 4168/L groups, respectively.

The kinetic BCF values for edible parts and whole fish corresponded well with the respective average steady-state (days 7 - 28) bioconcentration factors of 25.8 (edible parts) and 86.2 (whole fish) for 20 µg

[^{14}C]-KWG 4168/L and of 22.3 (edible parts) and 64.6 (whole fish) for 200 µg [^{14}C]-KWG 4168/L, respectively.

When exposure ceases, the residues are depurated very quickly with a half-life of approximately 13 to 19 hours (0.55 to 0.78 days, respectively).

I. Materials and Methods

A. Materials

Test Material	KWG 4168
Lot/Batch #:	Lager Nr. 8686/B
Radiochemical purity:	>99%
Description:	Clear, early liquid
Stability of test compound:	Not reported
Reanalysis/Expiry date:	Not reported
Density:	Not reported
Treatments	
Test rates:	20 and 200 µg a.s./L
Solvent/vehicle:	Triethyleneglycol
Analysis of test concentrations:	Exposure phase days: 0, 1, 3, 7, 10, 14, 21 and 28 Depuration phase days: 29, 31, 35, 38 and 42
Test organisms	
Species:	Bluegill sunfish (*Lepomis macrochirus*)
Source:	[redacted]
Acclimatisation period:	16-hour daylight photoperiod and observed for at least 14 days prior to testing. No mortality was noted 14 days prior to the test initiation and all unsuitable fish (*i.e.*, injured, deformed, *etc.*) were eliminated from inclusion in the test prior to assignment to test groups
Feeding:	During the acclimation and test periods, the fish received once daily *ad libitum* a standard fish-feed (Kronen-FB 50E, manufactured by Rheinkrone, D-4230 Wesel as batch no. WAJ 1). The feed was analysed for unwanted contaminants
Treatment for disease:	Not reported
Test design	
Test vessel:	100 litre test aquaria
Test water:	Aerated reconstituted water
Replication:	Single
No. of animals/vessel:	56

Duration of test:	28 days exposure and 13 days depuration phase
Environmental test conditions	
Temperature:	21 – 23°C
Dissolved oxygen:	82 – 118%
pH:	6.7 – 7.5
Photoperiod:	16-hour daylight photoperiod

B. Study Design

The objective of the bioconcentration study was to measure uptake and depuration of [^{14}C]-KWG 4168 by determining, if possible, its uptake rate constant (K_1), depuration rate constant (K_2) and steady-state bioconcentration factor (BCF).

The uptake phase was initiated by transferring groups of 56 randomly selected and previously acclimated fish to each of the control and test chambers. The initial loading was 2.9 g fish/L and 0.48 g fish/L/day (calculated from the mean bodyweights of sampled fishes over the whole exposure period). The fish were observed initially and every 24 hours on working days thereafter during the exposure period of 28 days for mortality and/or adverse behaviour. At the same intervals pH, temperature and dissolved oxygen were measured in all aquaria. Additionally the daily temperature fluctuation was controlled continuously in the control tank by a mercury-minimum-maximum-thermometer for detection of technical defects.

On day 28 of the exposure period, the addition of the [^{14}C]-KWG 4168 test material ceased. At the beginning of the depuration phase, the aquaria were cleaned mechanically, emptied by suction to a water height of ca. 5 cm, and filled with uncontaminated and temperated (22°C) diluent water. During that procedure the fish remained in the aquaria. The fish were then exposed to flowing uncontaminated diluent water for 14 days. The chemical and physical water parameters during depuration phase were recorded as described for the uptake phase.

Fish were sampled during the uptake phase on days 0, 1, 3, 7, 10, 14, 21 and 28 and during the depuration phase on days 29, 31, 35, 38 and 42. On each occasion, four fish from each chamber were collected and processed individually. The fish were dissected into edible (body muscle, skin, skeleton) and viscera / nonedible (head, fins, internal organs). Samples were transferred into weighed polystyrene vials suitable for further handling. After determining the wet weight of the samples they were frozen, lyophilised, reweighed, and homogenised.

On each sampling day, three samples of 7 mL of water were removed from each aquarium. The concentrations of ^{14}C calculated as [^{14}C]-KWG 4168 in water were calculated by liquid scintillation counting of triplicate 7 mL samples pipetted directly from each control and test tank. To each sample 7 mL scintillation cocktail (United Technologies Packard Instant Scint. Gel) were added.

The term uptake rate constant (K_1) as used in this report is the mathematically determined value that is used to define the uptake of test material by exposed test organisms. The depuration rate constant (K_2) is defined as the mathematically determined value of the depuration of test material from previously exposed test animals when placed in untreated dilution water. The steady-state bioconcentration factor (BCF) is the ratio of the test substance concentration in the whole fish (C_F) and the concentration in the test water (Cw) at steady-state (apparent plateau) or the ratio of K_1 and K_2.

II. Results and Discussion

The study was conducted to the HAMELINK, J.L., "Current Bioconcentration Test Methods and Theory" in: Aquatic Toxicology and Hazard Evaluation, ASTM publication STP 634, 149 -161, 1977. Validity criteria under the guideline used at the time that this test was conducted are not available

therefore the study has been assessed against the criteria in the current OECD 305 test guideline (2012) which have all been met:

- The water temperature variation is less than ± 2°C, because large deviations can affect biological parameters relevant for uptake and depuration as well as cause stress to animals
- The concentration of dissolved oxygen does not fall below 60% saturation
- The concentration of the test substance in the chambers is maintained within ± 20% of the mean measured values during the uptake phase
- The concentration of the test substance is below its limit of solubility in water, taking into account the effect that the test water may have on effective solubility (Actual: yes)
- The mortality or other adverse effects/disease in both control and treated fish is less than 10% at the end of the test; where the test is extended over several weeks or months, death or other adverse effects in both sets of fish should be less than 5% per month and not exceed 30% in all. Significant differences in average growth between the test and the control groups of sampled fish could be an indication of a toxic effect of the test chemical

A control analysis of stock-solutions containing 214 µg a.s./L and 1666 µg a.s./L showed that KWG 4168 was stable over a period of 4 weeks in triethyleneglycol which was used as solvent. Therefore, the stability of the ^{14}C-labelled test substance in the stock solutions can be assumed.

Water concentrations ranged through 28 days of the bioconcentration (uptake) phase from 17.7 µg a.s./L to 20.9 µg a.s./L for the nominal concentration of 20 µg a.s./L and from 181.3 µg a.s./L to 196.7 µg a.s./L for the nominal concentration of 200 µg a.s./L. The average water concentration (using the mean value for each sample) during the uptake phase was 18.9 (± 1.0) µg a.s./L for the nominal concentration of 20 µg a.s./L and 186.9 (± 5.7) µg a.s./L for the nominal concentration of 200 µg a.s./L. These concentrations compared well with the expected nominal concentrations of 20 µg a.s./L and 200 µg a.s./L for [^{14}C]-KWG 4168.

The specific radioactivity of [^{14}C]-KWG 4168 was 5416.8 dpm/µg test substance for the 20 µg a.s./L level and 548.4 dpm/µg test substance for the 200 µg a.s./L level.

Table CA 8.2.2.3/01-1 Measured concentrations of KWG 4168 in test medium

Sampling day	Control group			20 µg test substance / L				200 µg test substance / L			
	dpm in water per 7 mL sample			dpm in water per 7 mL sample			µg a.s./L water	dpm in water per 7 mL sample			µg a.s./L water
	1	2	3	4	5	6	Mean	7	8	9	Mean
Exposure											
0	1.4	1.5	0.5	676.7	672.4	670.7	17.73	761.7	774.2	732.8	196.70
1	-2.4	-3.6	-4.5	726.4	697.1	687.6	18.65	684.9	691.2	709.0	181.97
3	-2.8	-6.2	-4.8	749.5	713.4	711.6	19.13	707.9	693.3	729.7	185.15
7	-3.2	-4.5	-1.4	726.3	739.7	716.8	19.19	731.5	703.8	734.0	188.36
10	-2.0	-3.4	-4.5	704.9	734.7	716.6	19.04	752.3	735.4	734.9	193.85
14	-4.0	-1.4	-0.5	702.0	690.8	672.7	18.21	698.6	699.9	683.3	181.28
21	-3.3	-4.8	-6.9	678.4	706.0	683.6	18.31	726.2	693.3	673.8	183.07
28	0.4	-2.6	-4.5	803.4	782.9	796.0	20.88	719.7	722.1	694.7	184.87
Depuration											
29	-1.5	-1.4	-1.0	43.5	21.9	22.6	<1	22.9	8.0	5.7	12.2
34	-1.5	-2.6	-3.6	-0.1	13.4	-4.6	<1	-3.7	2.7	0.3	<1
35	-1.4	-4.6	-2.9	6.7	-3.7	-2.9	<1	-1.6	-0.4	-1.3	<1
38	-2.3	-4.0	-2.9	-3.8	-2.8	3.5	<1	-5.0	-1.5	-3.6	<1
42	-4.5	-4.7	-4.5	0.0	-3.7	7.3	<1	0.6	-0.2	-3.2	<1

Table CA 8.2.2.3/01-2 Fresh weight of whole fish (edible parts and viscera) (g)

Sampling day	Control mean	20 µg/L mean	200 µg/L mean
0	6.9	10.1	10.0
1	5.4	5.2	3.9
3	5.3	5.1	3.0
7	3.4	3.2	4.1
10	7.0	4.5	2.9
14	4.5	3.5	4.3
21	5.0	3.9	7.3
28	4.7	5.4	5.7
29	6.5	6.6	6.0
31	6.2	5.8	4.6
35	8.8	9.4	7.5
38	6.5	7.4	4.7
42	4.3	3.8	4.3

Table CA 8.2.2.3/01-3 Radioactivity in whole fish (dpm/g fresh weight)

Sampling day	Control mean	20 µg/L mean	200 µg/L mean
1	15.6	7700.5	6101.6
3	9.6	11767.3	8274.2
7	40.2	8842.3	8904.7
10	38.7	6460.7	4102.3
14	88.2i	9175.4	5463.1
21	280.9	11784.0	6490.5
28	105.8is	7959.1	8343.7
29	62.8	6208.5	4575.1
31	91.6	14408	764.3
35	106.0	1112.7	288.1
38	121.5	889.1	317.3
42	89.1	851.6	211.3

Table CA 8.2.2.3/01-4 Bioconcentration factors for whole fish (calculated from edible part and viscera)

Sampling day	20 µg/L mean	200 µg/L mean
1	78.0	61.8
3	117.3	82.5
7	87.0	87.6
10	63.2	40.0
14	89.9*	53.2
21	114.2	61.4
28	76.8	80.5
29	60.1	44 .1
31	13.2	6.6
35	9.8	1.8
38	7.5	1.9
42	7.4	1.2

Table CA 8.2.2.3/01-5 Uptake and depuration of radioactivity, whole fish (calculated from edible part and viscera / all values related to steady-state mean values)

Sampling day	% Radioactivity relative to fresh weight				% Radioactivity relative to dry weight			
	20 µg/L mean		200 µg/L mean		20 µg/L mean		200 µg/L mean	
	Uptake	Depurated	Uptake	Depurated	Uptake	Depurated	Uptake	Depurated
1	65.4	-	68.7	-	64.7	-	66.9	-
3	100.0	-	93.2	-	98.5	-	93.3	-
7	74.9	-	100.0	-	73.3	-	100.0	-

Sampling day	% Radioactivity relative to fresh weight				% Radioactivity relative to dry weight			
	20 µg/L mean		200 µg/L mean		20 µg/L mean		200 µg/L mean	
	Uptake	Depurated	Uptake	Depurated	Uptake	Depurated	Uptake	Depurated
10	54.6	-	45.8	-	52.3	-	44.4	-
14	77.3	-	60.6	-	75.6	-	59.2	-
21	97.8	-	70.0	-	100.0	-	70.4	-
28	66.8	-	92.9	-	67.1	-	94.2	-
29	-	47.7	-	49.1	-	49.1	-	51.4
31	-	88.5	-	92.4	-	92.4	-	92.8
35	-	91.4	-	97.9	-	97.9	-	98.2
38	-	93.5	-	97.8	-	97.8	-	97.8
42	-	93.5	-	98.6	-	98.6	-	98.7

Table CA 8.2.2.3/01-6 Summary of derived values from the kinetic modelling (using BIOFAC (2))

Parameter	20 µg [^{14}C]-KWG 4168/L		200 µg [^{14}C]-KWG 4168/L	
	Edible parts	Whole fish	Edible parts	Whole fish
Bioconcentration factor (BCF)	31 (± 9.2)	87 (± 8.7)	24 (± 10)	71 (± 23)
Time to reach 90% of steady state (days)	2.1 (± 0.42)	1.8 (± 0.15)	3.6 (± 1.0)	2.6 (± 0.59)
t(½) for clearance (days)	0.63 (± 0.13)	0.55 (± 0.05)	1.1 (± 0.31)	0.78 (± 0.18)
Uptake rate constant K_1 (1/day)	34 (± 7.5)	109 (± 6)	15 (± 4.4)	64 (± 15)
Clearance rate constant K_2 (1/day)	1.1 (± 0.22)	1.3 (± 0.11)	0.6 (± 0.18)	0.9 (± 0.20)

The BIOFAC calculated BCF values for edible parts and whole fish corresponded well with the respective average steady-state (days 7 - 28) bioconcentration factors of 25.8 (edible parts) and 86.2 (whole fish) for 20 µg [^{14}C]-KWG 4168/L and of 22.3 (edible parts) and 64.6 (whole fish) for 200 µg [^{14}C]-KWG 4168/L, respectively.

In viscera the following data were calculated:

- 20 ug [14C]-KWG 4168/1 (nominal):

BCF: 165 (± 48); Time to reach 90% of steady-state: 2.6 (± 0.52) days, t(1/2) for

Clearance: 0.78 (± 0.16) days, Uptake Rate Constant (K1): 147 (± 30) 1/days,

Clearance Rate Constant (K2): 0.89 (± 0.18) 1/days.

- 200 ug [14q-KWG 4168/1 (nominal):

BCF: 122 (± 33); Time to reach 90% of steady-state: 2.5* (± 0.46) days, t(1/2) for

Clearance: 0.75 (± 0.14) days, Uptake Rate Constant (K1): 112 (± 23) 1/days,

Clearance Rate Constant (K2): 0.92 (± 0.17) 1/days.

After 14 days in uncontaminated water for 20 ug [14C]-KWG 4168/1 (nominal) 92, 94 and 94 percent of the mean plateau radioactivity was depurated from edible portions, nonedible portions and whole fish, while for 200 ug [14C]-KWG 4168/1 (nominal) these were 99, 99 and 99 percent of the mean measured plateau radioactivity.

III. Conclusion

The objective of the bioconcentration study was to measure uptake and depuration of [^{14}C]-KWG 4168 in bluegill sunfish by determining, if possible, its uptake rate constant (K_1), depuration rate constant (K_2) and steady-state bioconcentration factor (BCF).

KWG 4168 was accumulated by bluegill sunfish with a maximum total residue bioconcentration factor of 87 for whole fish. Kinetic modelling yielded BCF values of 31 and 24 (edible parts) for the 20 µg [^{14}C]-KWG 4168/L and 200 µg [^{14}C]-KWG 4168/L groups, respectively and BCF values of 87 and 71 (whole fish) for the 20 µg [^{14}C]-KWG 4168/L and 200 µg [^{14}C]-KWG 4168/L groups, respectively.

The kinetic BCF values for edible parts and whole fish corresponded well with the respective average steady-state (days 7 - 28) bioconcentration factors of 25.8 (edible parts) and 86.2 (whole fish) for 20 µg [^{14}C]-KWG 4168/L and of 22.3 (edible parts) and 64.6 (whole fish) for 200 µg [^{14}C]-KWG 4168/L, respectively.

When exposure ceases, the residues are depurated very quickly with a half-life of approximately 13 to 19 hours (0.55 to 0.78 days, respectively).

Assessment and conclusion by applicant:

The study was conducted in 1995 to an ASTM test guideline. The current version of the OECD 305 test guideline requires that BCF values be reported which have been corrected to a lipid content of 5%. At the time of study conduct, measurement of fish lipid content was not a requirement and was not therefore conducted as part of this test. Thus, lipid data are not available to be able to calculate BCF values which have been standardised to a 5% lipid content. Under the current guideline, kinetic BCF values can also be corrected for growth dilution and expressed in addition to the standard kinetic BCF values, however, growth corrected kinetic BCF values have also not been determined.

The study was conducted to the HAMELINK, J.L.: "Current Bioconcentration Test Methods and Theory" in: Aquatic Toxicology and Hazard Evaluation, ASTM publication STP 634, 149 -161, 1977.

Validity criteria according to the current test guideline OECD 305 (2012) were met:

- The water temperature variation is less than ± 2°C, because large deviations can affect biological parameters relevant for uptake and depuration as well as cause stress to animals
- The concentration of dissolved oxygen does not fall below 60% saturation
- The concentration of the test substance in the chambers is maintained within ± 20% of the mean measured values during the uptake phase
- The concentration of the test substance is below its limit of solubility in water, taking into account the effect that the test water may have on effective solubility (Actual: yes)
- The mortality or other adverse effects/disease in both control and treated fish is less than 10% at the end of the test, where the test is extended over several weeks or months, death or other adverse effects in both sets of fish should be less than 5% per month and not exceed 30% in all. Significant differences in average growth between the test and the control groups of sampled fish could be an indication of a toxic effect of the test chemical

Taking the above points into consideration, the bioconcentration study is still considered to be acceptable on the basis that it was conducted to a recognised test guideline at the time of conduct. The data are considered to be adequate to demonstrate the low potential of spiroxamine to bioaccumulate in aquatic organisms with a kinetic BCF value of only 87. Furthermore, to conduct a new bioconcentration study would be considered to be unnecessary vertebrate testing therefore it is considered acceptable to use the available data to confirm the low bioconcentration potential and short clearance time of spiroxamine.

The study is therefore considered acceptable.

A BCF value of 87 has been taken for the risk assessment.

Data Point:	KCA 8.2.2.3/02
Report Author:	[redacted]
Report Year:	1997
Report Title:	[Cyclohexyl-1-14C] KWG 4168: Metabolism in the edible parts of bluegill sunfish
Report No:	PF4215
Document No:	M-006169-01-1
Guideline(s) followed in study:	US EPA 165-4 Pesticide Accumulation in Fish
Deviations from current test guideline:	Not conducted to a specific guideline
Previous evaluation:	yes, evaluated and classified DAR (1997), RAR (2010), RAR (2017) Validation not possible (The study as such is acceptable considering the respective EPA test method. However, the cited test is not designed to determine a BCF.)
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 gain information on the nature of the residue in the edible parts of the fish and to quantify the metabolites to the extent possible.

A 42-day study was conducted to evaluate the bioconcentration of [cyclohexyl-1-^{14}C] KWG 4168 by bluegill sunfish (*Lepomis macrochirus*). Mean water concentrations of 20 and 200 µg KWG 4168/L, respectively, were maintained for a 28-day exposure period.

I. Study Design

A 42-day study was conducted to evaluate the bioconcentration of [cyclohexyl-1-^{14}C] KWG 4168 by bluegill sunfish (*Lepomis macrochirus*). Mean water concentrations of 20 and 200 µg KWG 4168/L, respectively, were maintained for a 28-day exposure period. Please refer to M-006479-01-1 for a full summary of the test.

Extraction and sample processing

For the high dose group the combined fish samples (28.02 g) were given to a suction filter. After addition of the respective extraction solution the sample was homogenised for three minutes using a Polytron homogeniser. Then the solution was filtered by applying a controlled vacuum. The volumes of the filtrates were determined and their radioactivity content was measured by liquid scintillation counting of aliquots. The extraction residue was dried under vacuum and the radioactivity determined after combustion. The further purification procedure was conducted by liquid partition and elution via a hydrophobic polyaromatic resin (Amberlite XAD-4, Sigma).

For the low dose group the combined samples of the low dose group (33.34 g) were extracted in close analogy to the procedure described for the high dose group (see above). However, some of the liquid partition steps were omitted, since they proved to be unnecessary.

Measurement of radioactivity

Liquid samples were radioassayed by the following liquid scintillation counters; Beckman LS 6000 LL and LS 6500, Quench correction using the "H-number" Philips PW 4700, Quench correction using the "ESCR-number" LKB Rack Beta 1219 Spectral, Quench correction using the "SQP(E)-number".

Solid samples like extraction residues were weighed and combusted in an oxygen atmosphere using a Packard Instruments oxidiser.

II. Results and Discussion

The concentration of the total radioactivity in the edible parts of the fish after administration of 20 or 200 µg/L [^{14}C] KWG 4168 is given in the report on the bioconcentration and also summarised in the table below. Since the overall concentration is very low, all available samples from the 28-day bioconcentration and the 14-day depuration period (*i.e.* from day 1 to day 42) were combined separately for the two dose groups to gain as much material as possible for the metabolite identification. The metabolism was only investigated in the edible parts of the fish. The mean tissue residues at steady state are as follows:

Table CA 8.2.2.3/02-1 [^{14}C]KWG 4168: Radioactivity concentration in the edible parts of the fish samples (mean values corrected for outliers)

Time after dosing [days]	Dose level 20 µg/L		Dose level 200 µg/L	
	DPM/g dried fish	Equivalent concentration [µg/g]	DPM/g dried fish	Equivalent concentration [µg/g]
1	10215	1.89	5139	0.95
3	18702	3.45	8250	1.52
7	13293	2.45	17223	3.18
10	7657	1.41	4555	0.84
14	9699	1.79	7631	1.41
21	14932	2.76	7893	1.46
28	7984	1.47	7817	1.44
29	7045	1.30	4819	0.89
31	2142	0.40	725	0.13
35	1515	0.28	603	0.11
38	1355	0.25	545	0.10
42	997	0.18	418	0.08

Table CA 8.2.2.3/02-2 [^{14}C]KWG 4168: Extraction yields of the total radioactivity from the edible tissues and percentage of the radioactivity subjected to chromatographic analysis

Dose [µg/L]	Extraction rate [%]	% of organ radioactivity subjected to HPLC
20	84.20	80.02
200	91.36	86.39

Table CA 8.2.2.3/02-3 [^{14}C]KWG 4168: Relative distribution and concentration (µg a.s. equiv/g) of the identified metabolites in the edible parts of the high dosed Bluegill sunfish

Metabolite	% of HPLC		% of edible tissue	Concentration (ppm)
	Sample KNO 2403A	Sample KNO 2403B		
KNO 1634A	15.1	73.5	18.4	0.83
ECW 8046/ECW 80511	16.8	26.5	15.4	0.70
KWG 4168	28.8	-	22.2	1.00
Totally identified	60.7	100.0	56.0	2.53

Table CA 8.2.2.3/02-4 [^{14}C]KWG 4168: Relative distribution and concentration (µg a.s. equiv/g) of the identified metabolites in the edible parts of the ow dosed Bluegill sunfish

Metabolites	% of HPLC	% of edible tissue	Conc. (ppm)
	Sample KNO 2404A		
KNO 22302	6.8	5.4	0.031
ECW 8046/ECW 80511	15.5	12.4	0.072
KWG 4168	10.5	8.4	0.049

Metabolites	% of HPLC	% of edible tissue	Conc. (ppm)
Totally identified	32.8	26.2	0.152

III. Conclusion

A 42-day study was conducted to evaluate the bioconcentration of [cyclohexyl-1-^{14}C] KWG 4168 by bluegill sunfish (*Lepomis macrochirus*). Mean water concentrations of 20 and 200 µg KWG 4168/L, respectively, were maintained for a 28-day exposure period. The purpose of the experiments reported herewith was to gain information on the nature of the residue in the edible parts of the fish and to quantify the metabolites to the extent possible.

The mean tissue residues at steady state were as follows:

	20 µg [^{14}C] KWG 4168/L	**200 µg [^{14}C] KWG 4168/L**
Edible parts:	0.58 mg/kg	4.52 mg/kg
Non edible parts:	3.12 mg/kg	22.8 mg/kg
Whole fish:	1.64 mg/kg	13.4 mg/kg

The radioactivity was extracted almost completely from the edible tissues with mixtures of acetonitrile and tetrahydrofurane. After purification, 3 metabolites besides the unchanged parent compound were identified:

Metabolite	**% of total radioactive residue**	
	High dose	**Low dose**
KNO 22302 (alcohol-sulphate)	-	5.4
KNO 1634A (glucuronide)	18.4	-
ECW 80511/8046 (Carboxylic acid)	15.4	12.4
KWG 4168	22.2	8.4
Identification rate	56.0	26.2
Extraction rate	91.4	84.2

Assessment and conclusion by applicant:

The study was conducted to in order to provide additional information on the residues of the samples taken as part of the the fish bioconcentration test. Although the data are considered to be valid, the study did not follow a recognised test guideline and the validity of the results cannot be assessed. The results are also not necessary for the risk assessment of spiroxamine. As such the study is considered to be supporting information only.

CA 8.2.3 Endocrine disrupting properties

Data Point:	KCA 8.2.3/01
Report Author:	[redacted]
Report Year:	2008
Report Title:	Spiroxamine - fish screening assay (FSA) with fathead minnow
Report No:	EBKWX094
Document No:	M-304833-01-1
Guideline(s) followed in study:	OECD FSA test protocol (ENV/JM/TG/EDTA (2004) 1 REV2), including all standard operating procedures and guidance documents
Deviations from current test guideline:	Yes OECD 229 (2012) Fish were older than recommended (32 weeks instead of 20±2) The mean measured concentrations ranged from 29 to 84 percent of nominal for all test levels Each experimental unit was composed of four female and two male minnows instead of five of each, however four experimental units were used instead of tw
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

In order to address potential concerns over possible endocrine disruption, a fish screening assay (equivalent to a Fish short-term reproduction assay (FSTRA)) and a *Xenopus* eleutheroembryo thyroid assay (XETA) using spiroxamine have been conducted and have been submitted here. Full details are provided below. Please refer to the ED assessment for discussion of the results and an assessment against the ED criteria.

Executive Summary

This study was conducted in order to identify potential impacts of spiroxamine on endocrine biomarkers of reproductive performance in sexually mature fathead minnows over a 21-day exposure period. The assessment was based on the following biomarker endpoints: secondary sexual characteristics, vitellogenin plasma concentration and gonad histopathology. Fecundity and fertility of eggs were also evaluated, and growth, expressed as final length and weight, was measured as a non-endocrine-specific endpoint.

Mean measured test concentrations were 1.6, 6.3, 18.0, 56.4 and 58.8 µg a.s./L, along with a control.

A statistically significant and dose-dependent decrease of vitellogenin concentration in female blood plasma was observed in the 56.4 and 58.8 µg a.s./L test concentrations. All other endocrine-specific biomarkers, secondary sex characteristics and gonad histopathology showed no treatment-related effects up to and including 58.8 µg a.s./L, the highest dose tested.

No effects on growth as a non-endocrine-specific endpoint were observed.

Fecundity could not be evaluated in the study due to the very low control egg production during exposure. Measures of fertility were not affected by exposure to the test substance.

I. Materials and Methods

A. Materials

Test Material Spiroxamine technical (KWG 4168)

Lot/Batch #: EDTH004650

Purity: 95.1%

Description: Light brown clear oily liquid

Stability of test compound:	Not reported
Reanalysis/Expiry date:	14 August 2007
Density:	Not reported
Treatments	
Test rates:	Nominal: 2.50, 7.50, 22.5, 67.5 and 203 µg a.s./L Measured: 1.6, 6.3, 18.9, 56.4 and 58.8 µg a.s./L
Solvent/vehicle:	None
Analysis of test concentrations:	Yes, mean measured concentrations 29 – 84% of nominal
Test organisms	
Species:	Adult fathead minnow (*Pimephales promelas*), approx. 32 weeks old at day 0
Source:	[redacted]
Acclimatisation period:	Acclimated under similar conditions to the test for at least two weeks prior to test initiation
Feeding:	Fed *ad libitum* brine shrimp nauplii and ground flake food twice and once daily, respectively
Treatment for disease:	None reported
Test design	
Test vessel:	22 x 35 cm glass aquaria, with a water depth of 21 cm and approx. volume of 16 L
Test medium:	Reconstituted water
Replication:	Four replicates per concentration
No. of animals/vessel:	Two males and four females
Duration of test:	21 days
Environmental test conditions	
Temperature:	23.2 – 25.1°C
Dissolved oxygen:	93 – 106% saturation
pH:	6.6 – 6.9
Photoperiod:	16 h light : 8 h dark at 63 – 241 lux (mean 131 lux)

B. Study Design

This study was conducted in order to identify potential impacts of spiroxamine on endocrine biomarkers of reproductive performance in sexually mature fathead minnows over a 21-day exposure period. The assessment was based on the following biomarker endpoints: secondary sexual characteristics,

vitellogenin plasma concentration and gonad histopathology. Fecundity and fertility of eggs were also evaluated, and growth, expressed as final length and weight, was measured as a non-endocrine-specific endpoint.

Test fish were adult male and female fathead minnow (*Pimephales promelas*) with clear secondary sexual characteristics visible. Fish were approximately 32 weeks old at the beginning of the exposure phase on day 0. The fathead minnow had not been held under spawning conditions prior to the test, and so went through the first spawning cycle during the study period.

Glass aquaria used in the study were 22 x 35 cm, with a water depth of 21 cm, yielding an approximate chamber volume of 16 L. Test water was a reconstituted water aerated to oxygen saturation and periodically analysed for impurities. The test system was delivered at a flow rate of 7 L/h, approximately 10.5 water changes daily. Two stainless-steel 100 x 75 mm spawning substrates were added to each chamber.

Two males and four females were placed in one aquarium including two spawning substrates, consisting of two single half tiles constructed of stainless steel. They were exposed to various test item concentrations and a water control under continuous flow-through conditions for 21 days with four replicates for each test level. Nominal test concentrations were 2.63 (2.50), 7.88 (7.50), 23.7 (22.5), 71.0 (67.5) and 213 (203) µg test item (a.s.)/L. Mean measured concentrations of spiroxamine in the aquaria over the exposure period were 1.6, 6.3, 18.9, 56.4 and 58.8 µg a.s./L, equivalent to 64, 84, 84, 84 and 29% of nominal.

Before start of the test a 'pre-exposure' period over 15 days under 'non-exposure' conditions was initiated in the same aquaria as used in the test to check spawning activity of the selected breeding pairs. As a result, equilibration of the test system with the test compound was not possible prior to test start.

Fish were examined daily over the exposure period for mortality and behavioural or physical abnormalities. Observations of spawning activity in each test vessel were made daily. Eggs laid on the spawning substrates were removed, counted and discarded. Representative egg clutches were also checked for fertilisation success.

Fish were fed *ad libitum* during the course of the test with freshly hatched brine shrimp (*Artemia* spp.) nauplii and ground flake food (Tetramin®). Nauplii were added twice daily and ground flake food was added once daily. Food was withheld from fish for 12 hours prior to test termination on day 21.

The standard length was determined for each individual fish by measuring from the tip of the mouth to the tip of the caudal peduncle using an electronic digital caliper. Individual wet weight after blotting was also determined.

Observations of secondary sexual characteristics (SSCs) were made on working days of the course of exposure. Recorded characteristics were: colouration patterns, specialised SSCs (size of dorsal nape pads, number of nuptial tubercles in males and ovipositor in females) and territorial aggressiveness (assessed semi-quantitatively). Afterwards gross morphology including secondary sex characteristics

was assessed. The nuptial tubercles as male secondary sex characteristics located around eyes, between nares and/or around the mouth were counted in each individual fish, using a stereo microscope. Nuptial tubercle size was ranked as follows: (1) present = tubercle having a single point whose height is nearly equivalent to its diameter, (2) enlarged = tissue resembling an asterisk in appearance, having a large radial base with grooves or furrows emerging from the center and (3) pronounced = quite large and rounded with less definition in structure.

The measurements of blood plasma vitellogenin levels were made using a commercially produced enzyme-linked immunoabsorbant assay (ELISA) for fathead minnow VTG.

Three transverse sections of each fish were processed. The thickness of each slice and the distance between the three slices was dependent upon individual body anatomy (varying size of female and male fish). The dorsal fin was used as orientation. The sections were performed in a way that they contained the gonads (testes and deferent ducts or ovaries and oviducts) and the liver. The material was embedded

in Paraplast and cut at an approximate thickness of about 4 micrometers. The slides were stained with Hematoxylin and Eosin (H&E).

Analytical method

Samples of water were analysed using the validated analytical method 00623, report reference M-031628-01-1 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the OECD draft protocol were met:

- Control mortality or fish with signs of disease to not exceed 10% at test termination (actual: 0% mortality, 0.2% (one fish) was missing an eye, however this could be due to aggressive spawning behaviour of males rather than a symptom of the a.s.)
- Dissolved oxygen concentration to be ≥60% in all test vessels throughout the test (actual: 93 – 106%)

One validity criterion was not met:

- Water temperature to not differ by more than ±1.0°C between test vessels at any one time during the test (actual: a difference of 1.1 °C was observed between test concentrations 1.6 and 6.3 µg a.s./L on study day 20, however this was the only incidence of exceedance of the criterion)

Mean measured concentrations of spiroxamine in the aquaria over the exposure period were 1.6, 6.3, 18.9, 56.4 and 58.8 µg a.s./L, equivalent to 64, 84, 84, 84 and 29% of nominal. The mean measured values ranged from 29 to 84 percent of nominal for all test levels. In the lowest test level the mean measured concentration was at 64 percent of nominal, in the three mid test levels at 84 percent of nominal each, respectively, and in the highest test level only 29 percent of nominal was found. Therefore, the mean measured concentrations of the 2 highest test level resulted nearly in the same concentration.

No malfunctions of the dosing and diluter system were observed during the exposure, so the non-homogeneous analytical recoveries also in the newly prepared stock solutions may indicate that the test item could not been sufficiently dissolved. Adsorptivity of the test item may also had an influence on test media concentrations showing inconsistent results with high variations especially during the first two days of the test (equilibration of the system was not possible before start of exposure). The lowest and the highest test level were both throughout the whole exposure far too low and clearly deviated from the nominal values. According to the current OECD draft FSA guideline the test item concentrations should have been maintained within ±20 % of the mean measured values, which was not fulfilled for all test levels in this test due to high variabilities. But the mean concentrations of

the three mid test levels are approximately reflecting the nominal values and the effect threshold, was within this concentration range. The results of the study have been presented based on the mean measured test concentrations.

Table CA 8.2.3/01-1 Measured concentrations of spiroxamine during the test

	Nominal concentration (µg a.s./L)					
	Control	**2.50**	**7.50**	**22.5**	**67.5**	**203**
Measured test concentration (µg a.s./L)						
Mean	0.492	1.6	6.3	18.9	56.4	58.8
Min	-	1.1	4.7	10.8	29.9	31.5
Max	-	2.1	7.1	23.0	80.6	84.5
% of nominal	-	64	84	84	84	29

SD Standard deviation
LOQ Limit of quantification: 0.8 µg/L

No substance-related observations on mortality, behaviour or secondary sexual characteristics in either sex were observed over the 21-day exposure period.

Table CA 8.2.3/01-2 Effects of spiroxamine exposure on the mortality of fathead minnow at test termination

Mean measured concentration	**Control**				**1.6 µg a.s./L**				**6.3 µg a.s./L**			
Replicate	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Mortality (%)	0	0	0	0	0	0	16.7	0	0	0	0	0
Mean (%)	0				4.2				0			
SD	0				8.4				0			
Mean measured concentration	**18.9 µg a.s./L**				**56.4 µg a.s./L**				**58.8 µg a.s./L**			
Replicate	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Mortality (%)	16.7	0	0	0	0	0	0	0	0	16.7	16.7	0
Mean (%)	4.2				0				8.4			
SD	8.4				0				9.6			

The baseline fecundity determinations during the pre-exposure phase (without the test item (from day -15 until the start of the exposure phase on day 0) gave no indications of spawning failures in most cases. Fish were exchanged due to no or low egg production (< 1 egg per female and day) before start of exposure in only five of the total 24 replicates. Afterwards spawning success was improved in four of these replicates.

The number of eggs produced per female and day during exposure to spiroxamine did not indicate a statistically significant effect. Interpretation of this endpoint is difficult due to the very low egg production in the control group. Egg production in the two highest test levels was very low, however due to the comparably low control egg production, a definitive evaluation of this endpoint is not possible.

No significant effect on the fertilisation rate was observed in the assessed egg clutches of all test levels relative to the control fertility during the exposure phase.

Table CA 8.2.3/01-3 Effects of spiroxamine exposure on egg production of fathead minnow during the exposure phase

Mean measured concentration	**Control**				**1.6 µg a.s./L**				**6.3 µg a.s./L**			
Replicate	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Mean eggs/female	0.2	2.0	0.6	0	2.6	2.1	0.9	7.8	4.4	0	0.8	6.7
SD	1.0	6.8	2	0	7.6	4.6	2.1	11	11	0	2.1	13
Mean	0.7				3.3				3.0			
SD	2.4				6.4				6.4			
Mean measured concentration	**18.9 µg a.s./L**				**56.4 µg a.s./L**				**58.8 µg a.s./L**			
Replicate	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Mean eggs/female	10	1.1	5.5	2.4	0.9	0.1	0.2	0.1	0.3	2.1	1.1	0
SD	21	3.7	11	9.1	2.8	0.4	0.6	0.3	1.2	5.2	3.9	0
Mean	4.8				0.3				0.9			
SD	11.1				1.0				2.6			

No substance-related significant effects on fathead minnow growth, expressed as male or female final standard length or wet weight, were detected after exposure to spiroxamine.

Table CA 8.2.3/01-4 Effects of spiroxamine exposure on measures of fathead growth

Mean measured concentration	**Males**					
	Control	**1.6 µg a.s./L**	**6.3 µg a.s./L**	**18.9 µg a.s./L**	**56.4 µg a.s./L**	**58.8 µg a.s./L**
Mean length (mm)	49.6	49.5	49.3	49.4	49.5	50.4
SD	4.1	1.9	2.0	1.7	1.8	5.0
Mean wet weight (g)	2.71	2.56	2.54	2.69	2.48	2.69
SD	0.64	0.29	0.34	0.30	0.28	0.67
	Females					
Mean measured concentration	**Control**	**1.6 µg a.s./L**	**6.3 µg a.s./L**	**18.9 µg a.s./L**	**56.4 µg a.s./L**	**58.8 µg a.s./L**
Mean length (mm)	40.1	39.0	39.4	39.9	40.7	39.7
SD	2.0	0.8	2.3	1.2	1.9	1.5
Mean wet weight (g)	1.23	1.08	1.16	1.21	1.23	1.18
SD	0.24	0.09	0.18	0.20	0.20	0.16

At test termination the quantitative and qualitative assessment of nuptial tubercles as a specialised male secondary sexual characteristic in fathead minnows served as measurable biomarker endpoints. After exposure to spiroxamine, the number and score of nuptial tubercles in males was not affected in any test level, when compared to the control. In females no nuptial tubercles were observed.

Table CA 8.2.3/01-5 Mean number/score of nuptial tubercles in male fathead minnows at test termination

Mean measured concentration	**Males**					
	Control	**1.6 µg a.s./L**	**6.3 µg a.s./L**	**18.9 µg a.s./L**	**56.4 µg a.s./L**	**58.8 µg a.s./L**
Number of nuptial tubercles	13.1	8.7	9.5	11.8	10.3	7.8
SD	6.5	3.5	5.5	2.5	5.3	4.9
Score of nuptial tubercles*	16.8	10.3	11.9	17.4	14.1	9.9
SD	8.8	5.1	7.3	3.4	10.1	8.6

* score formula = (number of tubercles ranked as present x 1)+(number of tubercles ranked as enlarged x 2)+(number of tubercles ranked as pronounced x 3)

The vitellogenin concentration in blood plasma showed a significant reduction in females at the two highest test levels (56.4 and 58.8 µg a.s./L). With regard to vitellogenin contents in male blood plasma, detected at concentrations four orders of magnitude below the control female level, only a slight and insignificant decrease was observed in the highest test level.

Table CA 8.2.3/01-6 Effects of spiroxamine exposure on vitellogenin (VTG) concentration in blood plasma

Mean measured concentration	**Males**					
	Control	**1.6 µg a.s./L**	**6.3 µg a.s./L**	**18.9 µg a.s./L**	**56.4 µg a.s./L**	**58.8 µg a.s./L**
VTG (ng/mL)	1140.9	930.6	524.0	1172.7	656.2	142.1
SD	969.0	393.4	769.1	1548.4	856.7	66.7

	Females					
Mean measured concentration	**Control**	**1.6 µg a.s./L**	**6.3 µg a.s./L**	**18.9 µg a.s./L**	**56.4 µg a.s./L**	**58.8 µg a.s./L**
VTG (ng/mL)	10237556.2	10338738.8	5361114.7	4500759.3	1988465.4*	2612292.5*
SD	3275125.5	4058132.3	2890049.9	2574270.9	1944671.1	1645951.8

* Statistically significantly different to the control (α=0.05) (Dunnett's test)

The histopathological investigation of male and female gonads showed that they were unaffected by exposure to spiroxamine up to and including 58.8 µg a.s./L, regarding the developmental stage and degenerative changes.

Eggs or debris in the oviduct were frequently observed, indicating spawning activity. The incidence of inflammatory changes of the oviduct was slightly increased at 56.4 µg a.s./L.

The histopathological investigation of the liver tissue revealed also no concentration related effects up to and including 58.8 µg a.s./L.

Table CA 8.2.3/01-7 Summary of histopathological findings in males

Mean measured concentration (µg a.s./L)	**Control**	**1.6**	**6.3**	**18.9**	**56.4**	**58.8**
Total number of males	8	8	9	8	8	8
Number of males at testis stage:						
Juvenile	-	-	-	-	-	2
1 (early spermatogenic)	1	-	2	-	-	-
2 (mid spermatogenic)	7	6	7	8	6	4
3 (late spermatogenic)	-	1	-	-	2	2
Number of males with testis observations:						
Increased spermatogonia	1	3	1	-	2	-
Increased interstitial cells	1	1	-	1	1	-
Decreased spermatozoa in sperm (deferent) duct	-	-	1	-	-	2
Number of males with liver observations:						
Increased hepatic basophilia	-	1	1	-	-	-

Table CA 8.2.3/01-8 Summary of histopathological findings in females

Mean measured concentration (µg a.s./L)	**Control**	**1.6**	**6.3**	**18.9**	**56.4**	**58.8**
Total number of females	16	14	15	15	16	14
Total number intersex	0	1	0	0	0	0
Number of females at ovary stage:						
2 (mid development)	5	5	7	7	5	5
3 (late development)	8	9	6	6	10	7
4 (very late development)	3	1	2	2	1	2
Number of females with ovary observations:						
Vacuolation of ovarian stroma	2	3	2	2	2	5
Oocyte atresia immature	2	1	-	-	-	-
Oocyte atresia mature	5	4	5	4	8	6
Increased postovulatory follicles	8	6	8	6	6	6
Egg debris in oviduct	11	9	9	8	9	9
Inflammation in oviduct	-	-	-	-	3	1
Number of males with liver observations:						

Mean measured concentration (µg a.s./L)	Control	1.6	6.3	18.9	56.4	58.8
Decreased hepatic basophilia	-	-	-	-	2	1
Increased hepatic basophilia	4	2	4	5	3	4

III. Conclusion

A statistically significant and dose-dependent decrease of vitellogenin concentration in female blood plasma was observed in the 56.4 and 58.8 µg a.s./L test concentrations. All other endocrine-specific biomarkers, secondary sex characteristics and gonad histopathology showed no treatment-related effects up to and including 58.8 µg a.s./L, the highest dose tested.

No effects on growth as a non-endocrine-specific endpoint were observed.

Fecundity could not be evaluated in the study due to the very low control egg production during exposure. Measures of fertility were not affected by exposure to the test substance.

Assessment and conclusion by applicant:

The study was conducted to the OECD PSA test protocol (ENV/JM/TG/EDTA (2004) 1 REV2), including all standard operating procedures and guidance documents.

Validity criteria according to the OECD draft protocol were met:

- Control mortality or fish with signs of disease to not exceed 10% at test termination (actual: 0% mortality, 0.2% (one fish) was missing an eye, however this could be due to aggressive spawning behaviour of males rather than a symptom of the a.s.)
- Dissolved oxygen concentration to be ≥60% in all test vessels throughout the test (actual: 93 – 106%)

One validity criterion was not met:

- Water temperature to not differ by more than ±1.0°C between test vessels at any one time during the test (actual: a difference of 1.1°C was observed between test concentrations 1.6 and 6.3 µg ac./L on study day 20, however this was the only incidence of exceedance of the criterion)

The study has been re-assessed against the current OECD 229 test guideline validity criteria which are as follows:

- The mortality in the water (or solvent) controls should not exceed 10 per cent at the end of the exposure period;
- The dissolved oxygen concentration should be at least 60 per cent of the air saturation value (ASV) throughout the exposure period;
- The water temperature should not differ by more than ± 1.5 °C between test vessels at any one time during the exposure period and be maintained within the range of 25 ± 2°C for fathead minnow
- Evidence should be available to demonstrate that the concentrations of the test substance in solution have been satisfactorily maintained within 20% of the mean measured values;
- Evidence that fish are actively spawning in all replicates prior to initiating chemical exposure and in control replicates during the test.

The first three validity criteria from OECD 229 are essentially the same that were used for the acceptance criteria in the study report. The measured test concentrations have been discussed in the report and summary; measured concentrations were not maintained within 20% of the nominal value (particularly at the highest and lowest test concentrations) but the middle three treatment

concentrations were considered to have been maintained at levels around the nominal value. As the results have been based on mean measured test concentrations, the results are considered to accurately reflect the actual concentrations that fish were exposed to.

A 15-day pre-exposure period was included as part of the test and demonstrated that the fish were actively spawning prior to initiating chemical exposure.

Taking these points in to account, it is believed that the validity criteria of the current OECD 229 test guideline have been fulfilled therefore the study is considered to be valid.

A statistically significant and dose-dependent decrease of vitellogenin concentration in female blood plasma was observed in the 56.4 and 58.8 µg a.s./L test concentrations.

The data from this screening assay have been used for the assessment of ED potential only. As a result, it is not considered necessary to perform EC_{10} and EC_{20} calculations for the parameters assessed in this study as these values will not be used in the conventional risk assessment.

Data Point:	KCA 8.2.3/02
Report Author:	[redacted]
Report Year:	2021
Report Title:	Xenopus eleutheroembryonic thyroid assay (XETA) analysis report - Spiroxamine
Report No:	P-2019-0102
Document No:	M-762327-01-1
Guideline(s) followed in study:	OECD TG 248 (2019)
Deviations from current test guideline:	None
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 objective of the *Xenopus* Eleutheroembryonic Thyroid Assay (XETA) assay was to detect potential activity of the test item on the thyroid axis. According to OECD TG 248, the XETA detects thyroid active molecules acting through various mechanisms of action including TH receptors agonists and antagonists, modulators of TH metabolism (including deiodinase inhibitors), modulators of TH clearance (including UDPGT modulators), and potentially modulators of TH transport *via* interaction with TH plasma binding proteins.

The purpose of this test was to measure the capacity of spiroxamine to activate or inhibit the transcription of a genetic construct (THb/ZIP-GFP *X. laevis* eleutheroembryos) either directly through binding to the thyroid receptor (TR) or modifying the binding of thyroid hormones (TH) to the TR, or indirectly by modifying the amount of TH available to activate the TR and thereby transcription of the TH/bZIP-GFP construct.

Nominal concentrations of 0.025, 0.1, 0.4 and 1.6 mg a.s./L were evaluated for fluorescence. The highest test concentration was selected based upon the results of solubility and survival pre-tests showing that this concentration, corresponding to the LC_5, results in 5% mortality, and potentially corresponds to the Maximum Tolerated Concentration (MTC). The exposure levels of the nominal concentrations were verified analytically. Geometric mean measured concentrations were 3.52, 19.35, 102.90 and 434.15 µg a.s./L for nominal test concentrations 0.025, 0.1, 0.4 and 1.6 mg a.s./L, respectively, corresponding to a mean recovery of 21.5%.

No toxicity was observed in the eleutheroembryos in the controls. In the definitive test, the highest concentration of 434.15 µg a.s./L showed 33.3% cumulated mortality over the three runs in both unspiked and spiked modes; and therefore exceeds the MTC estimated from the survival pre-tests. The second highest nominal concentration (102.9 µg a.s/L) showed no sign of toxicity over the three runs.

Spiroxamine showed no indication of endocrine activity for the thyroid modality at the geomean test concentrations of 3.52, 19.35 and 102.90 µg a.s/L. A statistically significant increase in fluorescence was observed at the highest nominal test concentration, 1.6 mg a.s./L, however as this test concentration was above the MTC, it cannot be concluded that the increase in fluorescence is indicative of T-mediated endocrine activity.

It is therefore concluded that spiroxamine does not show endocrine activity on the thyroid axis in the XETA.

Materials and Methods

Materials

Test Material	Spiroxamine technical
Lot/Batch #:	AE 1344293-01-09
Purity:	92.3%
Description:	Colourless to pale brown liquid
Stability of test compound:	Not reported
Reanalysis/Expiry date:	31 March 2022
Density:	Not reported
Treatments	
Test rates:	Nominal: 0.025, 0.1, 0.4 and 1.6 mg a.s./L Measured: 3.52, 19.35, 102.90 and 434.15 µg a.s./L
Solvent/vehicle:	Ethanol (0.01%)
Analysis of test concentrations:	Yes, geometric mean recovery of 14.2 – 27.2% (mean 21.5%)
Test organisms	
Species:	*Xenopus laevis* eleutheroembryos, stage 45
Source:	In-house culture
Acclimatisation period:	None
Treatment for disease:	None
Test design	
Test vessel:	Plastic 6-well plates containing 8 mL test solution
Test medium:	Evian® water
Replication:	Three runs
No. of animals/run:	20 eleutheroembryos (split evenly over 2 wells)

Duration of test:	72 hours
Environmental test conditions	
Temperature:	21°C
Dissolved oxygen:	Not reported
Loading:	15.75 g/L (mean fresh weight 12.6 mg)
pH:	7 – 8
Photoperiod:	Constant darkness

Study Design

This study was conducted in order to detect potential activity of spiroxamine technical on the thyroid axis of *Xenopus laevis* eleutheroembryos. Test concentrations were selected based on the results of solubility and survival pre-tests, and were obtained *via* serial dilution of a 16 g/L stock solution, prepared daily.

Nominal concentrations were 0.025, 0.1, 0.4 and 1.6 mg a.s./L and the respective geometric mean measured concentrations were 3.52, 19.35, 102.90 and 434.15 µg a.s./L corresponding to a mean recovery of 21.5%. Endpoints are therefore based on geometric mean measured concentrations.

The exposure solutions were prepared by serial dilution of the 16 g/L stock solution with 100% ethanol in the Evian® water to reach the final concentration of solvent of 0.01%. For the unspiked mode, the exposure solutions were prepared by dilution of the 1.6 mg/L solution into the test medium (Evian® water + 0.01% ethanol). For the spiked mode, 100 mL of each exposure solution were spiked with 20 µL of a 16.25 mg/L solution of T3 hormone. Each spiked exposure solution contained 3.25 µg/L of T3 hormone in the exposure medium. There were additionally a negative control, a solvent control, a T3 (triiodothyronine at 3.25 µg/L) control and a T4 (thyroxine at 10 mg/L) saturation control.

The test media was renewed after 24 and 48 hours exposure.

Test vessels were plastic 6-well plates of a chemically inert material, containing 8 mL test solution per well. Each test group contained 20 *Xenopus laevis* eleutheroembryos per run, with three runs per group for a total of 60 eleutheroembryos. To each well was added 10 eleutheroembryos.

Eleutheroembryos in the "unspiked" test condition received no supplementation of thyroid hormones, whereas those in the "spiked" mode were supplemented with 3.25 µg/L of the thyroid hormone T3 (triiothyronine).

Mortality of the eleutheroembryos and observations of gross morphology and behaviour were recorded after 24, 48, and 72 hours exposure. The pH of the exposure solution containing the highest concentration of the test item was measured at the start of the test, and temperature was measured continually.

Anaesthetised eleutheroembryos were then rinsed and transferred to a 96-well plate, one per well, and imaged using a robotised imagery system to assess the fluorescence. Data were then analysed using Microsoft Excel and Graphpad Prism.

All the tested groups, stock solution and Evian® water + 0.01% Ethanol control groups were sampled in order to perform chemical analysis of the test item.

Results and Discussion

Validity criteria according to the OECD 248 (2019) "*Xenopus* Eleutheroembryo Thyroid Assay (XETA)" guideline were met:

- Control mortality to not exceed 10% at the end of the test (actual: 0%)

- Malformed organisms in the control to not exceed 10% (actual: 0)
- Initial pH of the exposure solutions to be between 6.5 and 8.5 for each renewal (actual: 7 – 8)
- Mean fluorescence of the T3 control group to be at least 20% higher than the mean fluorescence of the test medium control (actual: ≥58.8%)
- There should be a significant induction of fluorescence of at least 70% in the T4 control group compared to the test medium control (actual: ≥119.9%)
- The coefficient of variation of the fluorescence intensity in the test medium control to not exceed 30% (actual: ≤12%)

Geometric mean measured concentrations of test concentrations 0.025, 0.1, 0.4 and 1.6 mg a.s./L were 3.52, 19.35, 102.90 and 434.15 µg a.s./L, respectively, corresponding to a mean recovery of 21.5% (14.2 to 27.2%). The mean percent recovery in the stock solutions was 91.6%. The results of the test should therefore be considered with regard to the geometric mean measured concentrations. Addition of T3 in the "spiked" mode had no effect on the exposure concentrations.

Table CA 8.2.3/02-1 Mean measured concentrations of spiroxamine in test medium

Nominal test concentration (mg a.s./L)	Mean measured concentrations (µg a.s./L)[1]											
	0 h		24 h				48 h				72 h	
	Fresh	%	Spent	%	Fresh	%	Spent	%	Fresh	%	Spent	%
0.025	19.2	*77*	0.967	*4*	19.2	*77*	1.00	*4*	18.1	*72*	1.08	*4*
0.1	75.4	*75*	3.68	*4*	76.0	*76*	5.68	*6*	68.5	*68*	8.87	*9*
0.4	304	*76*	29.8	*7*	270	*68*	41.3	*10*	272	*68*	55.6	*14*
1.6	1063	*66*	129	*8*	1176	*74*	353	*22*	1038	*65*	151	*9*

[1] Figures rounded to 1 d.p.

Table CA 8.2.3/02-2 Geometric mean measured concentrations of spiroxamine in test medium

Nominal test concentration (mg a.s./L)	Geomean (µg a.s./L)	± Standard Error	Recovery (%)
0.025	3.52	1.13	14.2
0.1	19.35	1.62	19.0
0.4	102.90	12.60	25.7
1.6	434.15	191.00	27.2
		Mean:	21.5

Toxicity:

No morphological abnormalities were observed in any of the eleutheroembryos at any point during the test.

All eleutheroembryos exposed to the highest test concentration of 434.15 µg a.s./L died in both the unspiked and spiked modes of the first run. While there was no mortality in the second and third runs, toxic effects were nevertheless recorded at this same concentration after 24 hours of exposure (reduced mobility). Cumulated mortality over the three runs was 33.3%.

No signs of toxicity were observed at the second highest test concentration of 102.9 µg a.s./L, and one dead eleutheroembryo was recorded at the test concentration of 19.35 µg a.s./L (spiked mode) in the second run. Overall, the survival rate was greater than 90% in all groups in all runs and in the pooled data as required in the OECD TG 248, except for the 1.6 mg a.s/L nominal concentration which exceeds the Maximum Tolerated Concentration (MTC) and is therefore not included in the assessment for potential T-mediated effects.

Table CA 8.2.3/02-3 Survival and observations of eleutheroembryos – test run 1

Group (geomean)	Survival of eleutheroembryos[1]				
	24 h	48 h	72 h	Survival (%)	Malformations
Test medium	100	100	100	100	-
Test medium + solvent (Ethanol)	100	100	100	100	-
T3 3.25 µg/L + solvent (Ethanol)	100	100	100	100	-
T4 10 mg/L + solvent (Ethanol)	100	100	100	100	-
Spiroxamine – 3.52 µg/L	100	100	100	100	-
Spiroxamine – 19.35 µg/L	100	100	100	100	-
Spiroxamine – 102.9 µg/L	100	100	100	100	-
Spiroxamine – 434.15 µg/L	100	0	0	0[2]	-
Spiroxamine – 3.52 µg/L + T3	100	100	100	100	-
Spiroxamine – 19.35 µg/L + T3	100	100	100	100	-
Spiroxamine – 102.9 µg/L + T3	100	100	100	100	-
Spiroxamine – 434.15 µg/L + T3	100	0	0	0[2]	-

[1] 20 eleutheroembryos introduced
[2] Lethal concentration. 2 less mobile embryos after 24 hours of exposure

Table CA 8.2.3/02-4 Survival and observations of eleutheroembryos – test run 2

Group (geomean)	Survival of eleutheroembryos[1]				
	24 h	48 h	72 h	Survival (%)	Malformations
Test medium	100	100	100	100	-
Test medium + solvent (Ethanol)	100	100	100	100	-
T3 3.25 µg/L + solvent (Ethanol)	100	100	100	100	-
T4 10 mg/L + solvent (Ethanol)	100	100	100	100	-
Spiroxamine – 3.52 µg/L	100	100	100	100	-
Spiroxamine – 19.35 µg/L	100	100	100	100	-
Spiroxamine – 102.9 µg/L	100	100	100	100	-
Spiroxamine – 434.15 µg/L	100	100	100	100[2]	-
Spiroxamine – 3.52 µg/L + T3	100	100	100	100	-
Spiroxamine – 19.35 µg/L + T3	95	95	95	95	-
Spiroxamine – 102.9 µg/L + T3	100	100	100	100	-
Spiroxamine – 434.15 µg/L + T3	100	100	100	100[2]	-

[1] 20 eleutheroembryos introduced
[2] Toxic concentration. Lack of mobility observed in surviving individuals after 72 hours of exposure

Table CA 8.2.3/02-5 Survival and observations of eleutheroembryos – test run 3

Group (geomean)	Survival of eleutheroembryos[1]				
	24 h	48 h	72 h	Survival (%)	Malformations
Test medium	100	100	100	100	-
Test medium + solvent (Ethanol)	100	100	100	100	-
T3 3.25 µg/L + solvent (Ethanol)	100	100	100	100	-
T4 10 mg/L + solvent (Ethanol)	100	100	100	100	-
Spiroxamine – 3.52 µg/L	100	100	100	100	-
Spiroxamine – 19.35 µg/L	100	100	100	100	-
Spiroxamine – 102.9 µg/L	100	100	100	100	-
Spiroxamine – 434.15 µg/L	100	100	100	100[2]	-
Spiroxamine – 3.52 µg/L + T3	100	100	100	100	-
Spiroxamine – 19.35 µg/L + T3	100	100	100	100	-
Spiroxamine – 102.9 µg/L + T3	100	100	100	100	-
Spiroxamine – 434.15 µg/L + T3	100	100	100	100[2]	-

[1] 20 eleutheroembryos introduced
[2] Toxic concentration. All embryos less mobile after 24 hours of exposure

Fluorescence:

Unspiked

The highest test concentration, 434.15 µg/L resulted in a statistically significant increase of fluorescence, at 46.2%.

No statistically significant variation of fluorescence greater than 12% was induced by the test item at any of the test concentrations showing no mortality or sub-lethal toxic effects.

Spiked

The highest test concentration, 434.15 µg/L resulted in a statistically significant increase of fluorescence, at 22.1%.

No statistically significant variation of fluorescence greater than 12% was induced by the test item at any of the test concentrations showing no mortality or sub-lethal toxic effects.

Table CA 8.2.3/02-6 Normalised mean fluorescence of the control groups

Group	Normalised mean fluorescence	SEM[1]	Induction (%)
Test medium + solvent (Ethanol)	1.00	0.02	-
Test medium	1.00	0.02	0.3
T3 3.25 µg/L + solvent (Ethanol)	1.72	0.03	72***
T4 10 mg/L + solvent (Ethanol)	2.35	0.04	135***

[1] Standard Error of the Mean
*** Significantly different to the control (Mann-Whitney, p<0.001)

Table CA 8.2.3/02-7 Normalised mean fluorescence of the "unspiked" groups

Group (geomean)	Normalised mean fluorescence[1]	SEM[2]	Induction (%)
Spiroxamine – 3.52 µg/L	1.03	0.02	3
Spiroxamine – 19.35 µg/L	1.04	0.02	4
Spiroxamine – 102.9 µg/L	1.08	0.02	8.3**
Spiroxamine – 434.15 µg/L	1.46	0.02	46.2***

[1] Pooled and normalised to the solvent control
[2] Standard Error of the Mean
** Significantly different to the control (Dunnett's post-hoc test, p<0.01)
*** Significantly different to the control (Dunnett's post-hoc test, p<0.001)

Table CA 8.2.3/02-8 Normalised mean fluorescence of the "spiked" groups

Group (geomean)	Normalised mean fluorescence[1]	SEM[2]	Induction (%)
Spiroxamine – 3.52 µg/L	1.67	0.03	-2
Spiroxamine – 19.35 µg/L	1.77	0.03	3
Spiroxamine – 102.9 µg/L	1.83	0.03	6.7*
Spiroxamine – 434.15 µg/L	2.09	0.03	22.1***

[1] Pooled and normalised to the T3 control
[2] Standard Error of the Mean
* Significantly different to the control (Dunnett's post-hoc test, p<0.05)
*** Significantly different to the control (Dunnett's post-hoc test, p<0.001)

Conclusion

Mortality and sub-lethal effects of the test item was observed at the highest test concentration of 434.15 µg a.s./L in both the unspiked and spiked modes during the definitive test (33.3% cumulated mortality), which excludes this concentration for T-mediated endocrine evaluation in this test system. The second highest test concentration of 102.9 µg a.s./L (nom) showed no sign of toxicity or lethality.

No statistically significant variation of fluorescence greater than 12% was induced by the test item at any of the relevant test concentrations in both the "spiked" and "unspiked" modes, indicating no activation or inhibition of the thyroid axis.

It is therefore concluded that the test item spiroxamine (technical substance) does not show any activity on the thyroid axis at nominal concentrations of 0.025, 0.1 and 0.4 mg a.s./L, and geometric mean measured concentrations of 3.52, 19.35 and 102.9 µg a.s./L in the *Xenopus* eleutheroembryonic thyroid assay (XETA).

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 248, "*Xenopus* Eleutheroembryo Thyroid Assay (XETA)", adopted 18 June 2019.

Validity criteria according to the OECD 248 (2019) guideline were met:

- Control mortality to not exceed 10% at the end of the test (actual: 0%)
- Malformed organisms in the control to not exceed 10% (actual: 0)
- Initial pH of the exposure solutions to be between 6.5 and 8.5 for each renewal (actual: 7 – 8)
- Mean fluorescence of the T3 control group to be at least 20% higher than the mean fluorescence of the test medium control (actual: ≥58.8%)
- There should be a significant induction of fluorescence of at least 70% in the T4 control group compared to the test medium control (actual: ≥119.9%)
- The coefficient of variation of the fluorescence intensity in the test medium control to no exceed 30% (actual: ≤12%)

The study is therefore considered acceptable.

The results of the study lead to the conclusion that spiroxamine does not show any activity on the thyroid axis. The highest concentration tested did show a significant increase in the fluorescence but this concentration was well above the MTC and therefore the results are not included in the assessment of potential T-mediated effects. The study has been used as part of the ED assessment (refer to Appendix E and I, M-763155-01-1).

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:	1994
Report Title:	Acute toxicity of KWG 4168 (tech.) to waterfleas (Daphnia magna)
Report No:	HBF/DM 122
Document No:	M-006245-01-1
Guideline(s) followed in study:	OECD 202 (1984)
Deviations from current test guideline:	Yes OECD 202 (2004) Daphnids were 10/vessel instead of the recommended 5/vessel and only 3 replicates instead of 4 Temperature was measured in only one vessel and at the end of the study
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Available study data for the parent material clearly demonstrate that algae are the most sensitive organism group by at least two orders of magnitude therefore acute studies with the metabolites using aquatic invertebrates are not considered necessary. A non-GLP study is available for spiroxamine-N-oxide (M03) but acute *Daphnia* data are not available for the other relevant metabolites because these data are not considered to be required.

Executive Summary

The 48-hour acute toxicity of KWG 4168 to *Daphnia magna* was studied under static conditions. Test organisms were exposed to measured test concentrations of 0.23, 0.60, 0.82, 2.2, 4.4, 8.3 and 22 mg a.s./L for 48 hours. Immobilisation and sub-lethal effects were observed after 24 and 48 hours. The 48-hour EC_{50} was 9.1 mg a.s./L. The 48-hour NOEC based on immobilisation was 2.2 mg a.s./L.

I. Materials and Methods

A. Materials

Test Material KWG 4168

Lot/Batch #:	898 114 002
Purity:	97.8%
Description:	Colourless liquid
Stability of test compound:	Stable for the duration of the test, as shown by the results of the 48-hr analytical determination
Reanalysis/Expiry date:	27 January 1994
Density:	Not reported

Treatments

Test rates:	Nominal:	0.32, 1.00, 1.78, 3.16, 5.62, 10.0 and 31.6 mg a.s./L
	Measured:	0.23, 0.60, 0.82, 2.2, 4.4, 8.3 and 22 mg a.s./L
Solvent/vehicle:	Not reported	
Analysis of test concentrations:	Yes, mean initial measured concentrations 46.2 to 83.0% of nominal	

Test organisms

Species:	*Daphnia magna*, first instar (6 – 24 hrs old)
Source:	In-house culture, originally from Bundesgesundheitsamt, Berlin, Germany
Acclimatisation period:	None reported, however culture was raised in the laboratory
Feeding:	Not fed during the test
Treatment for disease:	None reported

Test design

Test vessel:	100-mL beakers containing 50 mL test solution, covered with plexi glass plates
Test medium:	M7-medium
Replication:	Three replicates
No. of animals/vessel:	Ten daphnids per vessel
Duration of test:	48 hours

Environmental test conditions

Temperature:	Test end: 19.9°C
Dissolved oxygen:	Test start: 8.7 – 8.8 mg/L (approx. 95.46 – 96.56% saturation) Test end: 8.6 – 8.8 mg/L (approx. 94.37 – 96.56% saturation)
pH:	Test start: 7.98 – 8.03 Test end: 7.93 – 8.06
Photoperiod:	16 h light : 8 h dark

B. Study Design

This study was conducted to assess the acute toxicity of KWG 4168 to the water flea *Daphnia magna* over 48 hours. The design of the study was based on the results of a preliminary non-GLP test.

First instar *Daphnia magna* were used in the test from an in-house culture, aged 6 to 24 hours. First instar daphnids were separated from older daphnids by sequential mesh screening.

Test vessels were 100-mL beakers containing 50 mL test solution, covered with a plexi glass plate. Beakers were held in a climatic chamber for 48 hours at 20 ± 1°C under a 16 hours light to 8 hours dark photoperiod.

Nominal concentrations of the test substance were prepared by dilution of stock solutions. Stock solutions had been treated in an ultrasonic bath for five minutes and stirred using a magnetic stirrer for 10 minutes. Nominal concentrations were 0.32, 1.00, 1.78, 3.16, 5.62, 10.0 and 31.6 mg a.s./L. Measured concentrations were 0.23, 0.60, 0.82, 2.2, 4.4, 8.3 and 22 mg a.s./L.

To each test concentration were added ten first instar *Daphnia magna* using a pipette. Three replicates were used per concentration.

After 24 and 48 hours, water fleas were assessed visually for survivors, *i.e.* animals with swimming movements within 15 seconds of gentle agitation of the test vessel, and any uncertainty was checked using a stereomicroscope.

Temperature, oxygen content and pH of the test water was determined using electronic measuring equipment. Temperature was determined at test end, and oxygen content and pH were determined both at test start and test end.

EC_{50} values were manually determined using probit analysis after the maximum-likelihood method.

Analytical method

Samples of water were analysed using the validated analytical method 00252 M001, report reference M-008490-02-2 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the OECD 202 guideline were met:

- Mortality/immobilisation in the control to not exceed 10% (actual: 0.0%)
- Dissolved oxygen concentration at test termination to be ≥3 mg/L in all test vessels (actual: 8.6 to 8.8 mg/L)

Test concentrations were determined at test start in all test concentrations. Mean measured concentrations were 46.2 to 83.0% of nominal, therefore results are based on mean measured concentrations.

Stability analysis of three test concentrations confirmed that test concentrations remained approximately constant throughout the test, with 48-hour mean measured concentrations 85.0 to 118.2% of the 0-hour concentrations.

Table CA 8.2.4.1/01-1 Measured concentrations of KWG 4168 during the test

Nominal test concentration (mg a.s./L)	Measured test concentration (mg a.s./L)							
	Test start (0 hr)				Test end (48 hr)			
	Rep 1	Rep 2	Mean	% of nominal	Rep 1	Rep 2	Mean	% of 0-hr concs
0.32	0.225	0.235	0.23	71.9	-		-	-
1.00	0.610	0.590	0.60	60.0	0.479	0.549	0.51	85.0
1.78	0.724	0.921	0.82	46.2	-		-	-
3.16	1.99	2.56	2.2	69.6	2.41	2.83	2.6	118.2
5.62	4.32	4.39	4.4	78.3	-		-	-
10.0	8.10	8.50	8.3	83.0	7.63	7.63	8.2	98.8
31.6	21.4	22.4	22	69.6	-		-	-
Mean				68.4				100.7

LOD Limit of determination: 0.05 µg/L

The results of the measured concentrations taken at 48 hours confirm that the measured concentrations were within 20% of the initial measured concentration. Thus, the results of this study have been based on initial measured test concentrations.

After 48 hours exposure to KWG 4168, cumulative immobility of *Daphnia magna* was 0, 0, 0, 0, 3, 23, 73 and 100% in the control and 0.23, 0.60, 0.82, 2.2, 4.4, 8.3 and 22 mg a.s./L test concentrations, respectively.

Table CA 8.2.4.1/01-2 Immobility of *Daphnia magna* after 48-hr exposure to KWG 4168

Measured concentration (mg a.s./L)	Cumulative immobility by replicate after 24 hours			Cumulative immobility by replicate after 48 hours			Cumulative immobility (%)	
	1	2	3	1	2	3	24 hours	48 hours
Control	0	0	0	0	0	0	0	0
0.23	0	0	0	0	0	0	0	0
0.60	0	0	0	0	0	0	0	0
0.82	0	0	0	0	0	0	0	0
2.2	0	0	0	0	1	0	0	3
4.4	1	1	1	1	3 [a]	3	10	23
8.3	3	4	1	8	8 [a]	6 [a]	27	73
22	10 [b]	10 [b]	10 [b]	10 [b]	10 [b]	10 [b]	100	100

[a] Animals almost motionless at the bottom of test vessels
[b] Animals observed in clusters at the water surface

The resulting NOEC and LOEC values after 48 hours exposure were 2.2 and 4.4 mg a.s./L, respectively. The 24- and 48-hour EC_{50} values were 9.3 and 6.1 mg a.s./L, respectively with corresponding 95% confidence intervals of 7.8 to 11.2 and 5.1 to 7.2 mg a.s./L.

Table CA 8.2.4.1/01-3 Summary of endpoints after 48-hour exposure to spiroxamine

Endpoint	NOEC	LOEC	EC_{10}	EC_{15}	EC_{50}
mg a.s./L	2.2	4.4	2.9	4.1	6.1

III. Conclusion

After 48 hours exposure to KWG 4168, the acute 48-hour EC_{50} to *Daphnia magna* was 6.1 mg a.s./L, with 95% confidence intervals of 5.1 to 7.2 mg a.s./L. The 48-hour NOEC and LOEC were 2.2 and 4.4 mg a.s./L, respectively.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 202 (1984), the most up-to-date version of which is the "*Daphnia* sp., acute immobilisation test", adopted 13 April 2004.

Validity criteria according to the most up-to-date OECD 202 guideline (2004) were met:

- Mortality/immobilisation in the control to not exceed 10% (actual: 0.0%)
- Dissolved oxygen concentration at test termination to be ≥3 mg/L in all test vessels (actual: 8.6 to 8.8 mg/L)

Only three replicates were tested instead of the recommended four replicates but groups of ten individuals were tested instead of groups of five. Thus, the total number of organisms tested was greater than the guideline recommendation but fewer replicates were used. However, the results are still considered to be sufficiently reliable for use in the risk assessment.

The study is therefore considered acceptable.

After 48 hours exposure to KWG 4168, the acute 48-hour EC_{50} to *Daphnia magna* was 6.1 mg a.s./L.

Data Point:	KCA 8.2.4.1/02
Report Author:	[redacted]
Report Year:	1996
Report Title:	Acute toxicity of 14-C-KWG 4168 (tech.) to water fleas (Daphnia magna)
Report No:	HBF/DM 148
Document No:	M-006476-01-1
Guideline(s) followed in study:	OECD-Guideline No. 202 'Guideline for Testing of Chemicals', 'Daphnia sp., Acute immobilisation Test and Reproduction Test, Part I, Adopted 4 April 1984' and EPA FIFRA Guideline 72-2
Deviations from current test guideline:	Yes OECD 202 (2004) Daphnids were 10/vessel instead of the recommended 5/vessel and only 3 replicates instead of 4
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The 48-hour acute toxicity of ^{14}C-KWG 4168 to *Daphnia magna* was studied under static conditions. Test species were exposed to nominal concentrations of 0.56, 1.0, 1.8, 3.2, 5.6, 10 and 18 mg a.s./L and a control and solvent control.

Based on mean measured concentrations, the EC_{50} (24 hours) was 10.6 mg a.s./L (95% confidence limits 8.9 - 12.7 mg a.s./L) and the EC_{50} (48 hours) was 6.8 mg a.s./L (95% confidence limits 5.7 - 8.1 mg a.s./L).

Based on mean measured concentrations, the no-observed-effect-concentration (NOEC) after 24 hours was 3.7 mg a.s./L and after 48 hours 2.1 mg a.s./L, the lowest-observed-effect-concentration (LOEC) after 24 hours was 6.5 mg a.s./L and after 48 hours 3.7 mg a.s./L.

I. Materials and Methods

A. Materials

	Unlabelled test substance	Labelled test substance
Test Material	KWG 4168	^{14}C-KWG 4168
Lot/Batch #:	17002/90	KML 2216
Active substance content:	96.3%	NA
Specific radioactivity:	NA	4.3 MBq (116 µCi)/mg
Radiochemical purity:	NA	99%
Description:	Colourless liquid	Not reported
Stability of test compound:	Mean recoveries of 66.6 – 121.8%	Not reported
Reanalysis/Expiry date:	Not reported	Not reported
Density:	Not reported	Not reported

Treatments

Test rates:	Nominal: 0.56, 1.0, 1.8, 3.2, 5.6, 10 und 18 mg a.s./L Mean measured: 0.43, 0.68, 1.3, 2.1, 3.7, 6.5 and 19.7 mg a.s./L
Solvent/vehicle:	Acetone
Analysis of test concentrations:	Yes (mean measured concentrations 66.6 – 99.2% of nominal)

Test organisms

Species:	*Daphnia magna* < 24 hours old
Source:	In-house culture, originally from the Bundesgesundheitsamt, Berlin, Germany
Acclimatisation period:	This strain was maintained in the laboratory for more than ten years (2 litre containers); the water in which they are kept is changed weekly (dilution water, see below). The organisms were kept in an environmental chamber under the test conditions 20 ± 1 °C, 16 : 8 hour light-dark cycle; the animals were fed with single cell green algae *Scenedesmus subspicatus* and occasionally some commercial ornamental fish food (TetraMin®) (aqueous suspension)
Feeding:	Not fed during the test
Treatment for disease:	Not reported

Test design

Test vessel:	100 mL beakers containing 50 mL test solution, covered with plexi glass plates
Test medium:	M7-medium
Replication:	Three per concentration
No. of animals/vessel:	Ten
Duration of test:	48-hours

Environmental test conditions

Temperature:	20 ± 1°C
Dissolved oxygen:	8.1 to 8.6 mg/L (8.6 mg/L = 99%)
pH:	7.92 to 8.28
Photoperiod:	16 h light : 8 h dark at 700 lux

B. Study Design

The exposure of *Daphnia magna* to ^{14}C-KWG 4168 (tech.) was conducted for 48-hours under static conditions in order to assess the acute toxicity of the active substance.

The test vessels consisted of 100 mL glass beakers (DIN 12332), labelled with study number, concentration and series number. Each test vessel contained 50 mL of the test solutions with ten animals per vessel, three replicates per concentration. The beakers were covered with a plexi glass plate and placed in a environmental chamber for 48 hours at 20 ± 1 °C and a 16:8 light-dark cycle. Light intensity

has been measured with a LMT "pocket-lux", type PO 449, and was about 700 lux. The water fleas were not fed and the test solutions were not aerated during the test.

Test concentrations were chosen based on the results of earlier acute toxicity tests. *Daphnia magna* (<24 hours old) were used in the test from an in-house culture.

Test vessels were held under a 16-hour light to 8-hour dark at 700 lux photoperiod at 20±1°C. The test medium was deionised water reconstituted to M7-medium.

Ten *Daphnia magna* were introduced into each test vessel, and there were three replicates per test concentration.

Nominal test concentrations were 0.56, 1.0, 1.8, 3.2, 5.6, 10 und 18 mg a.s./L along with a control and solvent control groups. Measured test concentration of the freshly prepared test medium gave recoveries of 66.6 to 99.2% of nominal, whereas, recoveries at 48 hours ranged from 66.7 to 121.8% of nominal.

Assessments of mortality and sub-lethal effects were made at 24 and 48 hours. *Daphnia* were taken as live when swimming movements were observed within approximately 15 seconds.

Analytical method

Samples of water were analysed using the validated analytical method 00252 M001, report reference M-008490-02-2 **(see Doc MCA Section 4)**.

II. Results and Discussion

Validity criteria according to the OECD 202 guideline were met:

- Mortality in the control and solvent control does not exceed 10% at the end of the test (actual = 0 and 0%)
- The dissolved oxygen concentration at the end of the test should be ≥3 mg/L in control and test vessels (actual = 8.1 – 8.6 mg/L)

The study is therefore considered acceptable.

The mean active substance contents analysed at the beginning and the end of the test were 66.2 to 109.5 % of the nominal concentrations (for an average 74.7 %). Therefore, the results of this test are reported as mean measured concentrations.

Moreover, the stability analyses at the end of the study showed, that 87.3 % to 98.4 % of the active substance was detected as KWG 4168 and WAK 6301 (KWG 4168-N-oxide), which is supposed to be in equilibrium with the active substance in water (for an average 92.9 %).

Table CA 8.2.4.1/02-1 Analysed concentrations of ^{14}C-KWG 4168 in test solutions at day 0 according to radioactivity measurements (1443945.34 dpm/L = 19.3 mg/L

Nominal		Mean measured radioactivity dpm/5mL	Calculated radioactivity dpm/L	Calculated concentration day 0 (mg a.s./L)	Percent of nominal concentration
Concentrations (mg a.s./L)	Radioactivity dpm/5mL				
Control	-	-0.20	<DL	-	-
Solvent control	-	-0.17	<DL	-	-
0.56	221.5	161.10	32220	0.43	76.9
1.0	397.5	266.10	53220	0.71	71.1
1.8	709.8	507.93	101586	1.4	75.4
3.2	1277.7	836.50	167300	2.2	69.9
5.6	2214.7	1437.77	287554	3.8	68.6
10	3946.8	2491.03	498206	6.7	66.6
18	7098.5	6678.73	1335746	17.9	99.2

<DL Lower than detection limit (53.6 dpm)

Table CA 8.2.4.1/02-2 Analysed concentrations of ^{14}C-KWG 4168 in test solutions after 48 hours according to radioactivity measurements (1443945.34 dpm/L = 19.3 mg/L)

Nominal		Mean measured radioactivity dpm/5mL	Calculated radioactivity dpm/L	Calculated concentration day 0 (mg a.s./L)	Percent of nominal concentration
Concentrations (mg a.s./L)	Radioactivity dpm/5mL				
Control	-	2.4	<DL	-	-
Solvent control	-	0.73	<DL	-	-
0.56	221.5	172.93	34586	0.46	82.6
1.0	397.5	274.17	54834	0.73	73.3
1.8	709.8	505.43	101086	1.4	75.1
3.2	1277.7	825.30	165060	2.2	68.9
5.6	2214.7	1403.93	280786	3.8	67.0
10	3946.8	2495.80	499160	6.7	66.7
18	7098.5	8203.63	1640726	21.9	121.8

<DL Lower than detection limit (53.6 dpm)

Table CA 8.2.4.1/02-3 Mean measured concentrations of ^{14}C-KWG 4168

Nominal concentrations (mg a.s./L)	Calculated concentrations day 0 (mg a.s./L)	Corrected concentrations 48 hours (mg a.s./L)	Mean of day 0 and day 2 (mg a.s./L)	% of nominal concentrations
0.56	0.43	0.42	0.43	76.1
1.0	0.71	0.64	0.68	67.6
1.8	1.4	1.2	1.3	72.0
3.2	2.2	2.0	2.1	66.9
5.6	3.8	3.6	3.7	66.2
10	6.7	6.3	6.5	64.9
18	17.9	21.6	19.7	109.5
			Average:	74.7

The control mortalities were below the 10 % value which is regarded as the limit for natural mortality. As the physico-chemical measurements show, the composition of the test water corresponds to the nominal values. The EC_{50} of the reference substance lies within the required range. Thus, the study conditions correspond to the standard.

Table CA 8.2.4.1/02-4 Water flea toxicity of ^{14}C-KWG 4168 (tech.) using *Daphnia magna*

Nominal concentrations (mg a.s./L)	Mean number of living animals after:		Number of immobilised animals (%) after:	
	24 hours	48 hours	24 hours	48 hours
Control	30	30	0	0
Solvent control	30	30	0	0
0.43	30	29	0	3 ± 6
0.68	30	30	0	0
1.3	29	28	3 ± 6	7 ± 12
2.1	30	30	0	0
3.7	29	26	3 ± 6	13 ± 15
6.5	29 [1] [3]	18 [3] [2] [1]	3 ± 6	40 ± 20
19.7	1 [1]	0	97 ± 6	100

[ ] Number of living animals with symptoms, if observed
Symptoms:
[1] quick trembling antennae movements [2] frequency of antenna movements clearly increased
[3] frequency of antenna movements clearly decreased

Table CA 8.2.4.1/02-5 Summary of endpoints after 48-hour exposure to spiroxamine

Endpoint	24-hours	48-hours
NOEC	3.7 mg a.s./L	2.1 mg a.s./L
LOEC	6.5 mg a.s./L	3.7 mg a.s./L
EC_{50}	10.6 mg a.s./L	6.8 mg a.s./L

III. Conclusion

Based on mean measured test concentrations, the EC_{50} (24 hours) was 10.6 mg a.s./L (95% confidence limits 8.9 - 12.7 mg a.s./L) and the EC_{50} (48 hours) was 6.8 mg a.s./L (95% confidence limits 5.7 - 8.1 mg a.s./L).

Based on mean measured concentrations, the no-observed-effect-concentration (NOEC) after 24 hours was 3.7 mg a.s./L and after 48 hours 2.1 mg a.s./L, the lowest-observed-effect-concentration (LOEC) after 24 hours was 6.5 mg a.s./L and after 48 hours 3.7 mg a.s./L.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 202, the most up-to-date version of which was adopted 13 April 2004

Validity criteria according to the OECD 202 guideline (2004) were met:

- Mortality in the control and solvent control does not exceed 10% at the end of the test (actual = 0 and 0%)
- The dissolved oxygen concentration at the end of the test should be ≥3 mg/L in control and test vessels (actual = 8.1 – 8.6 mg/L)

Only three replicates were tested instead of the recommended four replicates but groups of ten individuals were tested instead of groups of five. Thus, the total number of organisms tested was greater than the guideline recommendation but fewer replicates were used. However, the results are still considered to be sufficiently reliable for use in the risk assessment.

The study is therefore considered acceptable.

The 48-hour EC_{50} was determined to be 6.8 mg a.s./L.

Data Point:	KCA 8.2.4.1/03
Report Author:	[redacted]
Report Year:	1997
Report Title:	Acute toxicity of 14C-KWG 4168 (tech.) to water fleas (Daphnia magna) under flow-through test conditions
Report No:	HBF/DM 184
Document No:	M-006523-01-1
Guideline(s) followed in study:	OECD-Guideline No. 202 "OECD-Guideline for Testing Chemicals", 4 April 1984: "Daphnia spec. Acute Immobilisation Test and Reproduction Test
Deviations from current test guideline:	Yes OECD 202 (2004) Top concentration was not in solution, however, it was excluded from statistical analysis. Therefore, this had no impact on the study
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The 48-hour acute toxicity of ^{14}C-KWG 4168 to *Daphnia magna* was studied under flow-through conditions. Test organisms were exposed to nominal concentrations of 0.56, 1.0, 1.8, 3.2, 5.6, 10 and 18 mg a.s./L and a control and solvent control.

Based upon the mean measured concentrations, the EC_{50} (24 hours) for ^{14}C-KWG 4168 (tech.) was 3.8 mg a.s./L (95% confidence limits 3.4 - 4.3 mg a.s./L) and the EC_{50} (48 hours) was 3.0 mg a.s./L (95 %-confidence limits 3.1 to 4.4 mg a.s./L).

Based upon mean measured concentrations, the no-observed-effect-concentration (NOEC) after 24 and 48 hours was 1.4 mg a.s./L, the lowest-observed-effect-concentration (LOEC) was 2.4 mg a.s./L.

I. Materials and Methods

A. Materials

	Unlabelled test substance	Labelled test substance
Test Material	KWG 4168	^{14}C-KWG 4168
Lot/Batch #:	17002/90	10093/3
Active substance content:	96.5%	NA
Specific radioactivity:	NA	3.68 MBq (99.5 µCi)/mg
Radiochemical purity:	NA	>98%
Description:	Colourless liquid	Not reported
Stability of test compound:	Mean recovery: 75.5%	Not reported
Reanalysis/Expiry date:	07 August 1997	Not reported
Density:	Not reported	Not reported
Treatments		
Test rates:	Nominal: 0.56, 1.0, 1.8, 3.2, 5.6, 10 und 18 mg a.s./L Mean measured: 0.44, 0.73, 1.39, 2.39, 4.30, 7.25 and 8.80 mg a.s./L	
Solvent/vehicle:	DMF	
Analysis of test concentrations:	Yes, day 0 and 2 (mean measured concentrations 62.7 to 100.3% of nominal)	
Test organisms		
Species:	*Daphnia magna* < 24 hours old	
Source:	In-house culture, originally from the Bundesgesundheitsamt, Berlin, Germany	
Acclimatisation period:	This strain was maintained in the laboratory for more than ten years (2 litre containers); the water in which they are kept is changed weekly. The organisms were kept in a environmental chamber under the test conditions 20 ± 1 °C, 16 : 8 hour light-dark cycle; the animals were fed with single cell green algae *Scenedesmus subspicatus* and occasionally	

	some commercial ornamental fish food (TetraMin®) (aqueous suspension)
Feeding:	Not fed during the test
Treatment for disease:	Not reported
Test design	
Test vessel:	100 mL beakers containing 50 mL test solution, covered with plexi glass plates
Test medium:	M7-medium
Replication:	Four per concentration
No. of animals/vessel:	Ten
Duration of test:	48-hours
Environmental test conditions	
Temperature:	20 ± 1°C
Dissolved oxygen:	8.7 to 8.9 mg/L (8.7 mg/L = 96 %)
pH:	7.9 to 8.1
Photoperiod:	16 h light : 8 h dark at ~ 700 lux

B. Study Design

The exposure *of Daphnia magna* to ^{14}C-KWG 4168 (tech.) was conducted for 48-hours under flow-through conditions in order to assess the acute toxicity of the active substance.

The test vessels consisted of 100 mL glass beakers, labelled with study number, concentration and series number. Each test vessel contained 50 mL of the test solutions with ten animals per vessel, four replicates per concentration. The beakers were covered with a plexi glass plate and placed in a environmental chamber for 48 hours at 20 ± 1 °C and a 16:8 light-dark cycle. Light intensity was about 700 lux. The water fleas were not fed and the test solutions were not aerated during the test.

Test concentrations were chosen based upon historical toxicity information for the active substance. *Daphnia magna* (<24 hours old) were used in the test from an in-house culture.

Ten *Daphnia magna* were introduced into each test vessel, and there were four replicates per test concentration.

Nominal test concentrations were 0.56, 1.0, 1.8, 3.2, 5.6, 10 and 18 mg a.s./L along with a control and solvent control groups. Measured test concentration of the test medium gave recoveries of 62.7 to 100.3% of nominal.

Assessments of mortality and sub-lethal effects were made at 24 and 48 hours. *Daphnia* were taken as live when swimming movements were observed within approximately 15 seconds.

Analytical method

Samples of water were analysed using the validated analytical method 00252 M001, report reference M-008490-02-2 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the OECD 202 guideline were met:

- Mortality in the control and solvent control does not exceed 10% at the end of the test (actual = 2.5 and 2.5%)
- The dissolved oxygen concentration at the end of the test should be ≥3 mg/L in control and test vessels (actual = 8.7 – 8.9 mg/L)

The study is therefore, considered acceptable.

Measured concentrations of ^{14}C-KWG 4168 were 73 to 78 % (for an average of 75.5 %) of the nominal concentrations.

Table CA 8.2.4.1/03-1 Measured radioactivity in the test solutions

Nominal		Measured Radioactivity (dpm/10 mL)			
Concentrations (mg a.s./L)	**Radioactivity dpm/10mL**	**Day 0 (average)**	**Day 1 (average)**	**Day 2 (average)**	**Percent of nominal concentration**
Control	-	9.1	9.1	7.8	
Solvent control	-	10.3	7.9	8.6	-
0.56	200	199.1	206.4	196.4	100.3
1.0	200	183.0	185.9	184.0	92.1
1.8	200	191.8	192.3	190.7	95.8
3.2	200	188.6	180.0	179.3	91.3
5.6	200	197.2	193.3	187.2	96.3
10	200	190.4	197.1	189.1	94.6
18	200	133.2	118.7	124.6	62.7

LOD Detection limit (50.4 cpm, corresponding to 55.5 dpm)

The control mortalities were 2.5 % which was well below the 10 % value which is regarded as the limit for natural mortality. As the physico-chemical measurements show, the composition of the test water corresponds to the nominal values. The EC_{50} of the reference substance ($K_2Cr_2O_7$) lies within the required range. Thus, the study conditions and culture health correspond to the standard.

Table CA 8.2.4.1/03-2 Water flea toxicity of ^{14}C-KWG 4168 (tech.) using *Daphnia magna*

Mean measured concentration (mg a.s./L)	Mean number of living animals after:		Immobilised or dead water fleas (%) after:	
	24 hours	**48 hours**	**24 hours**	**48 hours**
Control	40	39	0	2.5 ± 5.0
Solvent control	40	39	0	2.5 ± 5.0
0.44	40	40	0	0
0.73	40	37	0	7.5 ± 9.6
1.4	38	37	0	7.5 ± 9.6
2.4	36 [1] 6,4	36	5.0 ± 5.8	15.0 ± 5.8
4.3	20 [3] [9] [1] 6,4,7,8	9 [3] [1] [3] 7,8	10.0 ± 8.2	77.5 ± 22.2
7.3**	0	0	100	100
8.8**	0	0	100	100

[ ] Number of living animals with symptoms, if observed
* These concentrations were used to calculate the EC_{50}
** In this concentration an oily layer of the test substance was observed on the water surface

Symptoms:
[1] quick, trembling antennae movements
[2] frequency of antenna movements clearly increased
[3] frequency of antenna movements clearly decreased
[4] hardly any movements perceivable
[5] swimming movements show coordination disturbances
[6] animals lie at the bottom
[7] animals cling to the water surface
[8] animals cling together in clusters

The highest test concentration (8.8 mg a.s./L) was observed to have an oily layer on the water surface. The KWG 4168 was not soluble at this concentration. Therefore, the highest concentration was excluded from statistical calculations.

Table CA 8.2.4.1/03-3 Summary of endpoints after 48-hour exposure to spiroxamine

Endpoint	24-hours	48-hours
NOEC	1.4 mg a.s./L	1.4 mg a.s./L
LOEC	2.4 mg a.s./L	2.4 mg a.s./L
EC_{50}	3.8 mg a.s./L	3.0 mg a.s./L

III. Conclusion

Based upon the mean measured concentrations, the EC_{50} (24 hours) for ^{14}C-KWG 4168 (tech.) was 3.8 mg a.s./L (95% confidence limits 3.4 - 4.3 mg a.s./L) and the EC_{50} (48 hours) was 3.0 mg a.s./L (95 %-confidence limits 3.1 to 4.4 mg a.s./L).

Based upon mean measured concentrations, the no-observed-effect-concentration (NOEC) after 24 and 48 hours was 1.4 mg a.s./L, the lowest-observed-effect-concentration (LOEC) was 2.4 mg a.s./L.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 202, the most up-to-date version of which was adopted 13 April 2004.

Validity criteria according to the OECD 202 guideline (2004) were met:

- Mortality in the control and solvent control does not exceed 10% at the end of the test (actual = 2.5 and 2.5%)
- The dissolved oxygen concentration at the end of the test should be ≥3 mg/L in control and test vessels (actual = 8.7– 8.9 mg/L)

The study is therefore considered acceptable.

The 48-hour EC_{50} was determined to be 3.0 mg a.s./L.

Metabolites

KWG 4168-N-oxide (M03)

Data Point:	KCA 8.2.4.1/04
Report Author:	[redacted]
Report Year:	1996
Report Title:	Orientating waterflea toxicity of N-oxide-KWG 4168
Report No:	HBF/ODM 122
Document No:	M-006702-01-1
Guideline(s) followed in study:	OECD-Guideline No. 202
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017) When compared to respective data on the active substance spiroxamine, the KWG-4168-N-Oxide is definitely less toxic. KWG 4168-N-oxide (WAK 6301) is therefore deemed to be ecotoxicologically irrelevant.
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

A non-GLP 48-hours acute toxicity test to *Daphnia magna* with KWG 4168-N-Oxide was conducted.

The no observed effect concentration (NOEC) after 48-hours was ≥100 mg/L and the lowest observed effect concentration (LOEC) after 48 hours was >100 mg/L. The 48 hour EC_{50} was >100 mg/L.

I. Materials and Methods

A. Materials

Test Material	N-oxide-KWG 4168
Lot/Batch #:	950209ELB01
Purity:	93%
Description:	Not reported
Stability of test compound:	Not reported
Reanalysis/Expiry date:	Not reported
Density:	Not reported
Treatments	
Test rates:	1.0, 10 and 100 mg/L
Solvent/vehicle:	None
Analysis of test concentrations:	No
Test organisms	
Species:	*Daphnia magna*
Source:	In-house culture
Acclimatisation period:	Not reported
Feeding:	Not reported
Treatment for disease:	Not reported
Test design	
Test vessel:	Not reported
Test medium:	Elendt
Replication:	3 replicates
No. of animals/vessel:	20
Duration of test:	48 hours
Environmental test conditions	
Temperature:	Not reported
Dissolved oxygen:	>90% O2
pH:	pH 8.0 ± 0.3
Photoperiod:	16:8 hours light-dark cycle

B. Study Design

The test was conducted according to the OECD Test Guideline 202. Test water (synthetic test water according to ELENDT, >90% O2, pH 8.0 ± 0.3) and test substance dispersions were prepared at the desired concentrations. 50 mL dispersion each were filled into a 100 mL beaker, with three beakers per concentration. Each beaker was stocked with 20 young *Daphnia magna*. After 48 hours exposure at 20 °C and 16:8 hours light-dark cycle the dead animals were counted.

II. Results

Table CA 8.2.4.1/04-1 Survival of the parent water fleas

Concentration (mg/L)	Mortality (%)
Control	0
1.0	10
10	3
100	3

III. Conclusion

The no observed effect concentration (NOEC) after 48-hours was ≥100 mg/L and the lowest observed effect concentration (LOEC) after 48 hours was >100 mg/L. The 48-hour EC_{50} was >100 mg/L.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 202. The results have been re-assessed to the current test guideline for *Daphnia magna* acute toxicity tests, OECD 202 (adopted, 13 April 2004)

Validity criteria according to the OECD 202 guideline (2004) were met:

- In the control, including the control containing the solubilising agent, not more than 10 percent of the daphnids should have been immobilised (actual: 0%);
- The dissolved oxygen concentration at the end of the test should be ≥ 3 mg/L in control and test vessels.

The test would appear to have met the validity criteria but as insufficient test details are available in the report and because no analytical verification of test concentrations was conducted, the study is therefore considered to be supporting information only.

A new GLP acute *Daphnia* study has not been conducted using KWG 4168-N-Oxide (M03) as this was not considered to be necessary. Available study data for the parent material clearly demonstrate that algae are the most sensitive organism group by at least two orders of magnitude therefore acute metabolite studies with aquatic invertebrates are not considered necessary. Furthermore, available algal data show that this metabolite is far less toxic than the parent material. Thus, this non-GLP study is considered sufficient to demonstrate the low toxicity of KWG 4168-N-Oxide (M03) to aquatic invertebrates.

The 48-hour EC_{50} for KWG 4168-N-Oxide (M03) was determined to be >100 mg/L.

CA 8.2.4.2 Acute toxicity to an additional aquatic invertebrate species

No acute data with an additional aquatic invertebrate species are available. Spiroxamine is a fungicide and does not display insecticidal activity therefore acute data with an additional species of aquatic invertebrate are 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:	1994
Report Title:	Influence of KWG 4168 (techn.) on the reproduction rate of water fleas
Report No:	HBF/RDM 50
Document No:	M-006401-01-1
Guideline(s) followed in study:	EEC XI/681/86 (1987) OECD 202 (II) (1984), now OECD 211 (2012)
Deviations from current test guideline:	Yes OECD 211 (2012) No analysis of aged test media. Only fresh test media was sampled.
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The 21-day chronic toxicity of KWG 4168 to *Daphnia magna* was studied under semi-static conditions. Test organisms were exposed to nominal test concentrations of 0.032, 0.10, 0.18, 0.32, 0.56, 1.0 and 3.2 mg a.s./L and a control. A delay in time to first brood was observed at concentrations ≥0.32 mg a.s./L. The most sensitive endpoint was reproduction. The 21-day NOEC based on reproduction was 0.10 mg a.s./L.

I. Materials and Methods

A. Materials

Test Material KWG 4168

Lot/Batch #: 898 114 002

Purity: 97.8%

Description: Colourless liquid

Stability of test compound: Mean recoveries of 51 – 75% of initial concentrations in parallel studies

Reanalysis/Expiry date: Not reported

Density: Not reported

Treatments

Test rates: Nominal: 0.032, 0.10, 0.18, 0.32, 0.56, 1.0 and 3.2 mg a.s./L

Solvent/vehicle: None

Analysis of test concentrations: Yes (mean measured concentrations 76 – 122% of nominal)

Test organisms

Species: *Daphnia magna*, approx. 6 – 24 h old

Source:	In-house culture, originally from the Bundesgesundheitsamt, Berlin, Germany
Acclimatisation period:	None reported
Feeding:	0.2 mg TOC-content green alga
Treatment for disease:	None reported
Test design	
Test vessel:	100-mL beakers containing 50 mL test solution, covered with plexi glass plates
Test medium:	M7-medium
Replication:	Ten replicates
No. of animals/vessel:	Individually held
Duration of test:	21 days
Environmental test conditions	
Temperature:	19.6 – 20.3°C
Dissolved oxygen:	Fresh: 8.7 – 9.9 mg/L (approx. 94.89 – 109.5% saturation) Spent: 8.0 – 9.3 mg/L (approx. 87.26 – 102.86% saturation)
pH:	Fresh: 8.00 – 8.15 Spent: 7.88 – 8.18
Photoperiod:	16 h light : 8 h dark at 1000 lux

B. Study Design

This study was conducted in order to assess the reproductive toxicity of KWG 4168 to the water flea *Daphnia magna* under semi-static conditions. Test concentrations were chosen based on the results of earlier acute toxicity tests.

First instar *Daphnia magna* were used in the test from an in-house culture, aged 6 to 24 hours. First instar daphnids were separated from older daphnids by sequential mesh screening. Test *Daphnia* were the young of an approximately 21-day old synchronous culture.

Test vessels were 100-mL glass beakers containing 50 mL test solution and covered with plexi glass plates. These were held under a 16-hour light to 8-hour dark 1000 lux photoperiod at 20 ± 1°C. The test medium was deionised water reconstituted to M7-medium.

One female *Daphnia magna* was introduced into each test vessel, and there were ten replicates per test concentration. Daphnids were transferred three times per week to freshly prepared test medium using a pipette and fed the green alga (*Scenedemus subspicatus*) to a quantity of 0.2 mg TOC-content.

Nominal test concentrations were 0.032, 0.10, 0.18, 0.32, 0.56, 1.0 and 3.2 mg a.s./L along with a control. Measured test concentration of the freshly prepared test medium gave recoveries of 76 to 122% of nominal.

Assessments of mortality and reproduction were made thrice weekly, at each test medium transfer. *Daphnia* were taken as dead when no swimming and/or antennae movements were observed within 15 seconds. Body length of the adult daphnids was determined at the end of the 21-day test. This was done by measuring from the head to the base of the spine using a binocular microscope with an eyepiece graticule.

Temperature was measured in one vessel of the control.

Analytical method

Samples of water were analysed using the validated analytical method 00252 M001, report reference M-008490-02-2 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the test guideline were met:

- Mortality of the parent animals in the control to not exceed 20% at the end of the test: (actual: 0%)
- Mean number of living offspring produced per parent animal surviving at the end of the test ≥60 (mean control value: 94.3)

Analytical results, corrected by the recovery rates, show measured test concentrations 76 to 122% of nominal. The stability of the active substance was ascertained with two parallel studies, with mean recoveries of 51 to 75% of initial concentrations. The results have been presented based on nominal concentrations.

Table CA 8.2.5.1/01-1 Measured concentrations of KWG 4168 during the test

Nominal test concentration (mg a.s./L)	Analysed concentrations (mg a.s./L)						Mean % of nominal
	Day 2	% of nominal	Day 9	% of nominal	Day 16	% of nominal	
Control	<LOD	-	<LOD	-	<LOD	-	-
0.032	0.063	195	0.026	82	0.029	90	122
0.10	0.125	125	0.065	65	0.076	76	89
0.18	0.154	85	0.128	71	0.128	71	76
0.32	0.321	100	0.333	104	0.308	96	100
0.56	0.615	110	0.603	108	0.679	121	113
1.0	0.935	97	0.919	92	0.826	83	91
3.2	2.91	91	2.91	91	2.91	91	91
Mean	-	115	-	88	-	90	97

LOD Limit of detection: 0.1 µg/L

No mortality of daphnids was observed in the control, 0.032, 0.10, 0.18, 0.32 and 1.0 mg a.s./L test concentrations after 21 days. When exposed to 0.56 and 3.2 mg a.s./L, mortality of 10 and 60% was observed, respectively. Compared to the control, the number of offspring produced was significantly reduced ($p<0.05$) at test concentrations of 0.18, 0.32, 0.56, 1.0 and 3.2 mg a.s./L.

At test termination, parent animals in the control had a body length of 4.34 mm indicating good female development. Adult body length was significantly reduced (p<0.05) at test concentrations of 1.0 and 3.2 mg a.s./L.

Table CA 8.2.5.1/01-2 Mortality of *Daphnia magna* over 21-day exposure to KWG 4168

Nominal concentration (mg a.s./L)	Cumulative mortality (%) by day									
	0	**2**	**5**	**7**	**9**	**12**	**14**	**16**	**19**	**21**
Control	0	0	0	0	0	0	0	0	0	0
0.032	0	0	0	0	0	0	0	0	0	0
0.10	0	0	0	0	0	0	0	0	0	0
0.18	0	0	0	0	0	0	0	0	0	0
0.32	0	0	0	0	0	0	0	0	0	0
0.56	0	0	0	0	0	0	0	10	10	10
1.0	0	0	0	0	0	0	0	0	0	0
3.2	0	0	0	0	10	30	40	50	60	60

A delay in time to first brood was observed at test concentrations 0.32, 0.56 and 1.0 mg a.s./L, with first brood observed 2, 2 and 7 days later than in the control. No offspring were observed at any time in the highest test concentration, 3.2 mg a.s./L.

Table CA 8.2.5.1/01-3 Number of offspring of *Daphnia magna* over 21-day exposure to KWG 4168

Nominal concentration (mg a.s./L)	Number of offspring by day											
	0	**2**	**5**	**7**	**9**	**12**	**14**	**16**	**19**	**21**	**Total**	**% of nominal**
Control	0	0	0	0	0	142	267	57	186	291	943	-
0.032	0	0	0	0	0	138	269	25	193	291	916	97
0.10	0	0	0	0	10	138	182	48	217	254	849	90
0.18	0	0	0	0	0	45	207	0	228	244	724	77
0.32	0	0	0	0	0	0	197	52	230	207	646	69
0.56	0	0	0	0	0	0	38	0	155	211	404	48
1.0	0	0	0	0	0	0	0	5	20	52	77	8
3.2	0	0	0	0	0	0	0	0	0	0	0	0

The mean body length of surviving adults was 102.0, 101.3, 100.6, 102.2, 101.7, 92.3 and 44.7% of the control at test concentrations 0.032, 0.10, 0.18, 0.32, 0.56, 1.0 and 3.2 mg a.s./L.

Table CA 8.2.5.1/01-4 Body length of surviving adult *Daphnia magna* after 21-day exposure to KWG 4168

Nominal concentration (mg a.s./L)	**Control**	**0.032**	**0.10**	**0.18**	**0.32**	**0.56**	**1.0**	**3.2**
Number	***10***	***10***	***10***	***10***	***10***	***9***	***10***	***4***
Mean (mm)	4.34	4.43	4.40	4.38	4.44	4.42	4.01	1.94
SD	0.100	0.068	0.107	0.124	0.132	0.203	0.211	0.165
CV (%)	2.31	1.53	2.42	2.83	2.97	4.6	5.28	8.50
% of control	-	102.0	101.3	100.6	102.2	101.7	92.3	44.7

SD: Standard Deviation; CV: Coefficient of Variation

The resulting NOEC and LOEC values based on reproduction after 21 days exposure were 0.10 and 0.18 mg a.s./L, respectively.

Table CA 8.2.5.1/01-5 Effects of spiroxamine exposure on the juvenile growth phase of the F_0 generation

Endpoint	NOEC	LOEC
mg a.s./L	0.10	0.18

III. Conclusion

The 21-day chronic toxicity of KWG 4168 to *Daphnia magna* was studied under semi-static conditions. A delay in time to first brood was observed at concentrations ≥0.32 mg a.s./L. The most sensitive endpoint was reproduction. The 21-day NOEC based on reproduction was 0.10 mg a.s./L.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 202 (II), the most up-to-date version of which is the OECD 211 "*Daphnia magna* reproduction test", adopted 02 October 2012.

Validity criteria according to the OECD 211 guideline (2012) were met:

- Mortality of the parent animals in the control to not exceed 20% at the end of the test: (actual: 0%)
- Mean number of living offspring produced per parent animal surviving at the end of the test ≥60 (mean control value: 94.3)

It is noted that analytical samples were only taken on freshly prepared test media on three occasions during the study. No samples of old test media were taken for analysis but the report does reference other parallel studies in which the stability of spiroxamine in test conditions has been demonstrated. The results of this study have been based on nominal concentrations which may therefore over-estimate the concentrations that were achieved in the test, thereby potentially over-estimating the NOEC value.

The study is considered acceptable but the results should be treated with caution due to the lack of chemical analysis for the old test media. Additional Daphnia reproduction studies using Spiroxamine are available and have determined slightly more conservative NOEC values than this study therefore the results of this study have not been used directly in the risk assessment.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

The 21-day NOEC based on reproduction was determined to be 0.10 mg a.s./L.

Data Point:	KCA 8.2.5.1/04
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10 and EC20 values for Daphnia magna with spiroxamine TG in a reproduction study
Report No:	0471836-ECO2
Document No:	M-761546-01-1
Guideline(s) followed in study:	Annex to Com. Reg. 283/2013
Deviations from current test guideline:	None
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 M-006401-01-1 on the effects of Spiroxamine TG on the reproduction of water fleas (*Daphnia magna*) study did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013. Effect concentrations with a 10% and 20% effect on survival, length and reproduction when compared to the control were re-calculated.

The resulting EC_{10} and EC_{20} values for survival at 21 d were 1.11 (95% CL: 0.58 – 2.11) and 1.62 (95% CL: 1.01 – 2.58) mg a.s./L, respectively. The resulting EC_{10} and EC_{20} values for length at 21 d were 1.13 (95% CL: 1.06 – 1.21) and 1.57 (95% CL: 1.49 – 1.64) mg a.s./L, respectively. The resulting EC_{10} and EC_{20} values for reproduction at 21d were 0.12 (95% CL: 0.06 – 0.17) and 0.20 (95% CL: 0.13 – 0.26) mg a.s./L, respectively.

I. Methods

The statistical evaluation was performed with statistical software ToxRat Standard v3.3.0.

Effect concentrations with 10 and 20% from the test item treatment when compared to the pooled controls were determined for survival, length and reproduction. A Probit function using linear maximum likelihood regression was used along with 95% EC_X confidence limits for length and a Weibull function using linear maximum likelihood regression was used along with 95% confidence limits for reproduction.

II. Results and Discussion

An explanation is given for regression analysis endpoints for length, reproduction and survival. These details can be found below.

Length at 21 days

Regarding the calculation of EC_{10} and EC_{20} values for length at 21 d, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.00, showing no significant deviation between fit and data, and a statistically significant concentration/response was found ($p(F) < 0.001$) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table.

Table CA 8.2.5.1/04-1 Results of the Probit analysis (max. likelihood regression) with length at 21 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (according to Fieller´s theorem)

Parameter	Length	
	EC_{10} (95 % confidence interval) [mg a.s./L]	EC_{20} (95 % confidence interval) [mg a.s./L]
Effect on length at 21 d	1.13 (1.06 – 1.21)	1.57 (1.49 – 1.64)

The resulting EC_{10} and EC_{20} values of 1.13 (95% CL: 1.06 – 1.21) and 1.57 (95% CL: 1.49 – 1.64) mg a.s./L, respectively, meet the goodness of fit criteria and therefore the estimated EC_{10} value is considered reliable for use within a risk assessment.

Reproduction at 21 d

Regarding the calculation of EC_{10} and EC_{20} values for reproduction at 21 d, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.00, showing no significant deviation between fit and data, and a statistically significant concentration/response was found ($p(F) < 0.001$) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table below.

Table CA 8.2.5.1/04-2 Results of the Weibull analysis (max. likelihood regression) with reproduction at 21 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (according to Fieller's theorem)

Parameter	Reproduction	
	EC_{10} (95 % confidence interval) [mg a.s./L]	EC_{20} (95 % confidence interval) [mg a.s./L]
Effect on reproduction at 21 d	0.12 (0.06 – 0.17)	0.20 (0.13 – 0.26)

The resulting EC_{10} and EC_{20} values of 0.12 (95% CL: 0.06 – 0.17) and 0.20 (95% CL: 0.13 – 0.26) mg a.s./L, respectively, meet the goodness of fit criteria and therefore the estimated EC_{10} value is considered reliable for use in the risk assessment.

Survival at 21 d

Regarding the calculation of EC_{10} and EC_{20} values for survival at 21 d, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 0.677, showing no significant deviation between fit and data, and a statistically significant concentration/response was found (p(F) = 0.013) for this parameter.

Table CA 8.2.5.1/04-3 Results of the Weibull (max. likelihood regression) with survival at 21 d: Selected effective concentrations (ECx) of the test item and their 95% confidence limits (by normal approximation)

Parameter	Survival	
	EC_{10} (95 % confidence interval) [mg a.s./L]	**EC_{20} (95 % confidence interval) [mg a.s./L]**
Effect on reproduction at 21 d	1.11 (0.58 – 2.11)	1.62 (1.01 – 2.58)

III. Conclusion

The resulting EC_{10} and EC_{20} values for survival at 21 d were 1.11 (95%CL: 0.58 – 2.11) and 1.62 (95%CL: 1.01 – 2.58) mg a.s./L, respectively. The resulting EC_{10} and EC_{20} values for length at 21 d were 1.13 (95%CL: 1.06 – 1.21) and 1.57 (95%CL: 1.49 – 1.64) mg a.s./L, respectively. The resulting EC_{10} and EC_{20} values for reproduction at 21d were 0.12 (95%CL: 0.06 – 0.17) and 0.20 (95%CL: 0.13 – 0.26) mg a.s./L, respectively.

Assessment and conclusion by applicant:

The statistical re-evaluation of the data has determined reliable EC_{10} and EC_{20} values for survival, length and reproduction. However, the lowest endpoint remains the NOEC of 0.10 mg a.s./L based on reproduction therefore this value shall be taken as the most critical endpoint from this study.

The values determined in the re-evaluation work are considered to be fully valid.

Data Point:	KCA 8.2.5.1/02
Report Author:	[redacted]
Report Year:	1998
Report Title:	Influence of 14C-KWG 4168 (technical) on the reproduction of water fleas under flow-through test conditions
Report No:	HBF/RDM 61
Document No:	M-006555-01-1
Guideline(s) followed in study:	OECD-Guideline No. 202 "OECD-Guideline for Testing Chemicals", 4 April 1984: "Daphnia spec., Acute Immobilisation Test and Reproduction Test"
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2016), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The 21-day chronic toxicity of ^{14}C-KWG 4168 to *Daphnia magna* was studied under flow-through conditions. Test organisms were exposed to nominal concentrations of 0.0056, 0.010, 0.018, 0.032, 0.056, 0.10 and 0.18 mg a.s./L plus a control and solvent control.

Test water samples were collected from all test concentrations including controls on seven occasions during the study and the radioactivity was measured by Liquid Scintillation Counts. The exposure concentrations were calculated from the measured dpm and the ratio of radiolabeled a.s. to unlabeled a.s. as determined in the corresponding stock solutions. The actual concentrations were 102.5 to 115.5 % (for an average: 108.1 %) of the nominal concentrations.

The no observed effect concentration (NOEC) with regard to reproduction was 0.034 mg a.s./L, whereas, the body length NOEC was 0.11 mg a.s./L and the body weight NOEC was 0.11 mg a.s./L.

I. Materials and Methods

A. Materials

	Unlabelled test substance	Labelled test substance
Test Material	Spiroxamine	[cyclohexyl-1-^{14}C]-KWG 4168
Lot/Batch #:	17002/96	10039/3
Active substance content:	96.5%	-
Specific radioactivity:	-	3.68 MBq (99.5 uCi)/rag
Radiochemical purity:	-	>98%
Description:	Colourless liquid	Not reported
Stability of test compound:	Sufficient based on expiration date	Sufficient based on expiration date
Reanalysis/Expiry date:	07 Aug 1997	Not reported
Density:	Not reported	Not reported

Treatments

Test rates:	Nominal: 0.0056, 0.010, 0.018, 0.032, 0.056, 0.10 und 0.18 mg a.s./L Mean measured: 0.0065, 0.011, 0.020, 0.034, 0.057, 0.11 and 0.19 mg a.s./L
Solvent/vehicle:	DMF
Analysis of test concentrations:	Yes, day 0, 2, 7, 9, 14, 16 and 21 (mean measured concentrations 102 % to 115.5% of nominal)
Test organisms	
Species:	*Daphnia magna* < 24 hours old
Source:	In-house culture, originally from the Bundesgesundheitsamt, Berlin, Germany
Acclimatisation period:	This strain was maintained in the laboratory for more than ten years (2 litre containers); the water in which they are kept is changed weekly. The organisms were kept in a environmental chamber under the test conditions 20 ± 1 °C 16 : 8 hour light-dark cycle, the animals were fed with single cell green algae *Scenedesmus subspicatus* and occasionally some commercial ornamental fish food (TetraMin®) (aqueous suspension)
Feeding:	The food suspension was freshly prepared two times per week from the algae stock suspension. During the study, the water fleas were fed with each cycle of test solution preparation. The food was living single cell green algae *(Scenedesmus subspicatus)*. The total organic carbon (TOC) content of this food suspension was determined photometrically. The daphnids were fed with 0.5 mg TOC per test vessel at each cycle of the test diluter system. The food suspension was continuously stirred to ensure homogeneity and equal delivery of food to all test chambers
Treatment for disease:	Not reported
Test design	
Test vessel:	The test beakers had holes of 3 cm diameter at the water level of 250 mL (water height: about 6 cm). Stainless steel screens (200 um mesh size) were secured to the outside of the beakers to prevent the loss of water fleas as the test solutions overflowed at each renewal.
Test medium:	M7-medium
Replication:	Four per concentration
No. of animals/vessel:	Five
Duration of test:	21-days
Environmental test conditions	
Temperature:	20 ± 1°C
Dissolved oxygen:	6.7 to 9.0 mg/L (8.9 mg/L = 98%)
pH:	7.5 to 8.0
Photoperiod:	16 h light : 8 h dark at ~700 lux

B. Study Design

The exposure *of Daphnia magna* to ^{14}C-KWG 4168 (technical) was conducted for 21-days under flow-through conditions in order to assess the chronic toxicity of the compound.

The test vessels consisted of beakers which had holes of 3 cm diameter at the water level of 250 mL, stainless steel screens (200 um mesh size) were secured to the outside of the beakers to prevent the loss of water fleas as the test solutions overflowed at each renewal. Each test vessel contained five animals per vessel, four replicates per concentration. The beakers were placed in a environmental chamber for 21 days at 20 ± 1 °C and a 16:8 light-dark cycle. Light intensity was about 700 lux. The water fleas were fed on media renewals days.

Test concentrations were chosen based upon historical toxicity information for the compound. *Daphnia magna* (<24 hours old) were used in the test from an in-house culture.

Nominal test concentrations were 0.0056, 0.010, 0.018, 0.032, 0.056, 0.10 and 0.18 mg a.s./L along with a control and solvent control groups. Mean measured test concentrations were 0.0065, 0.011, 0.020, 0.034, 0.057, 0.11 and 0.19 mg a.s./L.

Parent daphnids in the test vessels were observed daily, with exception of Saturday and Sunday of the first week (days 3 and 4). Observations for parent survival, neonate survival, and sublethal and behavioral effects were made and recorded. Starting with day 8, neonates were counted and removed daily. Prior to counting neonates, the parent daphnids were carefully removed from the beaker and placed in a quantity of the appropriate test solution. The test solution containing the neonates was then strained through a 0.20 mm plastic mesh. The neonates were retained on the mesh. The number of neonates was counted, the count recorded and the neonates discarded. The test solution, without neonates, and the parent daphnids were then returned to the original test beaker. The test beaker was then returned to the flow-through test system.

The stock solutions used for the flow-through test were prepared once per week; three were used for the definitive study. The new stock solutions were put into use in the diluter system on day -2, day 7, and day 14. The stock solutions were sampled for measured concentration on day 0, 7, 14 and 21. Both new and old stock solution was analysed to confirm stability during the course of the study. The measured concentrations in the stock solutions were determined by GLC (Gas Liquid Chromatography). Additionally the amount of 14C-KWG 4168 was measured by Liquid Scintillation Counts.

Test water samples were collected from all test levels including controls on seven occasions during the study. The exposure concentrations were calculated from the measured dpm and the ratio of radiolabeled a.s. to unlabeled a.s. as determined in the corresponding stock solutions.

Analytical method

Samples of water were analysed using the validated analytical method 00252 M001, report reference M-008490-02-2 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the test guideline were met:

- Mortality of the parent animals (female *Daphnia*) does not exceed 20% at the end of the test (actual: 5% and 0% for the control and solvent control, respectively)
- The mean number of living offspring produced per parent animal surviving at the end of the test is ≥60 (actual: ≥116)

The study is therefore considered acceptable.

Table CA 8.2.5.1/02-1 Summary of analytical results of water analyses

Nominal		Average measured radioactivity (dpm/10 mL)* and Calculated concentration (mg a.s./L)**							
Concentrations (mg a.s./L)	Radioactivity dpm/10mL	Day 0		Day 2		Day 7		Day 9	
		*	**	*	**	*	**	*	**
Control	-	5.4	-	5.7	-	7.2	-	5.6	-
Solvent control	-	6.7	-	8.1	-	4.6	-	4.3	-
0.0056	200	193.8	0.00535	211.4	0.00584	219.2	0.00605	218.3	0.00700
0.010	200	186.9	0.0096	220.6	0.0113	202.2	0.0104	204.2	0.0113
0.018	200	194.5	0.0181	195.5	0.0182	198.0	0.0185	216.8	0.0211
0.032	200	187.7	0.0263	204.5	0.0286	231.3	0.0324	191.3	0.0321
0.056	200	191.5	0.0496	215.8	0.0559	213.5	0.0553	199.3	0.0544
0.10	200	192.2	0.094	212.7	0.104	201.5	0.098	210.7	0.107
0.18	200	191.8	0.163	210.3	0.178	204.8	0.174	180.9	0.166

* Measured radioactivity (dpm/10 mL). Average of 4 replicates
** Calculated concentration (mg a.s./L)

Table CA 8.2.5.1/02-2 Continued summary of analytical results of water analyses

Nominal		Average measured radioactivity (dpm/10 mL)* and Calculated concentration (mg a.s./L)**					
Concentrations (mg a.s./L)	Radioactivity dpm/10mL	Day 14		Day 16		Day 21	
		*	**	*	**	*	**
Control	-	9.3	-	9.3	-	9.1	-
Solvent control	-	8.3	-	8.5	-	7.2	-
0.0056	200	222.5	0.00714	217.8	0.00707	209.5	0.00680
0.010	200	199.9	0.0111	209.3	0.0122	204.4	0.0119
0.018	200	197.8	0.0193	210.7	0.0224	206.5	0.0220
0.032	200	212.7	0.0357	203.4	0.0406	207.4	0.0414
0.056	200	215.2	0.0588	212.9	0.0639	213.3	0.0640
0.10	200	210.1	0.107	217.8	0.124	212.8	0.121
0.18	200	209.8	0.193	214.7	0.210	217.2	0.212

* Measured radioactivity (dpm/10 mL). Average of 4 replicates
** Calculated concentration (mg a.s./L)

The results of the study have been presented in terms of mean measured concentrations.

No mortalities higher than 5 % were observed in the parent animals of the control and solvent control. There was no mortality in any test concentration exceeding a mortality rate of 20% which is considered acceptable background mortality.

Table CA 8.2.5.1/02-3 Survival of the parent water fleas

Mean measured concentration (mg a.s./L)	Study day								
	0	1	2	5	6	7	8	9	10
Control	20	20	20	20	20	20	20	19	19
Solvent control	20	20	20	20	20	20	20	20	20
0.0065	20	20	20	20	20	20	20	20	19
0.011	20	20	20	20	20	20	20	20	20
0.020	20	20	20	20	20	20	19	19	19
0.034	20	20	20	20	20	20	20	20	20
0.057	20	20	20	20	20	20	20	20	20
0.11	20	20	20	20	20	19	19	19	19
0.19	20	20	19	19	19	19	19	19	19

Table CA 8.2.5.1/02-4 Survival of the parent water fleas (Continued)

Mean measured concentration (mg a.s./L)	Study day										
	11	12	13	14	15	16	17	18	19	20	21
Control	19	19	19	19	19	19	19	19	19	19	19
Solvent control	20	20	20	20	20	20	20	20	20	20	20
0.0065	19	19	19	19	19	19	19	19	19	19	19
0.011	20	20	20	20	20	20	20	20	20	20	20
0.020	19	19	19	19	19	19	19	19	19	19	19
0.034	20	20	19	19	19	19	19	19	19	19	19
0.057	20	20	20	20	20	20	20	20	20	20	20
0.11	19	19	19	19	19	19	19	19	19	19	19
0.19	19	19	19	19	19	19	19	19	19	19	19

A delay in time to first brood release was observed only in the highest test concentrations of 0.11 and 0.19 mg a.s./L. No neonates were observed in this study before day 7 in any test concentration including control and solvent control. No dead offspring or aborted eggs were found in any test levels throughout the study. Also, no abnormal behaviors of adult or juvenile organisms were observed.

In the control, the mean number of 8.3 offspring per parent per reproduction day (corresponding to a sum of offspring per parent of 116) represents an acceptable reproduction rate. Compared to the pooled control data, there was no biologically or statistically significant reduction of the number of offspring per parent per reproduction day at test concentrations from 0.0065 to 0.034 mg a.s./L (Dunnett's-test, p = 0.05). Concentrations from 0.057 to 0.19 mg a.s./L reduced the number of offspring per parent per reproduction day significantly compared to the pooled control data. Therefore, the NOEC for reproduction is 0.034 mg a.s./L.

Table CA 8.2.5.1/02-5 Number of juveniles produced

Day	Mean measured concentration (mg a.s./L)								
	Control	Solvent control	0.0065	0.011	0.020	0.034	0.057	0.11	0.19
7	0	0	0	0	0	0	0	0	0
8	23	6.5	4.25	7.75	5.75	3.5	6.25	1.5	0.5
9	43	8.75	7.25	9.5	8.25	8.25	5	3.25	0.5
10	38	40.25	46.75	46.5	42.75	47	20.75	24.25	18.5
11	10.75	16.75	4	13.5	2.5	14.75	10.25	7.25	2.25
12	14.5	19	18.75	28.5	36.5	59	10.5	27	15
13	95	108.5	99.5	94.75	73.5	58.75	45.5	53.75	50.5
14	27	38.75	36.75	48.25	8	18.75	19.75	13.75	5
15	26.25	27.25	24.75	48.25	29.5	39.75	18.75	26.5	20.5
16	63.25	99.5	93.75	94.25	86.25	75.5	64.75	74.75	49.75
17	57.75	52.5	43.5	48.75	54.75	12.25	31.25	25.75	29.25
18	33.5	37	34.75	46	48.25	38	34	22	28.75
19	66.5	100.5	75.5	88.5	57.25	107.5	53.5	100	19.5
20	109	51.25	59.75	61.25	61	19.75	53.5	22	57.25
21	48.25	21.75	39	41.75	42.5	29.75	36.5	29	7

Since the body length data of control and solvent control were not significantly different, the statistical comparison of the lengths of parent animals at the end of the study were performed with pooled control and solvent control data. The statistical comparison of the length of parent animals showed no significant reduction of body lengths at all concentrations except for the 0.19 mg a.s./L treatment where body length was reduced compared to the control (Dunnett's-test, p = 0.05). Therefore, the NOEC for the body length of the parent animals was 0.11 mg a.s./L.

Table CA 8.2.5.1/02-6 Body length (mm) of adult *Daphnia* after 21 days of exposure to KWG 4168

Animal No.:	Mean measured concentration (mg a.s./L)								
	Control	**Solvent control**	**0.0065**	**0.011**	**0.020**	**0.034**	**0.057**	**0.11**	**0.19**
1	4.20	4.40	4.55	4.20	4.65	4.55	4.30	4.30	3.95
2	4.50	4.60	4.25	4.30	4.50	4.50	4.35	4.55	4.00
3	4.30	4.55	4.45	4.40	4.50	4.45	4.35	4.65	3.30
4	4.60	4.30	4.50	4.30	4.10	4.50	4.30	4.50	4.10
5	*	4.40	4.35	4.35	4.10	4.35	4.25	4.15	*
6	4.50	4.50	4.35	4.35	4.40	4.55	4.00	4.35	3.75
7	4.40	4.35	4.25	4.30	4.35	4.55	4.25	4.35	3.80
8	4.55	4.45	4.20	4.45	4.50	4.35	4.20	4.30	3.80
9	4.25	4.30	3.80	4.00	4.25	4.20	4.10	4.50	3.90
10	4.80	4.20	*	4.35	4.45	4.30	4.00	4.35	3.60
11	4.30	4.30	4.15	4.50	4.00	4.25	4.60	4.30	3.85
12	4.50	4.45	4.60	4.30	4.30	4.40	4.60	4.40	3.90
13	4.55	4.55	4.30	4.35	4.40	4.35	4.30	4.55	3.75
14	4.45	4.55	4.40	4.35	4.55	4.50	4.35	4.30	3.80
15	4.25	4.80	4.15	4.40	*	4.35	4.25	4.20	3.75
16	4.35	4.15	4.30	4.35	4.10	4.30	4.55	4.80	3.70
17	4.25	4.45	4.35	4.40	4.30	4.50	4.50	4.30	3.45
18	4.25	4.35	4.00	4.28	4.45	4.65	4.40	4.40	3.00
19	4.55	4.30	4.25	4.60	4.55	4.25	4.65	4.45	3.65
20	4.60	4.25	4.30	4.55	4.55	*	4.05	*	3.85
Mean	4.429	4.410	4.289	4.353	4.379	4.392	4.303	4.405	3.732
SD	0.163	0.154	0.188	0.127	0.190	0.151	0.199	0.158	0.256
% of control	-	99.6	96.9	98.3	98.9	99.2	97.1	99.5	84.3
*	Animal died before the end of the study								

Since the body weight data of control and solvent control were significantly different, the statistical comparison of the dry weights of parent animals at the end of the study were performed with the solvent control data only. The statistical comparison of the dry weights of parent animals showed no significant reduction of body weights at the concentrations from 0.0065 to 0.11 mg a.s./L, except at 0.020 mg a.s./L. This exception was not considered in the assignment of the NOEC since higher concentrations did not show a reduction in body weight (Dunnett's-test, p = 0.05). The highest test concentration 0.19 mg a.s./L reduced the body weights significantly compared to the solvent control data. Therefore, the NOEC for the dry weight of the parent animals is 0.11 mg a.s./L.

Table CA 8.2.5.1/02-7 Dry weight (mg) of adult *Daphnia* after 21 days of exposure to KWG 4168

Animal No.:	Mean measured concentration (mg a.s./L)								
	Control	**Solvent control**	**0.0065**	**0.011**	**0.020**	**0.034**	**0.057**	**0.11**	**0.19**
1	0.712	1.200	1.284	1.148	0.810	1.166	1.228	1.112	0.730
2	0.714	0.952	1.084	1.208	1.060	1.242	1.206	1.050	1.012
3	1.072	1.224	1.048	0.378	1.086	1.134	1.234	1.310	0.650
4	0.874	1.178	1.344	1.126	0.884	1.172	1.086	1.022	0.786
5	*	1.156	1.042	1.104	0.726	1.180	1.038	1.052	*

Animal No.:	Mean measured concentration (mg a.s./L)								
	Control	Solvent control	0.0065	0.011	0.020	0.034	0.057	0.11	0.19
6	1.220	1.168	1.140	1.212	0.958	1.014	0.758	1.082	0.722
7	0.876	1.102	1.038	1.250	1.198	1.020	0.970	1.218	0.694
8	0.826	1.218	1.060	1.208	0.874	0.684	1.176	1.076	0.754
9	1.020	1.030	0.776	1.248	0.762	1.082	0.862	1.034	0.566
10	0.850	0.718	*	1.204	1.146	1.002	0.622	1.180	0.778
11	1.016	1.104	1.002	1.228	0.730	1.014	1.096	1.104	0.702
12	1.116	1.240	1.250	1.126	0.808	1.152	0.998	1.166	0.798
13	1.000	1.294	0.826	1.170	1.040	1.154	1.046	1.326	0.696
14	0.994	1.250	1.154	1.180	1.120	1.246	1.036	1.170	0.762
15	0.936	1.330	0.958	1.130	*	0.852	1.022	0.848	0.532
16	0.738	0.976	0.932	0.992	0.742	1.224	1.404	0.910	0.896
17	1.002	1.044	1.178	1.218	1.032	1.216	0.904	1.046	0.666
18	0.656	0.718	1.054	0.906	1.072	1.272	1.338	1.154	0.428
19	1.024	1.154	1.114	1.264	1.174	1.194	0.850	1.164	0.734
20	0.938	1.162	0.990	1.268	1.076	*	0.858	*	0.806
Mean	0.925	1.111	1.067	1.128	0.963	1.106	1.037	1.107	0.717
SD	0.150	0.167	0.142	0.198	0.164	0.148	0.195	0.148	0.123
% of control	-	120.0	115.3	121.9	104.0	119.5	112.0	119.6	77.5
*	Animal died before the end of the study								

Table CA 8.2.5.1/02-8 Effects of KWG 4168 exposure on the Daphnia

Endpoint	Reproduction – number of offspring (mg a.s./L)	Growth – body length (mg a.s./L)	Growth – body dry weight (mg a.s./L)
NOEC	0.034	0.11	0.11
LOEC	0.057	0.19	0.19

III. Conclusion

The 21-day chronic toxicity of ^{14}C-KWG 4168 to *Daphnia magna* was studied under flow-through conditions. Test organisms were exposed to nominal concentrations of 0.0056, 0.010, 0.018, 0.032, 0.056, 0.10 and 0.18 mg a.s./L plus a control and solvent control.

The no observed effect concentration (NOEC) with regard to reproduction was 0.034 mg a.s./L, whereas, the body length NOEC was 0.11 mg a.s./L and the body weight NOEC was 0.11 mg a.s./L.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 202 (1984), therefore, the test was assessed to the current test guideline for *Daphnia magna* reproduction tests OECD 211 (adopted: 02 October 2012).

Validity criteria according to the OECD 211 guideline (2012) were met:

- Mortality of the parent animals (female *Daphnia*) does not exceed 20% at the end of the test (actual: 5% and 0% for the control and solvent control, respectively)
- The mean number of living offspring produced per parent animal surviving at the end of the test is ≥60 (actual: ≥116)

The study is therefore considered acceptable.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

The NOEC with regard to reproduction was determined to be 0.034 mg a.s./L.

Data Point:	KCA 8.2.5.1/05
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10 and EC20 values for Daphnia magna with 14C-spiroxamine TG in a reproduction study
Report No:	0471836-ECO6
Document No:	M-760409-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
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 M-006555-01-1 on the effects of ^{14}C-Spiroxamine TG on the reproduction of water fleas (*Daphnia magna*) study did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013. As there were no effects > 5% on the parameter survival, it was not possible to determine reliable EC_x values.

Due to a scattering of data around the lower doses of the response curve, the low amount of variance explained by the models, and poor-fitting linear and non-linear regression models, it was not possible to calculate reliable EC_{10} values for any of the parameters tested.

I. Methods

The statistical evaluation was performed with statistical software ToxRat Professional v3.3.0.

Concentrations with 10 and 20% effects from the test item treatment when compared to the pooled controls were calculated for reproduction, but due to lack of a dose response, these could not be determined for length. Although ECx values and 95% confidence interval were calculated for the parameter dry weight, as confidence intervals were large, spanning more than 2 concentrations and the dose response curve is not covered by the data, it was not possible to determine reliable ECx values.

A Logit function using linear maximum likelihood regression was used along with 95% EC_x confidence limits (calculated using normal approximation) for dry weight.

II. Results and Discussion

An explanation is given for regression analysis endpoints for length, dry weight and reproduction. These details can be found below.

Length at 21 days

Due to the significant amount of data scattering around the lower doses of the response curve and due to the low amount of variance (<30%) explained by all tested regression models, no reliable EC_{10} and EC_{20} can be calculated.

Dry weight at 21 d

Regarding the calculation of EC_{10} and EC_{20} values for dry weight at 21 d, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.00, showing no significant deviation between fit and data, and a statistically significant concentration/response was found (p(F) = 0.047) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table and figure below.

Table CA 8.2.5.1/05-1 Results of the Logit analysis (max. likelihood regression) with reproduction at 21 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (by normal approximation)

Parameter	Dry weight	
	EC_{10} (95 % confidence interval) [mg a.s./L]	**EC_{20} (95 % confidence interval) [mg a.s./L]**
Effect on dry weight at 21 d	0.117 (0.075 – 0.181)	0.152 (0.114 – 0.203)

The resulting EC_{10} and EC_{20} values of 0.117 (95% CL: 0.075 – 0.181), 0.152 (95% CI: 0.114 – 0.203) mg a.s./L, respectively, meet the goodness of fit criteria. However, with a p(F) value of 0.047, the dose response is only marginally significant and only 57.8% of the variance is explained by the dose/response function (r^2 = 0.578). Along with a poor-fitting concentration-curve (see below), the estimated EC_{10} value is therefore not considered reliable for use in the risk assessment.

Reproduction (cumulative offspring per survived parent) at 21 d

Regarding the calculation of EC_{10} and EC_{20} values for reproduction at 21 d, the criteria for goodness of fit were met as the P(Chi^2) value was 0.999, showing no significant deviation between fit and data, and a statistically significant concentration/response was found (p(F) = 0.026) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table and figure below.

Table CA 8.2.5.1/05-2 Results of the Probit (max. likelihood regression) with reproduction at 21 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (by normal approximation)

Parameter	Reproduction	
	EC_{10} (95 % confidence interval) [mg a.s./L]	**EC_{20} (95 % confidence interval) [mg a.s./L]**
Effect on reproduction at 21 d	0.032 (0.011 – 0.095)	0.068 (0.036 – 0.132)

The resulting EC_{10} and EC_{20} values of 0.032 (95% CL: 0.011 – 0.095) and 0.068 (95% CI: 0.036 – 0.132) mg a.s./L, respectively, meet the goodness of fit criteria. However, as the confidence intervals are large, spanning more than 6 concentrations and the obtained endpoints do not visually fit with the raw data from the study, it was concluded that the estimated EC_{10} value is not considered reliable for use within a risk assessment.

III. Conclusion

It was not considered possible to determine reliable EC_x values for any parameter.

Assessment and conclusion by applicant:

The statistical re-evaluation of the data has determined EC_{10} and EC_{20} values for dry weight and reproduction but these values were not considered to be reliable. The lowest EC_{10} and EC_{20} values of 32 and 68 µg a.s./L, respectively were determined for reproduction. Thus, the EC_{10} of 32 µg a.s./L is lower than the NOEC of 34 µg a.s./L for this parameter but as the EC_{10} is not considered reliable, the NOEC of 34 µg a.s./L shall remain the critical endpoint determined from this study.

The NOEC values determined in the re-evaluation work are considered to be fully valid.

Data Point:	KCA 8.2.5.1/03
Report Author:	[redacted]
Report Year:	1996
Report Title:	Influence of 14C-KWG 4168 (techn.) on the reproduction rate of water Fleas
Report No:	HBF/RDM 55
Document No:	M-006466-01-1
Guideline(s) followed in study:	OECD 202 (II) (1984), now OECD 211 (2012) EPA FIFRA 72-4 EEC XI/681/86 (1987)
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The 21-day chronic toxicity of ^{14}C-KWG 4168 to *Daphnia magna* was studied under semi-static conditions. Test organisms were exposed to mean measured test concentrations of 0.015, 0.027, 0.047, 0.085, 0.15, 0.27 and 0.47 mg a.s./L plus a control and solvent control. The 21-day NOEC based on reproduction was determined to be 0.047 mg a.s./L.

I. Materials and Methods

A. Materials

Test Material	KWG 4168	^{14}C-KWG 4168
Lot/Batch #:	17002/90	KML 2216
Purity:	96.3%	99%
Description:	Colourless liquid	Not reported
Stability of test compound:	Stable under the conditions of the test	Not reported
Reanalysis/Expiry date:	08 August 1995	Not reported
Density:	Not reported	Not reported

Treatments

Test rates:	Nominal: 0.018, 0.032, 0.056, 0.10, 0.18, 0.32 and 0.56 mg a.s./L
Solvent/vehicle:	Acetone
Analysis of test concentrations:	Yes, mean measured concentrations 104.7% in both the fresh and spent solutions

Test organisms

Species:	*Daphnia magna*, <24 h old
Source:	In-house culture, originally from the Bundesgesundheitsamt, Berlin, Germany
Acclimatisation period:	None
Feeding:	Green algae *Scenedesmus subspicatus* at 0.8 mg C/vessel daily

Treatment for disease:	None reported
Test design	
Test vessel:	250 mL glass beakers containing approx. 200 mL test solution at a depth of approximately 8 cm
Test medium:	M7-medium
Replication:	Mortality: three replicates Reproduction: ten replicates
No. of animals/vessel:	Mortality: five animals Reproduction: individually held
Duration of test:	21 days
Environmental test conditions	
Temperature:	19.7 – 20.2°C
Dissolved oxygen:	Fresh: 8.4 – 8.9 mg/L (approx. 91.8 – 98.2% saturation) Spent: 5.2 – 10.4 mg/L (approx. 56.8 – 114.8% saturation)
pH:	Fresh: 7.96 – 8.32 Spent: 7.32 – 8.70
Photoperiod:	16 h light : 8 h dark at approx. 700 lux

B. Study Design

This study was conducted in order to assess the reproductive toxicity of ^{14}C-KWG 4168 to the water flea *Daphnia magna* under semi-static conditions. Test concentrations were chosen based on the results of earlier toxicity tests.

Nominal test concentrations were 0.018, 0.032, 0.056, 0.10, 0.18, 0.32 and 0.56 mg a.s./L along with a control and solvent control. Mean measured concentrations were 0.015, 0.027, 0.047, 0.085, 0.15, 0.27 and 0.47 mg a.s./L, respectively, equivalent to 82.2 to 85.2% of nominal.

Test vessels were 250 mL glass beakers containing approximately 200 mL test solution at a depth of approximately 8 cm. Test solutions were renewed every 48 hours during the week, and after 72 hours over weekends. Beakers were covered with plexi glass plates and placed in an environmental chamber for 21 days at 20 ± 1°C under a 16 hour light : 8 hour dark photoperiod at approximately 700 lux.

Daphnia were first instar at less than 24 hours old. Three replicates of five animals were assessed for the mortality assessment and ten replicates of individually held daphnids were used for the reproductive and sub-lethal effects assessment. Test animals were transferred to newly prepared test media with one or two drops of the old test solution.

Immediately after removal of the parent animal, neonates from each reproductive vessel were counted by straining the test solution through a 0.20 mm mesh. Observations for survival, sub-lethal and behavioural effects to the parent daphnids were made daily. At test termination, the body lengths and dry weights of the parent animals were determined.

Body length measurements were made under a binocular microscope from the apex of the helmet to the base of the posterior spine using an eyepiece graticle. Body weight was then determined individually using a microbalance after 48-hours drying at 60°C.

Temperature was measured in one vessel of the control. Oxygen content and pH values in the control and test concentrations were determined in freshly prepared test solutions.

Samples of the test solution were taken four times during the study and analysed for the content of the active substance. A stability assessment of the test substance was also made on days 7, 14 and 21.

Analytical method

Samples of water were analysed using the validated analytical method 00252 M001, report reference M-008490-02-2 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the test guideline were met:

- Mortality of the parent animals in the controls to not exceed 20% at the end of the test: (actual: 0 and 7% in the control and solvent control, respectively)
- Mean number of living offspring produced per parent animal surviving at the end of the test in the controls to be ≥60 (actual: mean total offspring 139.7 and 143.3 in the control and solvent control, respectively)

Analytical results, measured on four occasions in the fresh solutions, ranged from 96.9 to 112.4% of nominal, with a mean recovery of 104.7%. After 72 hours of exposure, measured radioactivity was 98.8 to 110.1% of nominal, with a mean recovery of 104.7%. The results achieved in this study have been presented in terms of mean measured test concentrations.

Table CA 8.2.5.1/03-1 Measured concentrations of spiroxamine in fresh solutions during the test

Nominal test concentration (mg a.s./L)	Analysed concentrations (mg a.s./L)							
	Day 0	% of nominal	Day 4	% of nominal	Day 11	% of nominal	Day 18	% of nominal
Control	-	-	-	-	-	-	-	-
Solvent control	-	-	-	-	-	-	-	-
0.018	0.0178	98.6	0.0188	104.3	0.0202	112.4	0.0199	110.7
0.032	0.0323	100.8	0.0339	105.9	0.0341	106.6	0.0354	110.7
0.056	0.0561	100.2	0.0601	107.2	0.0607	108.3	0.0629	112.4
0.10	0.0986	98.6	0.105	105.0	0.105	104.8	0.109	109.2
0.18	0.178	98.9	0.186	103.2	0.190	105.5	0.201	111.4
0.32	0.310	96.9	0.331	103.5	0.333	104.2	0.346	108.2
0.56	0.546	97.4	0.571	102.0	0.557	99.4	0.587	104.9

Table CA 8.2.5.1/03-2 Measured concentrations of spiroxamine in test solutions after 72 hours

Nominal test concentration (mg a.s./L)	Analysed concentrations (mg a.s./L)					
	Day 7	% of nominal	Day 14	% of nominal	Day 21	% of nominal
Control	-	-	-	-	-	-
Solvent control	-	-	-	-	-	-
0.018	0.0188	104.5	0.0193	107.0	0.0191	106.2
0.032	0.0336	104.9	0.0340	106.2	0.0352	109.8
0.056	0.0584	104.2	0.0597	106.6	0.0617	110.1
0.10	0.1033	103.3	0.105	104.9	0.106	106.3
0.18	0.184	102.5	0.189	104.8	0.195	108.5
0.32	0.320	100.0	0.329	102.7	0.342	107.0
0.56	0.553	98.8	0.557	99.4	0.568	101.4

Table CA 8.2.5.1/03-3 Mean measured concentrations of ^{14}C-KWG 4168 calculated from the 0-hour, 72-hour and stability analysis solutions

Nominal test concentration (mg a.s./L)	Mean 0-hr concentrations (mg a.s./L)	Mean 72-hr concentrations[1], (mg a.s./L)	Mean analysed concentrations (mg a.s./L)	% of nominal
Control	-	-	-	-
Solvent control	-	-	-	-
0.018	0.0192	0.0104	0.015	82.2
0.032	0.0339	0.0199	0.027	84.1
0.056	0.0599	0.0349	0.047	84.6
0.10	0.1044	0.0651	0.085	84.8
0.18	0.189	0.118	0.15	85.2
0.32	0.330	0.215	0.27	85.2
0.56	0.565	0.369	0.47	83.4
Mean:				84.2

[1] Corrected by stability analysis

There was no mortality observed in the control. In the solvent control and all test concentrations no mortality >20% was observed which is considered acceptable background mortality. In the control, the mean number of 140 newborn waterfleas/adult was high. Compared to the pooled control data, there was no biologically and statistically significant reduction of the sum of offspring per parent and the number of offspring per parent and reproduction day at the test concentrations of 0.015 to 0.047 mg a.s./L. In the concentrations from 0.085 to 0.47 mg a.s./L, the sum of offspring per parent and the number of offspring per parent and reproduction day was significantly reduced (α=0.05) compared to the pooled controls.

Table CA 8.2.5.1/03-4 Mortality of *Daphnia magna* over 21-day exposure to KWG 4168

Mean measured concentrations (mg a.s./L)	Cumulative mortality (%) by day															
	0	1	2	3	4	7	8	9	10	11	14	15	16	17	18	21
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Solvent control	0	0	0	0	0	0	0	0	0	0	7	7	7	7	7	7
0.015	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.027	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.047	0	0	0	0	0	7	7	7	7	7	7	7	7	7	7	7
0.085	0	0	0	0	0	0	0	0	0	0	7	7	7	7	7	7
0.15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.27	0	0	0	0	0	0	0	0	0	0	13	13	13	13	13	13
0.47	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table CA 8.2.5.1/03-5 Number of offspring of *Daphnia magna* over 21-day exposure to spiroxamine

Mean measured concentration (mg a.s./L)	Total offspring ±SD		Number of offspring/adult/reproductive day	
	Mean ± SD	% of controls	Mean ± SD	% of controls
Control	139.7 ± 26.9	-	10.5 ± 2.0	-
Solvent control	143.3 ± 25.4	102.6	10.9 ± 1.5	103.4
0.015	135.5 ± 26.3	97.0	10.1 ± 1.5	96.2
0.027	153.2 ± 17.9	109.7	11.4 ± 1.6	108.0
0.047	124.1 ± 38.1	88.8	9.5 ± 2.5	90.5
0.085	99.7 ± 22.4	71.4*	8.1 ± 1.8	76.4*
0.15	76.1 ± 23.9	54.5*	6.7 ± 2.2	63.8*
0.27	51.1 ± 13.4	36.6*	6.4 ± 1.6	60.8*
0.47	29.6 ± 15.6	21.2*	7.2 ± 3.3	68.5*

* Significantly reduced compared to the pooled controls (Dunnett's t-test, α=0.05)

At the end of the study, the parent animals in the control had a body length of 4.89 mm indicating well developed females of *Daphnia magna*. Compared to the pooled control data, at the concentrations from 0.015 to 0.047 mg a.s./L no significant decrease in body length was recorded. In the concentrations higher than 0.047 mg a.s./L the body length was significantly reduced (p=0.05) compared to the pooled control data.

Table CA 8.2.5.1/03-6 Body length of *Daphnia magna* after 21-day exposure to spiroxamine

Mean measured concentration (mg a.s./L)	**Control**	**Solvent control**	**0.015**	**0.027**	**0.047**	**0.085**	**0.15**	**0.27**	**0.47**
Number	***10***	***10***	***10***	***10***	***10***	***10***	***10***	***10***	***10***
Mean (mm)	4.89	4.90	4.86	4.86	4.77	4.59	4.52	4.24*	3.99*
SD	0.08	0.10	0.09	0.11	0.17	0.27	0.20	0.17	0.30
% of control	-	100.2	99.5	99.5	97.5	93.9*	92.5*	86.7*	81.7*

SD Standard deviation
* Significantly reduced compared to the pooled controls (Dunnett's t-test, α=0.05)

The statistical comparison of the dry weights of the parent animals at the end of the study showed no significant reduction of body weights at the concentrations from 0.015 to 0.085 mg a.s./L. At the concentrations from 0.15 to 0.47 mg a.s./L the body weights were significantly reduced compared to the pooled control data (Dunnett's test, p=0.05).

Table CA 8.2.5.1/03-7 Dry weight of *Daphnia magna* after 21-day exposure to spiroxamine

Mean measured concentration (mg a.s./L)	**Control**	**Solvent control**	**0.015**	**0.027**	**0.047**	**0.085**	**0.15**	**0.27**	**0.47**
Number	***10***	***10***	***10***	***10***	***10***	***10***	***10***	***10***	***10***
Mean (mm)	1.204	1.288	1.217	1.113	1.212	1.139	0.992	0.815	0.635
SD	0.109	0.160	0.178	0.173	0.111	0.187	0.104	0.175	0.202
% of control	-	106.9	101.1	92.4	100.7	94.6	82.4*	67.7*	52.7*

SD Standard deviation
* Significantly reduced compared to the pooled controls (Dunnett's t-test, α=0.05)

The resulting NOEC and LOEC values are summarised in the table below:

Table CA 8.2.5.1/03-8 Summary of endpoints of *Daphnia magna* after 21 days exposure to spiroxamine exposure

Endpoint	**NOEC (mg a.s./L)**	**LOEC (mg a.s./L)**
Sum of offspring/parent	0.047	0.085
Number of offspring/parent/day	0.047	0.085
Body length of parent animals	0.047	0.085
Dry weight of parent animals	0.085	0.15

III. Conclusion

The 21-day chronic toxicity of ^{14}C-KWG 4168 to *Daphnia magna* was studied under semi-static conditions. Test organisms were exposed to mean measured test concentrations of 0.015, 0.027, 0.047, 0.085, 0.15, 0.27 and 0.47 mg a.s./L plus a control and solvent control. The 21-day NOEC based on reproduction was determined to be 0.047 mg a.s./L.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 202 (II), the current version of which is the OECD 211 "*Daphnia magna* reproduction test", adopted 02 October 2012.

Validity criteria according to the OECD 211 guideline (2012) were met:

- Mortality of the parent animals in the controls to not exceed 20% at the end of the test: (actual: 0 and 7% in the control and solvent control, respectively)
- Mean number of living offspring produced per parent animal surviving at the end of the test in the controls to be ≥60 (actual: mean total offspring 139.7 and 143.3 in the control and solvent control, respectively)

The study is therefore considered acceptable.

The 21-day NOEC based on reproduction was determined to be 0.047 mg a.s./L.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.2.5.1/06
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10 and EC20 values for Daphnia magna with 14C-spiroxamine TG in a reproduction study
Report No:	0471836-ECO21
Document No:	M-761544-01-1
Guideline(s) followed in study:	Annex to Com. Reg. 283/2013
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary

The report M-006466-01-1 on the effects of ^{14}C-Spiroxamine TG on the reproduction of water fleas (*Daphnia magna*) study did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013. Effect Concentrations with a 10% and 20% effect on dry weight and reproduction when compared to the control were re-calculated. As there were no effects on the parameter survival, it was not possible to determine reliable ECx values.

The resulting EC_{10}, and EC_{20} values for length at 21 d were 0.194 (95% CL: 0.168 – 0.219) and 0.523 (95% CL: 0.453 – 0.629) mg a.s./L, respectively. The resulting EC_{10} and EC_{20} values for dry weight at 21d were 0.076 (95% CL: 0.036 – 0.111) and 0.150 (95% CL: 0.099 – 0.192) mg a.s./L, respectively. The resulting EC_{10} and EC_{20} values for reproduction at 21d were 0.039 (95%CL: 0.028 – 0.049) and 0.065 (95%CL: 0.052 – 0.077) mg a.s./L, respectively.

I. Methods

The statistical evaluation was performed with statistical software ToxRat Professional v3.3.0.

Effect concentrations with 10 and 20% effects from the test item treatment when compared to the pooled controls were calculated but due to lack of a dose response, these could not be determined for survival. A Probit function using linear maximum likelihood regression was used along with 95% EC_X confidence

limits for length and reproduction while a Logit function using linear maximum likelihood regression was used along with 95% confidence limits for dry weight.

II. Results and Discussion

An explanation is given for regression analysis endpoints for length, dry weight and reproduction. These details can be found below.

Length at 21 days

Regarding the calculation of EC_{10} and EC_{20} values for length at 21 d, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.00, showing no significant deviation between fit and data, and a statistically significant concentration/response was found ($p(F) < 0.001$) for this parameter.

The resulting EC_{10}, and EC_{20} and values and the respective confidence intervals are represented in the following table.

Table CA 8.2.5.1/06-1 Results of the Probit analysis (max. likelihood regression) with length at 21 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (according to Fieller's theorem)

Parameter	Length	
	EC_{10} (95 % confidence interval) [mg a.s./L]	**EC_{20} (95 % confidence interval) [mg a.s./L]**
Effect on length at 21 d	0.194 (0.168 – 0.219)	0.523 (0.453 – 0.629)

The resulting EC_{10} and EC_{20} values of 0.194 (95% CL: 0.168 – 0.219) and 0.523 (95% CL: 0.453 – 0.629) mg a.s./L, respectively, meet the goodness of fit criteria and therefore the estimated EC_{10} value is considered reliable for use within a risk assessment.

Dry weight at 21 d

Regarding the calculation of EC_{10} and EC_{20} values for dry weight at 21 d, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.00, showing no significant deviation between fit and data, and a statistically significant concentration/response was found ($p(F) < 0.001$) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table below.

Table CA 8.2.5.1/06-2 Results of the Logit analysis (max. likelihood regression) with reproduction at 21 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (according to Fieller's theorem)

Parameter	Dry weight	
	EC_{10} (95 % confidence interval) [mg a.s./L]	**EC_{20} (95 % confidence interval) [mg a.s./L]**
Effect on dry weight at 21 d	0.076 (0.036 – 0.111)	0.150 (0.099 – 0.192)

The resulting EC_{10} and EC_{20} values of 0.076 (95% CL: 0.036 – 0.111), and 0.150 (95%CI: 0.099 – 0.192) mg a.s./L, respectively, meet the goodness of fit criteria and therefore the estimated EC_{10} value is considered reliable for use in the risk assessment.

Reproduction (cumulative offspring per survived parent) at 21 d

Regarding the calculation of EC_{10} and EC_{20} values for reproduction at 21 d, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.000, showing no significant deviation between fit and data, and a statistically significant concentration/response was found ($p(F) < 0.001$) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table and figure below.

Table CA 8.2.5.1/06-3 Results of the Probit (max. likelihood regression) with reproduction at 21 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (according to Fieller's theorem)

Parameter	Reproduction	
	EC_{10} (95 % confidence interval) [mg a.s./L]	**EC_{20} (95 % confidence interval) [mg a.s./L]**
Effect on reproduction at 21 d	0.039 (0.028 – 0.049)	0.065 (0.052 – 0.077)

The resulting EC_{10} and EC_{20} values of 0.039 (95% CL: 0.028 – 0.049) and 0.065 (95% CI: 0.052 – 0.077) mg a.s./L, respectively, meet the goodness of fit criteria and the estimated EC_{10} value is considered reliable for use within a risk assessment.

III. Conclusion

The resulting EC_{10}, and EC_{20} values for length at 21 d were 0.194 (95% CL: 0.168 – 0.219) and 0.523 (95% CL: 0.453 – 0.629) mg a.s./L, respectively. The resulting EC_{10} and EC_{20} values for dry weight at 21d were 0.076 (95% CL: 0.036 – 0.111) and 0.150 (95% CL: 0.099 – 0.192) mg a.s./L, respectively. The resulting EC_{10} and EC_{20} values for reproduction at 21d were 0.039 (95% CL: 0.028 – 0.049) and 0.065 (95% CL: 0.052 – 0.077) mg a.s./L, respectively.

Assessment and conclusion by applicant:

The statistical re-evaluation of the data has determined EC_{10} and EC_{20} values for length, dry weight and reproduction and these values are considered to be reliable. The lowest EC_{10} and EC_{20} values of 39 and 65 µg a.s./L, respectively were determined for reproduction. Thus, the EC_{10} of 39 µg a.s./L is lower than the NOEC of 47 µg a.s./L for this parameter. The EC_{10} of 39 µg a.s./L shall therefore be taken as the critical endpoint determined from this study.

The values determined in the re-evaluation work are considered to be fully valid.

CA 8.2.5.2 Reproductive and development toxicity to an additional aquatic invertebrate species

No reproductive data with an additional aquatic invertebrate species are available. Spiroxamine is a fungicide and does not display insecticidal activity therefore chronic data with an additional species of aquatic invertebrate are not required. However, sediment-water toxicity studies with *Chironomus* and *Lumbriculus* are available and have been summarised under point CA 8.2.5.4.

CA 8.2.5.3 Development and emergence in *Chironomus riparius*

Spiroxamine is not an insecticide nor is it an IGR therefore a chronic study with *Chironomus* is not triggered. However, in accordance with the Aquatic Guidance Document (EFSA, 2013), a study with a sediment-dwelling organism has been triggered based on the environmental fate water-sediment study which demonstrates that spiroxamine is present in the sediment at ≥10% of the applied radioactivity on Day 14. Furthermore, the chronic *Daphnia* NOEC is <0.1 mg a.s./L therefore, taking these two points together, a study with a sediment-dwelling organism is automatically required. A water-sediment toxicity study with *Chironomus* has been summarised under point CA 8.2.5.4.

CA 8.2.5.4 Sediment dwelling organisms

Data Point:	KCA 8.2.5.4/01
Report Author:	[redacted]
Report Year:	1998
Report Title:	Influence of 14C-KWG 4168 (techn.) on development and emergence of larvae of Chironomus riparius in a water-sediment stystem
Report No:	HBF/CH 21
Document No:	M-006549-01-1
Guideline(s) followed in study:	Proposal for a BBA-Guideline: "Effects of plant protection products on the development of sediment-dwelling larvae of Chironomus riparius in a water-sediment system" (1995)
Deviations from current test guideline:	Yes OECD 219 (2004) Replication of vessels not as per current guidance which recommends at least four replicates per control and test group. Three-litre glass beakers were used as test-vessels, however, 600 mL glass beakers are recommended by the current guidance. However, the larger vessels were not likely to have a negative impact on the organisms based on space available per larvae The composition of the artificial sediment is not as per current guidance however, it was prepared following OECD 207 guidance at the time of testing. Based on the control emergence rate this did not impact the validity of the test
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

A water-sediment study using *Chironomus* is available and has been summarised below. According to the Aquatic Guidance Document (EFSA, 2013), the preferred species for fungicides is *Lumbriculus* therefore a new study using *Lumbriculus* has been conducted and has also been summarised below.

Executive Summary

^{14}C-KWG 4168 (tech.) was tested to assess the potential impact on the maturation of the sediment dwelling life stage of *Chironomus riparius*. Test organisms were exposed to nominal concentrations of 0.1, 0.18, 0.32, 0.56, 1.0, 1.8, 3.2 and 5.6 mg a.s./L and a control and solvent control.

Three times during the study (on days 0, 7 and 28), the radioactivity was measured in all test solutions. Based on the ratio between analysed concentration of KWG 4168 technical and the radioactivity measurements in the stock solutions, the concentrations of test compound in the overlying water were calculated as a.s.-equivalents. The day 0 analytical samples were taken 1 hour after application. These results indicated that 89 to 105 % (for an average 99 %) of the nominal concentration was parent KWG 4168. The initial test concentrations were prepared correctly as verified by the measured concentration analysis. The nominal initial concentrations were used to calculate EC-values. On day 7 after application, the percent parent KWG 4168 in the test solutions was 52 to 65 % (for an average 58 %) of the initial concentrations. On Day 28 after application, the percent parent KWG 4168 was 38 to 49 % (for an average 44 %) of nominal concentrations.

The EC$_{50}$ with regard to development of male and female midges ~5.6 mg initial nominal a.s./L (EC$_{50}$ > 5.6 mg/L). The NOEC with regard to emergence rate was determined to be 5.6 mg a.s./L.

I. Materials and Methods

A. Materials

Test Material KWG 4168 (tech.)

Lot/Batch #:	07002/96
Active substance content:	96.3%
Specific activity:	3.68 MBq or 99.5 µCi/mg (radiochemical purity = > 98%)
Description:	Clear yellow liquid
Stability of test compound:	Sufficient based on expiration date
Reanalysis/Expiry date:	10 March 1998
Density:	Not reported
Treatments	
Test rates:	0.10, 0.18, 0.32, 0.56, 1.0, 1.8, 3.2 and 5.6 mg a.s./L
Solvent/vehicle:	Dimethylformamide (DMF)
Analysis of test concentrations:	Day 0, 7 and 28
Test organisms	
Species:	*Chironomus riparius*
Source:	Obtained from a culture maintained at the University of Sheffield (UK)
Acclimatisation period:	For the breeding phase the midges are kept in plastic cages (60 x 60 x 55 cm) with plastic gauze on the sides. A basin (45 x 55 x 10 cm) made of inert plastic is secured to the bottom of each breeding cage. The bottom of the basins are covered with a thin layer of "Kieselgur" (silica). Reconstituted water M7 (Elendt, 1990) was added to the basin to a depth of approximately 3 cm. The water in the basin was aerated gently. To start the culture in a cage, 2 - 4 egg masses were placed into the prepared basin. The hatched larvae were fed with green algae and an aqueous suspension of a vegetable fish food (Tetra Phyll®). After 2 - 3 weeks the adults emerged. After mating, female adults laid the egg masses on the water surface. The egg masses were taken to start a new culture or to perform toxicity tests. The culture conditions were 20 + 2°C and 16:8 hours light-dark-cycle with a 30 minutes dusk and dawn period.
Feeding:	With a commercial ornamental fish food extract (trade name Tetra Phyll®). The food was prepared as an aqueous suspension (1 g Tetra Phyll per 200 mL culture media). An appropriate amount of this suspension (about 1 mg Tetraphyll® /larvae/day) was added to each test container on days: -1, 0, 1, 4, 5, 6, 7, 8, 11, 12, 13, 14, 15, 18, 19, 20, 21, 22 and 25
Treatment for disease:	NA
Test design	
Test vessel:	3 L glass beakers
Test medium:	Artificial sediment and Elendt M7 medium
Replication:	Three per control and solvent control, single replicate for the test concentrations

No. of animals/vessel:	25
Duration of test:	28 days
Environmental test conditions	
Temperature:	20 ± 2°C (measured = 18.8 to 20.7°C)
Dissolved oxygen:	6.7 to 10.0 mg/L
pH:	6.6 to 7.7
Photoperiod:	16:8 hours light-dark cycle (including half hour dusk and dawn) mean light intensity – 3800 lux

B. Study Design

KWG 4168 (tech.) was tested to assess the potential impact on the maturation of the sediment-dwelling life stage of *Chironomus riparius.*

Each test vessel contained twenty-five animals, three replicates were prepared for the control and solvent control, whereas, single replicate vessels were prepared for the test concentration groups. The test was conducted at 18.8 to 20.7°C and a 16:8 light-dark cycle (including half hour dusk and dawn). Mean light intensity was about 3800 lux.

The bottom of the test containers (3L glass beakers) were covered with a 2-cm deep layer of sediment. Prior to adding the test water, the sediment was covered with a plastic sheet. This was done to avoid a separation of the sediment ingredients when the water was poured into the test vessel. The test water was slowly poured into the beaker (the beakers were filled with 2.65 L water) and then the sheet was carefully removed. The height of the water was 20 cm. Gentle aeration was provided through a glass Pasteur pipette situated about 2.5 cm above the sediment layer. Test beakers were covered by clear plastic plates to prevent evaporation and prevent emerged midges from escaping.

The range of test concentrations were selected to determine the EC_{15}. The following initial nominal test concentrations were chosen 0.10, 0.18, 0.32, 0.56, 1.0, 1.8, 3.2 and 5.6 mg a.s./L. For biological evaluations, three replicates were prepared of the control and solvent control and one replicate for each test concentration. For chemical analysis of the active substance additional parallel replicates were prepared for analytical purposes only (0.18, 1.0, and 5.6 mg a.s./L: 2 replicates). These analytical replicates were prepared and maintained under identical conditions to the biological test chambers; the analytical replicates also contained organisms and got food. Each of these extra beakers was used for all analyses (water, pore water, and sediment).

Dimethylformamide (DMF) was used to prepare the stock solutions and the solvent control. The solvent load in the solvent control and all test concentrations was 0.1 mL/L. The dilution water control consisted of water only; no solvent and no compound.

Nominal test concentrations were 0.10, 0.18, 0.32, 0.56, 1.0, 1.8, 3.2 and 5.6 mg a.s./L along with control and solvent control groups.

The test vessels were observed at least three times per week to make a visual assessment of any behavioural differences compared to the control. The sex, time to emergence and number of emerged or not fully emerged adults was recorded daily during the period of emergence. As only fully emerged adults are relevant for the endpoints of this study, larvae which did not mature were not evaluated.

To determine number and sex of emerged adults, the covering plates of each test container were carefully removed. The midges, which mostly stayed at the sides of the vessels, were enumerated and the sex of each was determined (male midges have feathered antennae). The midges were removed and discarded from the test vessels at the end of each observation period.

Analytical method

Samples of water were analysed using the validated analytical method 00252 M001, report reference M-008490-02-2 (**see Doc MCA Section 4**).

II. Results and Discussion

The study was deemed to be valid in the report as 90% of the larvae inserted into the control and solvent control groups had emerged.

Three times during the study (on days 0, 7 and 28), the radioactivity was measured in all test solutions. Based on the ratio between analysed concentration of KWG 4168 technical and the radioactivity measurements in the stock solutions, the concentrations of test compound in the overlying water were calculated as a.s.-equivalents. The day 0 analytical samples were taken 1 hour after application. These results indicated that 89 to 105 % (for an average 99 %) of the nominal concentration was parent KWG 4168. The initial test concentrations were prepared correctly as verified by the measured concentration analysis. The nominal initial concentrations were used to calculate EC-values. On day 7 after application, the percent parent KWG 4168 in the test solutions was 52 to 65 % (for an average 58 %) of the initial concentrations. On Day 28 after application, the percent parent KWG 4168 was 38 to 49 % (for an average 44 %) of nominal concentrations.

Table CA 8.2.5.4/01-1 Summary of analysis of ^{14}C-KWG 4168 (tech.) in the overlying water

Nominal concentration (mg/L)	Day 0			Day 7			Day 28		
	Measured radioactivity (dpm/10 mL)	Measured a.s. equivalent (mg/L)	% of nominal ^{14}C-KWG 4168 (tech.) Conc.	Measured radioactivity (dpm/10 mL)	Measured a.s. equivalent (mg/L)	% of nominal ^{14}C-KWG 4168 (tech.) Conc.	Measured radioactivity (dpm/10 mL)	Measured a.s. equivalent (mg/L)	% of nominal ^{14}C-KWG 4168 (tech.) Conc.
Control	5.8	-	-	5.1	-	-	4.3	-	-
S. control	5.4	-	-	4.5	-	-	2.4	-	-
0.10	1761.9	0.10	101.3	949.4	0.055	54.6	818.0	0.047	47.0
0.18	1806.1	0.19	105.2	950.4	0.10	55.3	836.0	0.088	48.7
0.32	1829.9	0.33	104.5	1036.7	0.19	59.2	803.6	0.15	45.9
0.56	1739.4	0.50	88.7	1014.8	0.29	51.8	789.0	0.23	40.2
1.0	1864.3	1.0	102.3	1095.4	0.60	60.1	834.4	0.46	45.8
1.8	1841.1	1.7	95.1	1107.6	1.03	57.2	850.9	0.79	44.0
3.2	1822.2	3.1	97.3	1159.7	1.98	61.8	799.6	1.36	42.6
5.6	1861.9	5.4	92.0	1241.3	3.62	64.6	725.1	2.11	37.8
-		Average:	98.9	-	Average:	58.1	-	Average:	44.0
		Min:	88.7		Min:	51.8		Min:	37.8
		Max:	105.2		Max:	64.6		Max:	48.7

Pore water samples were taken from concentrations 0.18, 1.0 and 5.6 mg/L and the radioactivity was measured in these samples. The pore water samples were taken from the parallel replicates at these test concentrations. The parallel replicates were included in the test system to provide samples for pore water and sediment analysis on Day 7 and Day 28. These replicates did also contain test organisms. The results are given in the table below.

Table CA 8.2.5.4/01-2 Analytical results of pore water

Initial Nominal concentration (mg/L)	Volume (mL) day 7	Measured radioactivity on day 7 (dpm/10mL)		average	Total radioactivity (Bq)	Volume (mL) Day 28	Measured radioactivity on day 28 (dpm/10mL)		average	Total radioactivity (Bq)
0.18	155	161.8	159.9	161	42	115	267.0	246.7	257	49

1.0	140	58.9	50.9	55	13	115	262.7	269.4	266	51
5.6	100	32.6	31.5	32	5	100	110.3	114.5	112	19

The total radioactivity Bq (Bequerel) was calculated: average dpm/10 mL x volume / 10 / 60

The wet weights of the sediment before vacuum filtration, and the dry weights of the sediment after vacuum filtration were recorded. Dry weights were calculated from the wet weight and the volume of filtered pore water. The total radioactivity in the sediment was calculated.

Table CA 8.2.5.4/01-3 Analytical results of sediment

Initial Nominal concentration (mg/L)	Study day	Sediment weight before vacuum filtration (g)	Volume of pore water removed from the sediment (mL)	Calculated sediment weight after vacuum filtration (g)	Measured radioactivity in the sediment (dpm/g)	(dpm/g)	(dpm/g)	Average	Total radioactivity in the sediment (Bq)
0.18	Day 7	444.7	155	289.7	290.5	351.4	319.9	320.6	1548
1.0		436.6	140	296.6	411.4	324.6	391.4	374.8	1853
5.6		412.2	100	312.2	696.9	1006.0	688.7	795.2	4138
0.18	Day 28	410.9	115	295.9	744.8	651.5	637.1	677.8	3343
1.0		409.5	115	294.5	611.9	674.1	703.1	663.0	3254
5.6		379.8	100	279.8	624.8	672.6	634.3	643.9	3003

The total radioactivity Bq (Bequerel) calculated: average dpm/g/60 x dry weight (g)

At study days 0, 7 and 28, samples of the overlying water were analysed with Thin Layer Chromatography (TLC) in order to differentiate between the radioactivity of the active substance and metabolites. The results are calculated from the amount of radioactivity applied to the beakers in the concentrations 0.18, 1.0 and 5.6 mg/L and the percentage of active substance and metabolites. The analyses were performed in two different ways: the results obtained by the first method (silica gel) are reported here. Results obtained by the reversed phase method are very similar and confirm these results very well.

Table CA 8.2.5.4/01-4 Measurement of radioactivity in the sediment

Initial nominal concentration (mg a.s./L)	Study day	Measured radioactivity (average) (dpm/10 mL)	Calculated total radioactivity in water (Bq)	Percentage of a.s. obtained from the thin layer analysis (%)	Calculated radioactivity as a.s. (Bq)	Calculated radioactivity as metabolites (Bq)
0.18	Day 0	1820.9	8042	100	8042	0
1.0		1926.0	8507	100	8507	0
5.6		1900.2	8392	96.84	8127	265
0.18	Day 7	925.9	4089	36.91	1509	2580
1.0		1114.1	4920	40.23	1979	2941
5.6		1208.6	5338	61.96	3307	2030
0.18	Day 28	836.0	3692	1.73	64	3628
1.0		834.4	3685	4.98	184	3502
5.6		725.1	3203	27.34	876	2327

The total radioactivity Bq (Bequerel) was calculated: average dpm/10 mL x 265 / 60

The results achieved in the study have been presented in terms of the nominal overlying water concentrations.

The %-emergence of midges in the control and solvent control fulfilled the guideline requirements: 90 % of the inserted larvae maturated to adults. The x2-test established no difference of sex in emerged midges at any test concentration (p = 0.05). Because it was not possible to introduce the same number of female and male organisms as larvae into each test beaker, the emergence rates of male and female numbers were pooled for the statistical analysis.

Since the rate of development (male and female midges) was not influenced at any test concentration, except for the delay of 17.5 % (related to the pooled control results) at the highest test concentration of 5.6 mg a.s./L, the EC_{15} for numbers the development rate was ~5.6 mg initial nominal a.s./L (EC_{50} > 5.6 mg/L).

The NOEC with regard to emergence rate and development rate was determined to be > 5.6 mg a.s./L.

Table CA 8.2.5.4/01-5 Summary of numbers of emerged midges

Initial nominal concentration (mg a.s./L)	Number of emerged midges	Emergence (%) of inserted larvae	% male emergence	% female emergence
Control	68	90.7*	42.6	57.4
Solvent control	67	89.3*	49.3	50.7
0.10	23	92.0	52.2	47.8
0.18	22	88.0	45.5	54.5
0.32	22	88.0	45.5	54.5
0.56	25	100	56.0	44.0
1.0	19	76.0	52.6	47.4
1.8	19	76.0	47.4	52.6
3.2	24	96.0	54.2	45.8
5.6	20	80.0	50.0	50.0

*related to three beakers with 25 larvae each; in all other cases related to 1 beaker

Table CA 8.2.5.4/01-6 Mean development time of *Chironomus riparius* exposed to spiroxamine

Initial nominal concentration (mg a.s./L)	Replicate	Mean development time (days)	Mean development rate (1/d)
Control	1	17.8	0.056
	2	16.3	0.061
	3	16.1	0.062
	Mean	16.7 ± 0.91	0.060 ± 0.003
Solvent control	1	16.1	0.062
	2	16.5	0.061
	3	16.4	0.061
	Mean	16.3 ± 0.20	0.061 ± 0.001
0.10	-	16.1	0.062
0.18	-	16.2	0.062
0.32	-	17.1	0.058
0.56	-	16.3	0.061
1.0	-	16.6	0.060
1.8	-	16.8	0.060
3.2	-	16.6	0.060
5.6	-	19.4	0.052

The results of this study are summarised in the table below (mg a.s./L, based on nominal initial concentrations):

Table CA 8.2.5.4/01-7 Effects of KWG 4168 exposure on *Chironomus riparius*

Endpoint	EC_{15}	95% confidence limits	EC_{50}
Emergence rate (mg a.s./L)	> 5.6	Not calculated	> 5.6

Development rate (mg a.s./L)	~ 5.6	Not calculated	> 5.6

III. Conclusion

^{14}C-KWG 4168 (tech.) was tested to assess the potential impact on the maturation of the sediment dwelling life stage of *Chironomus riparius.* Test organisms were exposed to nominal concentrations of 0.1, 0.18, 0.32, 0.56, 1.0, 1.8, 3.2 and 5.6 mg a.s./L and a control and solvent control.

The EC_{15} with regard to development of male and female midges ~5.6 mg initial nominal a.s./L (EC_{50} >5.6 mg/L). The NOEC with regard to emergence rate was determined to be 5.6 mg a.s./L.

Assessment and conclusion by applicant:

An assessment has been made against the validity criteria in the current OECD 219 (2004) test guideline:

- The emergence in the control and solvent control must be at least 70% at the end of the test (actual: 90.7 and 89.3%, respectively)
- *C. riparius* emergence to adults from control vessels should occur between 12 and 23 days after their insertion into the vessels (actual: between days 15 and 21)
- At the end of the test, pH and the dissolved oxygen concentration should be measured in each vessel. The oxygen concentration should be at least 60% of the air saturation value (ASV) at the temperature used, and the pH of overlying water should be in the 6-9 range in all test vessels (actual: Dissolved oxygen: 9.2 to 10.0 mg/L; pH: 7.6 – 7.7)
- The water temperature should not differ by more than ± 1.0°C. The water temperature could be controlled by isothermal room and in that case the room temperature should be confirmed in an appropriate test vessel (actual: 1.9°C, however, the control emergence would suggest that this temperature variation had no impact on the organisms)

The study was conducted in 1998 and therefore followed the BBA test guideline in place at the time. Several differences exist between this test guideline and the current OECD 218 and 219 test guidelines, most notably the number of replicates used and the size of the test vessels. This study tested only a single replicate of 25 organisms at each test substance concentration as opposed to the four replicates of 20 organisms (total: 80 organisms per treatment). The artificial sediment is also different to that currently recommended.

All these points taken into consideration, the results are still considered to be suitable for use in the risk assessment as the study met the requirements of the test guideline at the time and the results largely fulfil the validity criteria of the current OECD test guideline. The study is therefore, considered acceptable.

The EC_{15} with regard to development of male and female midges ~5.6 mg initial nominal a.s./L (EC_{50} > 5.6 mg/L). The NOEC with regard to emergence rate was determined to be 5.6 mg a.s./L.

This study used an overlying water spike test method. However, due to the potential concern for spiroxamine to affect sediment dwelling organisms, a study in which the test vessels were dosed *via* the sediment may have been preferable. It should be noted that a recent water-sediment study conducted using *Lumbriculus* is available and has been summarised in KCA 8.2.5.4/03 below.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.2.5.4/02
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10 and EC20 values for Chironomus riparius with 14C-spiroxamine TG in a chronic study
Report No:	0471836-ECO5
Document No:	M-760403-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
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 M-006549-01-1 on the effects of ^{14}C-Spiroxamine TG on the development and emergence of the non-biting midge (*Chironomus riparius*) study did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013. Due to a lack of dose response, it was not possible to calculate reliable ECx values for either of the parameters tested.

I. Methods

The statistical evaluation was performed with statistical software ToxRat Professional v3.3.0.

Effect concentrations with 10 and 20% from the test item treatment when compared to the pooled controls were calculated for cumulative emergence and development rate, but due to lack of a dose response, these could not be determined for either parameter.

II. Results and Discussion

An explanation is given for regression analysis endpoints for cumulative emergence and development rate. These details can be found below.

Cumulative emergence at 28 days

Due to the lack of a significant dose response on the emergence, when compared to the pooled control, it was not possible to calculate EC_{10} and EC_{20} values.

Development rate at 28 d

Due to the lack of a significant dose response on the development rate, when compared to the pooled control, it was not possible to calculate EC_{10} and EC_{20} values.

Development rate for males at 28 d

Due to the lack of a significant dose response on the development rate in males, when compared to the pooled control, it was not possible to calculate EC_{10} and EC_{20} values.

Development rate for females at 28 d

Due to the lack of a significant dose response on the development rate in females, when compared to the pooled control, it was not possible to calculate EC_{10} and EC_{20} values.

Sex ratio at 28 d

According to the obtained results due to the $p(Chi^2)$ being above the chosen alpha, no effects were detected on sex ratio differences at the study termination.

III. Conclusion

Due to a lack of dose response, it was not possible to calculate reliable EC_x values for any of the parameters tested.

Assessment and conclusion by applicant:

The statistical re-evaluation of the data confirmed that due to a lack of a significant dose-response it was not possible to determine reliable EC_{10} and EC_{20} values for emergence and development rate.

The NOEC of 5.6 mg a.s./L shall remain the critical endpoint determined from this study.

The values determined in the re-evaluation work are considered to be fully valid.

Data Point:	KCA 8.2.5.4/03
Report Author:	[redacted]; [redacted]
Report Year:	2020
Report Title:	Spiroxamine technical - Effects on Lumbriculus variegatus in a sediment-water system - exposed via spiked sediment
Report No:	143051255
Document No:	M-688127-01-1
Guideline(s) followed in study:	Regulation 1107/2009 (Europe) OECD Guideline for the Testing of Chemicals 225: "Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment", adopted October 16, 2007
Deviations from current test guideline:	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 28-day chronic toxicity of spiroxamine technical to *Lumbriculus variegatus* was studied under static exposure conditions. Test organisms were exposed to an untreated control, solvent control and spiroxamine technical at nominal concentrations of 6.25, 12.5, 25, 50 and 100 mg a.s./kg dry sediment. The test concentrations correspond to time weighted average concentrations of 4.44, 8.88, 16.7, 35.5 and 74.2 mg a.s./kg dry sediment.

The endpoints of this study were the total number of living individuals per replicate and their biomass.

The 28-day EC_{10}, EC_{50}, NOEC and LOEC values for total number of worms were determined to be 27.6, 74.2, 35.5 and 74.2 mg a.s./kg dry sediment, respectively. For weight based on the dry weight of the worms, the EC_{10}, EC_{50}, NOEC and LOEC values were determined to be 20.4, 40.3, 16.7 and 35.5 mg a.s./kg dry sediment, respectively. For individual weight, the EC_{10}, EC_{50}, NOEC and LOEC values were determined to be 7.12, 41.5, 16.7 and 35.5 mg a.s./kg dry sediment, respectively.

The analytical determination showed that the test item was not stable during the 28-day exposure period. Since the recovery deviates from the nominal concentrations by more than ±20% of the nominal concentrations, the effect concentrations refer to time weighted average concentrations.

I. Materials and Methods

A. Materials

Test Material Spiroxamine technical

Lot/Batch #: AE 1344293-01-07

Purity:	97.0%
Description:	Light-yellow liquid
Reanalysis/Expiry date:	04 June 2019
Treatments	
Test rates:	Nominal: 6.25, 12.5, 25, 50 and 100 mg a.s./kg dry sediment TWA: 4.44, 8.88, 16.7, 35.5 and 74.2 mg a.s./kg dry sediment
Solvent/vehicle:	Acetone
Analysis of test concentrations:	Yes, 60 -79% of nominal
Test organisms	
Species:	*Lumbriculus variegatus*, 14 days old at test initiation
Source:	In-house laboratory culture
Acclimatisation period:	2 days
Feeding:	None, because the sediment contained a food source *i.e. Urtica* powder
Test design	
Test vessel:	Glass beakers of 250 mL volume covered with a pierced lid
Test medium:	Formulated sediment and reconstituted water
Replication:	4 replicates per test concentration and the control, 6 replicates for the solvent control
No. animals/vessel:	10 animals per replicate
Duration of test:	28 days
Environmental test conditions	
Temperature:	20.3 – 21.1°C
Dissolved oxygen:	83.0 – 99.0% of the air saturation value
pH:	7.3 – 8.3
Photoperiod:	16 hours light, 8 hours dark (light intensity 210 to 400 lux)

B. Study Design

This study was conducted in order to assess the potential impact of spiroxamine technical on the endobenthic sediment-ingesting oligochaete *Lumbriculus variegatus*. Test organisms were exposed to spiroxamine technical for 28 days to assess the impact on reproduction and biomass of adult worms.

Lumbriculus variegatus were exposed to spiroxamine technical at nominal test concentrations of 6.25, 12.5, 25, 50 and 100 mg a.s./kg dry sediment, an untreated control and a solvent control. The worms had been through a synchronisation phase of 14 days so that at test initiation, they were of the same reconstituted stage.

Test vessels comprised of glass beakers of 250 mL covered with a pierced lid to allow for aeration and prevention of organism escape. Each beaker was filled with moist, formulated sediment which consisted of 75% quartz sand, 20% kaolinite clay, 7.5% sphagnum moss peat and 0.75% $CaCO_3$ to a depth of 1.5

cm (approximately 60 g wet weight and 43 g dry weight). Test water was then added to each vessel to a depth of 6 cm.

Ten synchronised oligochaetes were allocated randomly to each test vessel at test initiation. Observations of intoxication (*e.g.* leaving sediment unusual swimming) were made on days 1, 4, 8, 11, 12, 14, 18, 20, 21, 22, 25 and 28. The total dry weight of the living worms per replicate was determined at test termination after placing the worms in a drying oven at 60ºC to dry. Observations of mortality and reproduction were also made at test termination by determining the total number of living and dead individuals per replicate. Missing worms and worms that were unresponsive to gentle mechanical stimulus were considered dead.

The test vessels were held in a controlled environment room maintained at a temperature within 20.3 to 21.2 ºC. At test initiation, test termination and once a week during the test, pH, dissolved Oxygen content and water temperature were measured. pH in the overlying test water ranged from 7.3 to 8.3, oxygen content ranged from 83 to 99% of the air saturation value in the overlying test water and the water temperature was maintained within 19.3 to 20.7 ºC. The test vessels were held under a photoperiod of 16 hours light, 8 hours dark (light intensity 210 to 400 lux).

The worms were not fed during the test because the sediment contained a food source i.e. *Urtica* powder.

The 28-day EC_{50}, EC_{20}, EC_{10} and the 95% confidence limits for worm number, dry weight and individual dry weight were calculated by probit analysis. For the determination of the NOEC and LOEC values, Student t-tests were performed to compare the untreated control with the solvent control (two-sided, $\alpha = 0.05$). Both controls were pooled and the Williams t-test (one-sided smaller, $\alpha = 0.05$) was used to determine the NOEC and LOEC values for worm number, dry weight and individual dry weight after 28 days.

Analytical method

Samples of water and sediment were analysed using the validated analytical method M-688127-01-1, report reference M-688127-01-1 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the OECD 225 guideline (2007) were met.

- The average number of living worms per replicate in the controls should have increased by a factor of at least 1.8 at the end of exposure compared to the number of worms per replicate at the start of exposure (actual: 2.6 in untreated control and 2.1 in the solvent control)
- The pH of the overlying water should be between 6 and 9 throughout the test (actual: 7.3 – 8.3)
- The oxygen concentration in the overlying water should not be below 30% of air saturation value at test temperature during the test (actual: ≥91%)

Analytical results are summarised in the table below.

Table CA 8.2.5.4/03-1 Summary of analytical results

Treatment group (mg a.s. kg)	Overlying water % of nominal[1]	Pore water % of nominal[1]	Sediment % of nominal[1]	Total recovery
Test start (Day 0)				
Control	n.a.	n.a.	n.a.	n.a.
Solvent control	n.a.	n.a.	n.a.	n.a.
25	2	0.1	77	79
100	4	0.2	81	85
Test end (Day 28)				
Control	n.a.	n.a.	n.a.	n.a.
Solvent control	n.a.	n.a.	n.a.	n.a.
25	<LOQ	0.04	60	60

Treatment group (mg a.s. kg)	Overlying water % of nominal[1]	Pore water % of nominal[1]	Sediment % of nominal[1]	Total recovery[2]
100	2	0.11	60	62

[1]: mean value of all measured samples per treatment group
[2]: sum of recovery in overlying water (if applicable), pore water (if applicable) and sediment
LOQ: Limit of Quantification (= 100µg test item/L)
n.a.: not applicable

For the 25 mg a.s./kg treatment the time weighted average recovery was 67% and for the 100 mg a.s./kg treatment the time weighted average recovery was 74%. This gave an overall time weighted average recovery of 71%. The value of 71% was used to determine the TWA concentrations for the 50, 12.5 and 6.25 mg a.s./kg treatments. The test concentrations therefore correspond to time weighted average concentrations of 4.44, 8.88, 16.7, 35.5 and 74.2 mg a.s./kg dry sediment.

The number of worms after 28 days was not statistically significantly different between the untreated control and the solvent control (Student t-test, two sided, $\alpha = 0.05$) so both controls were pooled. The number of worms after 28 days was not statistically significantly different compared to the pooled control up to and including the test concentration of 35.5 mg a.s./kg dry sediment. At 74.2 mg a.s./kg dry sediment, the number of worms was statistically significantly reduced compared to the pooled control (Williams t-test, one-sided smaller, $\alpha = 0.05$).

Table CA 8.2.5.4/03-2 Summary of reproduction data

TWA test concentration (mg a.s./kg dry sediment)	Mean number of worms		% of pooled control
	0 days after exposure	28 days after exposure	
Control	10	26	Mean of the pooled controls = 23
Solvent control	10	21.2	
4.44	10	17.8	76.8
8.88	10	25.5	110.4
16.7	10	28	121.2
35.5	10	21.3	92
74.2	10	10.5	45.5*

* statistically significantly different compared to the pooled controls (Williams t-test, $\alpha = 0.05$)

The dry weight of worms after 28 days was not statistically significantly different between the untreated control and the solvent control (Student t-test, two sided, $\alpha = 0.05$) so both controls were pooled. The dry weight after 28 days was not statistically significantly different compared to the pooled control up to and including the test concentration of 16.7 mg test item/kg dry sediment. At 35.5 and 74.2 mg a.s./dry sediment, the dry weight of worms was statistically significantly reduced compared to the pooled control (Williams t-test, one-sided smaller, $\alpha = 0.05$).

Table CA 8.2.5.4/03-3 Summary of biomass data after 28 days exposure

TWA test concentration (mg a.s./kg dry sediment)	Mean number of worms (28 days after exposure)	Mean dry weight (mg)	% of pooled control	Mean individual dry weight (mg)	% of pooled control
Control	26	30.8	Mean of pooled control (mg): 31.6	1.184	Mean of pooled control (mg): 1.453
Solvent control	21.2	32.3		1.632	
4.44	17.8	31	98.2	1.859	128
8.88	25.5	28.1	88.9	1.108	76.3
16.7	28	31.8	100.7	1.141	78.6
35.5	21.3	18.1	57.4.*	0.858	59.1
74.2	10.5	4.5	14.3*	0.434	29.9

TWA test concentration (mg a.s./kg dry sediment)	Mean number of worms (28 days after exposure)	Mean dry weight (mg)	% of pooled control	Mean individual dry weight (mg)	% of pooled control

* statistically significantly different compared to the pooled controls (Williams t-test, one-sided smaller, α = 0.05)

The results achieved in the study have been summarised in the table below for each of the parameters assessed.

Table CA 8.2.5.4/03-4 Summary of biomass data after 28 days exposure

Parameter	Number of worms [mg a.s./kg dry sediment]	Weight (biomass) [mg a.s./kg dry sediment]	Individual weight (biomass) [mg a.s./kg dry sediment]
28-day EC_{50}	74.2 (n.d.)	40.3 (27.3 – 61.1)	41.5 (24.0 – >74.2)
28-day EC_{20}	38.8 (n.d.)	25.7 (6.46 – 13.4)	13.0 (<4.44 – 22.8)
28-day EC_{10}	27.6 (n.d.)	20.4 (<4.44 – 29.1)	7.12 (<4.44 – 14.7)
28-day NOEC	35.5	16.7	16.7
28-day LOEC	74.2	35.5	35.5

n.d. not determinable
Values in parentheses refer to 95% confidence limits
Values refer to time weighted average concentrations

III. Conclusion

The influence of spiroxamine technical on the development of the freshwater oligochaete *Lumbriculus variegatus* was assessed in a static dose-response test.

For total number of worms, the 28-day EC_{50} was estimated to be 74.2 mg a.s./kg dry sediment. The 28-day NOEC and LOEC values were determined to be 35.5 and 74.2 mg a.s./kg dry sediment, respectively. The EC_{10} was 27.6 mg a.s./kg dry sediment.

For weight based on dry weight of the worms, the EC_{50} was determined to be 40.3 mg a.s./kg dry sediment. The 28-day NOEC and LOEC values were determined to be 16.7 and 35.5 mg a.s./kg dry sediment, respectively. The EC_{10} was 20.4 mg a.s./kg dry sediment.

For individual weight, the EC_{50} was determined to be 41.5 mg a.s./kg dry sediment. The NOEC and LOEC values for individual weight were determined to be 16.7 and 35.5 mg a.s./kg dry sediment, respectively. The EC_{10} was 7.12 mg a.s./kg dry sediment.

Assessment and conclusion by applicant:

This is a new study that has not been previously submitted or evaluated.

Validity criteria according to the OECD 225 guideline (2007) were met.

- The average number of living worms per replicate in the controls should have increased by a factor of at least 1.8 at the end of exposure compared to the number of worms per replicate at the start of exposure (actual: 2.6 in untreated control and 2.1 in the solvent control)
- The pH of the overlying water should be between 6 and 9 throughout the test (actual: 7.3 – 8.3)
- The oxygen concentration in the overlying water should not be below 30% of air saturation value at test temperature during the test (actual: ≥ 91%)

The study is therefore considered acceptable.

The most sensitive parameter in the test was biomass which gave a NOEC of 16.7 mg a.s./kg dry sediment. However it is noted that the EC_{10} of 7.12 mg a.s./kg dry sediment, determined for individual weight biomass, is lower than the NOEC value therefore the EC_{10} value of 7.12 mg a.s./kg dry sediment has been taken as the critical endpoint determined from this study.

CA 8.2.6 Effects on algal growth

For procedural reasons studies listed in the Table 8.2.6-1 below are included in the current dossier as available data or information previously submitted but not necessarily evaluated. However, these reports have been fully superseded by newer studies. Consequently, no summaries of the reports have been included in the dossier.

Table CA 8.2.6-1: Studies previously submitted and not relied upon for the risk assessment

Data Point	Document No.	Date	Title
KCA 8.2.6/01	M-296921-01-5	2009	Evaluations in aquatic risk assessments based on studies with aquatic plants: choice of biomass or growth rate

CA 8.2.6.1 Effects on growth of green algae

Data Point:	KCA 8.2.6.1/01
Report Author:	[redacted]
Report Year:	1994
Report Title:	Influence of KWG 4168 on the growth of the green alga, Scenedesmus subspicatus
Report No:	AJO422694
Document No:	M-096228-01-1
Guideline(s) followed in study:	EEC Directive 79/831/E, Annex V, C.3. Algal Inhibition Test, Revised Version No. L383 A/179 (1992) ISO Guideline 8692: 1989 (E) "Water Quality - Fresh Water Algal Growth Inhibition Test with Scenedesmus subspicatus and Selenastrum capricornutum" (1989) OECD Guideline 201 "Alga Growth Inhibition Test" (1984)
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In a 72-hour toxicity study, cultures of *Scenedesmus subspicatus* were exposed to KWG 4168 at mean measured test concentrations of 0.00023, 0.00038, 0.00084, 0.0012, 0.0028, 0.0042, 0.0068, 0.014 and 0.024 mg a.s./L under static conditions.

The NOE_rC and E_rC_{50} values for growth rate were 0.0012 and 0.012 mg a.s/L, respectively. The percent growth inhibition in the treated algal culture as compared to the control ranged from 1.7 to 65.4%. The NOE_bC and E_bC_{50} values for biomass were 0.0012 and 0.0032 mg a.s/L, respectively. The percent growth inhibition in the treated algal culture as compared to the control ranged from 1.9 to 93.3%.

I. Materials and Methods

A. Materials

Test Material	KWG 4168
Lot/Batch #:	898114002
Purity:	97.50%
Description:	Colourless liquid
Stability of test compound:	Not reported
Reanalysis/Expiry date:	25 January 1995
Density:	Not reported
Treatments	
Test rates:	Nominal: 0.00032, 0.00056, 0.0010, 0.0018, 0.0032, 0.0056, 0.010, 0.018 and 0.032 mg a.s./L Measured: 0.00023, 0.00038, 0.00084, 0.0012, 0.0028, 0.0042, 0.0068, 0.014 and 0.024 mg a.s./L
Solvent/vehicle:	None
Analysis of test concentrations:	Yes, mean measured concentrations 68.2 – 88.5% of nominal
Test organisms	
Species:	Green alga *Scenedesmus subspicatus*, strain 86/81 SAG
Source:	Collection of Algal Cultures, Universität Göttingen, Göttingen, Germany
Test design	
Test vessel:	300 mL Erlenmeyer flasks containing 150 mL solution
Test medium:	Nutrient solution prepared with slight modification to that described in OECD 201
Replication:	None reported
Initial cell density:	1×10^4 cells/mL
Duration of test:	72 hours
Environmental test conditions	
Temperature:	23 ± 2°C
pH:	7.98 – 9.86
Photoperiod:	Continuous lighting at 8000 lux

B. Study Design

This study was conducted in order to assess the growth of the green alga *Scenedesmus subspicatus* when exposed to KWG 4168 over 72 hours.

Test concentrations were prepared from a stock solution and 150 mL of the solution was used in 300-mL Erlenmeyer flasks during the test. Nominal test concentrations were 0.00032, 0.00056, 0.0010, 0.0018, 0.0032, 0.0056, 0.010, 0.018 and 0.032 mg/L. Corresponding measured test concentrations were 0.00023, 0.00038, 0.00084, 0.0012, 0.0028, 0.0042, 0.0068, 0.014 and 0.024 mg/L.

Test media were inoculated with enough 2 or 3-day old pre-culture to give an initial cell density of 1×10^4 cells/mL.

Incubation was at 23 ± 2°C and under continuous light at 8000 lux. Sedimentation of the cells or test substance was prevented by intermittent turning of the pole on which test flasks were suspended.

Cell numbers were determined microscopically on each day of the test using a Thoma counting chamber.

Analytical method

Samples of water were analysed using the validated analytical method 00252 M001, report reference M-008490-02-2 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the OECD 201 guideline in place at the time of study conduct were achieved because the cell density in the control cultures increased by a factor of at least 16 by the end of the test.

The recoveries of treated, cell-free test vessels during the study were 68.2 to 88.5% of nominal, with a mean recovery of 76.2%. The results of the study have been presented based on mean measured test concentrations.

Table CA 8.2.6.1/01-1 Nominal and measured concentrations in treated, cell-free vessels at day 0

Nominal concentration (µg a.s./L) (*µg test item/L*)	Mean measured concentration (µg a.s./L)	% of nominal
0.31 (*0.32*)	0.226	72.9
0.55 (*0.56*)	0.375	68.2
0.98 (*1.0*)	0.843	86.0
1.76 (*1.8*)	1.21	68.8
3.12 (*3.58*)	2.76	88.5
5.46 (*4.26*)	4.16	76.2
9.75 (*7.17*)	6.79	69.6
17.6 (*14.7*)	14.2	80.7
31.2 (*25.2*)	23.5	75.3
-	Mean:	76.2

Limit of Detection (LOD): 0.001 mg/L

After 24 hours exposure, some cells were spherical and enlarged in the 0.014 and 0.024 mg a.s./L test concentrations. After 48 hours, some cells were spherical and enlarged in the 0.0042 mg a.s./L test concentration, and all cells were spherical and enlarged in the 0.0012, 0.0028, 0.0068, 0.014 and 0.024 mg a.s./L test concentrations.

Table CA 8.2.6.1/01-2 Cell density during the toxicity phase

Mean measured concentration (mg a.s./L)	Number of cells ($x10^4$/mL)[1] by time (h)		
	24	48	72
Control	4.00 ± 1.00	27.42 ± 4.40	149.00 ± 11.90
0.00023	3.17 ± 1.00	29.67 ± 3.70	136.50 ± 4.90
0.00038	5.67 ± 1.00	25.17 ± 2.40	125.33 ± 13.20
0.00084	3.83 ± 0.80	34.50 ± 7.90	131.33 ± 10.20
0.0012	4.17 ± 0.30	25.50 ± 4.80	127.33 ± 6.70
0.0028	4.33 ± 1.00	16.67 ± 5.40	94.67 ± 2.90
0.0042	4.33 ± 0.30	11.50 ± 3.10	55.75 ± 3.20

Mean measured concentration (mg a.s./L)	Number of cells (x10^4/mL)[1] by time (h)		
	24	48	72
0.0068	3.50 ± 1.50	6.00 ± 1.50	16.17 ± 4.60
0.014	2.67 ± 1.80	3.17 ± 1.90	7.17 ± 0.80
0.024	2.83 ± 0.60	4.67 ± 0.80	5.67 ± 0.80

[1] Mean of two samples of three replicates (six replicates for the control)

Statistically significant inhibition of area under the growth curve (biomass) could be observed after 48 hours in the 0.014 and 0.024 mg a.s./L test concentrations. After 72 hours, significant inhibition could be observed in the 0.0028, 0.0042, 0.0068, 0.014 and 0.024 mg a.s./L test concentrations.

Table CA 8.2.6.1/01-3 Area under the growth curve and inhibition of treated cultures

Mean measured concentration (mg a.s./L)	24 h		48 h		72 h	
	Area	% inhibition	Area	% inhibition	Area	% inhibition
Control	36	-	389	-	2484	-
0.00023	26	27.8	398	-1.8	2346	5.6
0.00038	56	-55.6	402	-3.3	2184	12.1
0.00084	34	5.6	470	-20.8	2436	1.9
0.0012	38	-5.6	370	4.9	2180	12.2
0.0028	40	-11.1	268	31.1	1580	36.4*
0.0042	40	-11.1	206	47.0	999	59.8*
0.0068	30	16.7	120	69.2	362	85.4*
0.014	20	44.4	66	83.0*	166	93.3*
0.024	22	38.9	88	77.4	188	92.4*

* Statistically significantly different to the control (Dunnett's test, p=0.05, one-sided)

Statistically significant inhibition of growth rate could be observed after 24 hours in the 0.00023, 0.0068, 0.014 and 0.024 mg a.s./L test concentrations. After 48 hours, significant inhibition could be observed in the 0.0028, 0.0042, 0.0068, 0.014 and 0.024 mg a.s./L test concentrations. After 72 hours, significant inhibition could be observed in the 0.0028, 0.0042, 0.0068, 0.014 and 0.024 mg a.s./L test concentrations.

Table CA 8.2.6.1/01-4 Growth rate and inhibition of treated cultures

Mean measured concentration (mg/L)	24 h		48 h		72 h	
	Rate	% inhibition	Rate	% inhibition	Rate	% inhibition
Control	1.36	-	1.65	-	1.67	-
0.00023	1.11	18.2*	1.69	-2.5	1.64	1.7
0.00038	1.72	-26.8	1.61	2.4	1.61	3.5
0.00084	1.33	2.1	1.76	-6.8	1.63	2.5
0.0012	1.43	-4.9	1.61	2.3	1.62	3.1
0.0028	1.45	-6.6	1.39	16.0*	1.52	9.0*
0.0042	1.46	-7.8	1.21	26.7*	1.34	19.6*
0.0068	1.19	12.8*	0.89	46.4*	0.92	45.0*
0.014	0.81	40.6*	0.48	70.8*	0.66	60.7*
0.024	1.03	24.3*	0.77	53.6*	0.58	65.4*

* Statistically significantly different to the control (Dunnett's test, p=0.05, one-sided)

The endpoints derived from the results of this study have been summarised below:

Table CA 8.2.6.1/01-5 Summary of derived endpoints

Biomass		Growth rate	
E_bC_{50} (95% CI)	0.0032 mg a.s./L (0.0020 to 0.0052 mg a.s./L)	E_rC_{50} (95% CI)	0.012 mg a.s./L (0.0081 to 0.020 mg a.s./L)

LOE_bC	0.0028 mg a.s./L	LOE_rC	0.0028 mg a.s./L
NOE_bC	0.0012 mg a.s./L	NOE_rC	0.0012 mg a.s./L

III. Conclusion

In a 72-hour toxicity study, cultures of *Scenedesmus subspicatus* were exposed to KWG 4168 at mean measured test concentrations of 0.00023, 0.00038, 0.00084, 0.0012, 0.0028, 0.0042, 0.0068, 0.014 and 0.024 mg a.s./L under static conditions.

The NOE_rC and E_rC_{50} values for growth rate were 0.0012 and 0.012 mg a.s./L, respectively. The percent growth inhibition in the treated algal culture as compared to the control ranged from 1.7 to 65.4%. The NOE_bC and E_bC_{50} values for biomass were 0.0012 and 0.0032 mg a.s./L, respectively. The percent growth inhibition in the treated algal culture as compared to the control ranged from 1.9 to 93.3%.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 201 (1984), the current version of which is the OECD 201 "Freshwater alga and cyanobacteria, growth inhibition test", adopted 28 July 2011.

Validity criteria according to the OECD 201 guideline (2011) have been re-assessed and the outcome presented below.

1) The cell density increase in the control culture to be at least a factor of 16 (actual 149);
2) The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, and 2-3) in the control culture to be ≤35% (actual 24.4%);
3) The coefficient of variation of the average specific growth rates over the whole test period should be ≤7% (actual 1.61%).

The validity criteria according to the current test guideline have been met but please note that the two coefficient of variation criteria are only met if replicate 2 (out of the 6 replicates in the control) is excluded from the analysis. Replicate 2 has been excluded due to a technical error with this replicate during conduct of the study.

The validity criteria have been achieved therefore the study is considered to be acceptable.

The E_rC_{50} value was determined to be 0.012 mg a.s./L.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.2.6.1/10
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10, EC20 and EC50 values for Scenedesmus subspicatus with KWG 4168 in analgal growth inhibition test
Report No:	0471836-ECO24
Document No:	M-761401-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
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 M-006228-01-1 on the effects of exposure to KWG 4168 on the growth of algae (*Scenedesmus subspicatus*) did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values as well as EC_{50} values have been calculated in accordance with the Annex to Com. Reg. 283/2013 for yield and growth rate.

The resulting EC_{10}, EC_{20} and EC_{50} values for yield at 72 h were 0.84, 1.44 and 3.28 µg a.s./L, respectively. The resulting EC_{10}, EC_{20} and EC_{50} values for growth rate at 72 h were 1.56, 3.51 and 11.90 µg a.s./L, respectively.

I. Methods

The statistical evaluation was performed with statistical software ToxRatPro v3.3.0.

Effect concentrations with 10, 20 and 50% from the test item treatment when compared to the control were determined for yield and growth rate after 72 hours exposure. A Weibull regression was performed, with confidence limits for the EC-x values estimated according to Fieller's theorem.

II. Results and Discussion

Yield at 72 hours

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for yield at 72 h, a statistically significant concentration/response was found ($p(F) < 0.001$) for this parameter. The resulting EC_{10}, EC_{20} and EC_{50} values and the respective confidence intervals are represented in the following table below.

Table CA 8.2.6.1/10-1 Results of the Weibull analysis of yield at 72 h: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Yield		
	EC_{10} (95 % confidence interval) [µg a.s./L]	**EC_{20} (95 % confidence interval) [µg a.s./L]**	**EC_{50} (95 % confidence interval) [µg a.s./L]**
Effect on yield at 72 h	0.84 (0.57 – 1.09)	1.44 (1.11 – 1.73)	3.28 (2.91 – 3.66)

The resulting EC_{10}, EC_{20} and EC_{50} values of 0.84 (95%CL: 0.57 – 1.09), 1.44 (95%CL: 1.11 – 1.73) and 3.28 (95%CL: 2.91 – 3.66) µg a.s./L, respectively, meet the goodness of fit criteria showing a significant concentration/response relationship, and therefore the estimated ECx values are considered reliable.

Growth rate at 72 hours

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for growth rate at 72 h, a statistically significant concentration/response was found (p(F) <0.001) for this parameter.

The resulting EC_{10}, EC_{20} and EC_{50} values and the respective confidence intervals are represented in the following table below.

Table CA 8.2.6.1/10-2 Results of the Weibull analysis of growth rate at 72 h: Selected effective concentrations (EC_x) of the test item and their 95% confidence limits

Parameter	Growth rate		
	EC_{10} (95 % confidence interval) [µg a.s./L]	**EC_{20} (95 % confidence interval) [µg a.s./L]**	**EC_{50} (95 % confidence interval) [µg a.s./L]**
Effect on growth rate at 72 h	1.56 (0.97 – 2.18)	3.51 (2.58 – 4.39)	11.90 (10.32 – 13.77)

The resulting EC_{10}, EC_{20} and EC_{50} values of 1.56 (95% CL: 0.97 – 2.18), 3.51 (95% CL: 2.58 – 4.39) and 11.90 (95%CL: 10.32 – 13.77) µg a.s./L, respectively, meet the goodness of fit criteria by showing a significant concentration/response relationship, and therefore the estimated EC_x values are considered reliable.

III. Conclusion

The resulting EC_{10}, EC_{20} and EC_{50} values for yield at 72-hours were determined to be 0.84, 1.44 and 3.28 µg a.s./L, respectively. The resulting EC_{10}, EC_{20} and EC_{50} values for growth rate at 72-hours were determined to be 1.56, 3.51 and 11.90 µg a.s./L.

Assessment and conclusion by applicant:

The statistical re-evaluation of the data has determined reliable EC_{10}, EC_{20} and EC_{50} values for both growth rate and yield. The E_rC_{50} determined in this re-evaluation work of 11.9 µg a.s./L is considered to be the same as the E_rC_{50} determined in the original study report of 0.012 mg a.s./L (12 µg a.s./L) therefore the original E_rC_{50} from the study report remains the critical endpoint determined from this study.

The values determined in the re-evaluation work are considered to be fully valid.

Data Point:	KCA 8.2.6.1/02
Report Author:	[redacted]
Report Year:	1998
Report Title:	Toxicity of 14C-KWG 4168 to the green alga Selenastrum capricornutum
Report No:	108058
Document No:	M-006533-01-1
Guideline(s) followed in study:	American society for testing and materials (ASTM). 1990. Standard guide for conducting static 96-hour toxicity tests with microalgae. ASTM Standard E1218, Philadelphia, PA.
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The objective of the study was to determine the growth effects of ^{14}C-KWG 4168 to the green alga, *Selenastrum capricornutum*.

The cultures of *Selenastrum capricornutum* were exposed to ^{14}C-KWG 4168 at initial measured test concentrations of 0.55, 1.05, 2.01, 4.12 and 8.14 µg a.s./L under static conditions.

The 96-hour EC_{50} and EC_{25} for growth rate were calculated to be >8.14 µg a.s./L, the highest concentration tested.

The 96-hour EC_{50} and EC_{25} for area under the growth curve were calculated to be 5.5 and 2.1 µg a.s./L, respectively.

The 96-hour EC_{50} and EC_{25} for cell density were calculated to be 5.7 and 2.5 µg a.s./L, respectively.

I. Materials and Methods

A. Materials

Test Material ^{14}C-KWG 4168

Lot/Batch #: C-681B

Purity: 98.2% (specific activity 29.6 mCi/mMole)

Description: Not reported

Stability of test compound: Not reported

Reanalysis/Expiry date: Not reported

Density: Not reported

Treatments

Test rates: Nominal: 0.5, 1.0, 2.0, 4.0 and 8.0 µg a.s./L
Initial measured: 0.55, 1.05, 2.01, 4.12 and 8.14 µg a.s./L

Solvent/vehicle: Methanol

Analysis of test concentrations: Yes, measured concentrations 94 – 111% of nominal

Test organisms

Species:	Green alga, *Selenastrum capricornutum* (now *Raphidocelis subcapitata*)
Source:	Carolina Biological Supply
Test design	
Test vessel:	250-ml borosilicate glass culture flasks filled with approximately 100 mL of test solution and capped with sterile glass closures
Test medium:	Freshwater Nutrient Media (ASTM, 1990)
Replication:	Three replicate vessels were prepared for each concentration and used to determine daily cell density. The highest test concentration had four replicates: 3 replicates for cell density determinations and one additional replicate that was only used to provide a sufficient volume of solution for Day 4 measured concentration analysis
Initial cell density:	1 x 10^4 cells/mL
Duration of test:	96 hours
Environmental test conditions	
Temperature:	24.4 to 25.0°C
Conductivity:	85 – 87 µmhos/cm
pH:	7.5 – 9.2
Photoperiod:	Continuous lighting at ~4300 lux

B. Study Design

This study was conducted in order to determine the growth effects of ^{14}C-KWG 4168 to the green alga, *Selenustrum capricornutum* over 96 hours.

Test concentrations were prepared from a stock solution, and 100 mL of the solution was added to each 250-mL Erlenmeyer flask at the start of the test. Nominal test concentrations were 0.5, 1.0, 2.0, 4.0 and 8.0 µg a.s./L. Corresponding initial measured test concentrations were 0.55, 1.05, 2.01, 4.12 and 8.14 µg a.s./L.

Test media were inoculated with enough 3-day old pre-culture to give a density of 1 x 10^4 cells/mL.

Incubation was at 24.4 to 25.0°C under continuous light at ~4300 lux. Sedimentation of the cells or test substance was prevented by placing test vessels on a shaker table set at 100 revolutions per minute (rpm).

Each day, density was determined in three replicates at each test concentration using a light microscope and an Improved Neubauer hemocytometer.

II. Results and Discussion

The study was considered to be valid at the time of conduct but no specific criteria have been cited in the report.

Analytical recoveries of treated test vessels during the study was 94 to 105% of nominal. The results of the study have been presented in terms of the initial measured test concentration.

Table CA 8.2.6.1/02-1 Measured test concentrations based upon LSC during the exposure of *Selenastrum capriocornutum*

^{14}C-KWG 4168 Nominal concentration (µg/L)	Measured concentration (µg/L)			
	Day 0	Percent of nominal	Day 4[a]	Percent of nominal
Control	<0.08	-	<0.08	-
Solvent control	<0.08	-	<0.08	-
0.5	0.55	111	0.47	94
1.0	1.05	105	1.01	101
2.0	2.01	101	1.91	96
4.0	4.12	103	3.98	99
8.0	8.14	102	8.07	101
Lab recovery[1]	1.66	104	1.67	104

[1] Lab recovery based upon a lab spike of 1.6 µg ^{14}C-KWG 4168/L media

[a] These values indicate that 94 to 101% of the nominal amount of total radioactivity was still present in the test solutions after 4 days. It was determined by radio-TLC that the radioactivity was present in the form of metabolites (68%) and parent compound (32%). The KWG 4168 was not stable under test conditions

The growth curves clearly show decreased growth in the 4.12 and 8.14 µg a.s./L test levels as compared to the controls. The controls, 0.55, 1.05 and 2.01 µg a.s./L level exhibit similar growth through Day 4.

Table CA 8.2.6.1/02-2 Measured algal cell densities during the ^{14}C-KWG 4168 *Selenastrum capricornutum*

Measured concentration (µg/L)	Mean cell density (cells/mL) x 10^4			
	Day 1	Day 2	Day 3	Day 4
Control	3.08	15.85	77.83	195.63
Solvent control	3.21	18.64	94.25	222.75
0.55	3.29	20.11	100.33	219.50
1.05	3.16	14.09	82.33	225.33
2.01	3.10	11.60	51.58	159.67
4.12	3.15	11.09	48.84	127.50
8.14	3.17	10.40	37.96	127.50

The endpoints derived from the results have been summarised below:

Table CA 8.2.6.1/02-3 Summary of derived endpoints

Biomass (Area under the growth curve)			
LOEC	4.12 µg a.s./L	EC_{25} (95% CI)	2.1 µg a.s./L
NOEC	2.01 µg a.s./L	EC_{50} (95% CI)	5.5 µg a.s./L
Growth rate			
LOEC	4.12 µg a.s./L	EC_{25} (95% CI)	>8.14 µg a.s./L
NOEC	2.01 µg a.s./L	EC_{50} (95% CI)	>8.14 µg a.s./L
Cell density			
LOEC	4.12 µg a.s./L	EC_{25} (95% CI)	2.5 µg a.s./L
NOEC	2.01 µg a.s./L	EC_{50} (95% CI)	5.7 µg a.s./L

III. Conclusion

The objective of the study was to determine the growth effects of ^{14}C-KWG 4168 to the green alga, *Selenastrum capricornutum*.

The cultures of *Selenastrum capricornutum* were exposed to ^{14}C-KWG 4168 at initial measured test concentrations of 0.55, 1.05, 2.01, 4.12 and 8.14 µg a.s./L under static conditions.

The 96-hour EC_{50} and EC_{25} for growth rate were calculated to be >8.14 µg a.s./L, the highest concentration tested.

The 96-hour EC_{50} and EC_{25} for area under the growth curve were calculated to be 5.5 and 2.1 µg a.s./L, respectively.

The 96-hour EC_{50} and EC_{25} for cell density were calculated to be 5.7 and 2.5 µg a.s./L, respectively.

Assessment and conclusion by applicant:

The study was conducted to the American society for testing and materials (ASTM). 1990. Standard guide for conducting static 96-hour toxicity tests with microalgae. ASTM Standard E1218, Philadelphia, PA.

The study has therefore been assessed against the validity criteria according to the OECD 201 guideline (2011).

Validity criteria according to OECD 201 (2011) were not consistently met:

- Cell density of control cultures to increase by at least 16x (actual: 209)
- Mean coefficient of variation for section-by-section specific growth rates in control cultures to be ≤35% (actual: 46.2% in the control and solvent control, respectively)
- Coefficient of variation of average specific growth rates in control cultures over the test period to be ≤7% (actual: 6.18% in the control and solvent control, respectively)

The control growth rate data do not meet the mean coefficient of variation criterion of ≤35%. However, it is noted that if Replicate 1 is excluded for being an outlier then the validity criteria are all met.

The results have been based on initial measured concentrations whereas it would have been expected to base the results on nominal concentrations on the basis that the recoveries remained within 80 – 120% of nominal for the duration of the test. The initial measured concentrations are very similar to the nominal concentrations therefore this is not considered to be a significant deviation from the OECD test guideline recommendation.

On balance the study is considered to be acceptable and provides the lowest endpoint for a green algal species. The 96-hour E_rC_{50} was determined to be >8.14 µg a.s./L, the highest concentration tested.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.2.6.1/11
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10, EC20 and EC50 values for Selenastrum capricornutum with 14C-KWG 4168 in an algal growth inhibition test
Report No.:	0471836-EC027
Document No:	M-761427-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
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 M-006533-01-1 on the effects of exposure to ^{14}C-KWG 4168 on the growth of algae (*Selenastrum capricornutum*) did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values, as well as EC_{50} values, have been calculated in accordance with the Annex to Com. Reg. 283/2013.

The resulting EC_{10}, EC_{20} and EC_{50} values for yield at 96 h were 1.29, 2.18 and 5.90 µg a.s./L, respectively. For growth rate after 96 h, the EC_{10} and EC_{20} values were 4.93 and 10.51 µg a.s./L, respectively. An EC_{50} value could not be reliably determined due to value being beyond the tested concentrations.

I. Methods

The statistical evaluation was performed with statistical software ToxRatPro v3.3.0.

Effect concentrations with 10, 20 and 50% from the test item treatment when compared to the pooled control were determined for yield and growth rate after 96 hours exposure. A Probit regression was performed withconfidence limits for the EC_x values estimated according to Fieller's theorem.

II. Results and Discussion

Yield at 96 hours

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for growth rate at 96 h, a statistically significant concentration/response was found (p(F) <0.001).

The resulting EC_{10}, EC_{20} and EC_{50} values and the respective confidence intervals are represented in the following table and figure below.

Table CA 8.2.6.1/11-1 Results of the Probit analysis of yield at 96 h: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Yield		
	EC_{10} (95 % confidence interval) [µg a.s./L]	**EC_{20} (95 % confidence interval) [µg a.s./L]**	**EC_{50} (95 % confidence interval) [µg a.s./L]**
Effect on yield at 96 h	1.29 (0.82 – 1.72)	2.18 (1.62 – 2.66)	5.90 (5.01 – 7.25)

The resulting EC_{10}, EC_{20} and EC_{50} values of 1.29 (95% CL: 0.82 – 1.72), 2.18 (95% CL: 1.62 – 2.66) and 5.90 (95%CL: 5.01 – 7.25) µg a.s./L, respectively, meet the goodness of fit criteria and therefore the estimated ECx values are considered reliable.

Growth rate at 96 hours

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for growth rate at 96 h, a statistically significant concentration/response was found (p(F) <0.001).

The resulting EC_{10}, EC_{20} and EC_{50} values and the respective confidence intervals are represented in the following table and figure below.

Table CA 8.2.6.1/11-2 Results of the Probit analysis of growth rate at 96 h: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Growth rate		
	EC_{10} (95 % confidence interval) [µg a.s./L]	**EC_{20} (95 % confidence interval) [µg a.s./L]**	**EC_{50} (95 % confidence interval) [µg a.s./L]**
Effect on growth rate at 96 h	4.93 (4.29 – 5.55)	10.51 (9.04 – 13.08)	n.d.

n.d.: not determined since value is beyond the tested concentrations

The resulting EC_{10} and EC_{20} values of 4.93 (95%CL: 4.29 – 5.55) and 10.51 (95%CL: 9.04 – 13.08) µg a.s./L, respectively, meet the goodness of fit criteria and therefore are considered reliable. The EC_{50} value could not be reliably determined since it is beyond the tested concentrations and was therefore considered to be >8.14 µg a.s./L.

III. Conclusion

The resulting EC_{10}, EC_{20} and EC_{50} values for yield at 96-hours were determined to be 1.29, 2.18 and 5.90 µg a.s./L, respectively. For growth rate after 96 h, the EC_{10} and EC_{20} values were 4.93 and 10.51 µg a.s./L, respectively. An EC_{50} value could not be reliably determined due to value being beyond the tested concentrations and was therefore considered to be >8.14 µg a.s./L.

Assessment and conclusion by applicant:

The statistical re-evaluation of the data has determined reliable EC_{10}, EC_{20} and EC_{50} values for yield and reliable EC_{10} and EC_{20} values for growth rate. A reliable EC_{50} value for growth rate could not be determined.

The E_rC_{50} determined in this re-evaluation work is the same as that determined in the original study report. Thus, the E_rC_{50} of >8.14 µg a.s./L shall be taken as the critical endpoint determined from this study.

The values determined in the re-evaluation work are considered to be fully valid.

Data Point:	KCA 8.2.6.1/03
Report Author:	[redacted]
Report Year:	2006
Report Title:	Desmodesmus subspicatus growth inhibition test with Spiroxamine
Report No:	EBKWX077
Document No:	M-273962-01-1
Guideline(s) followed in study:	Draft Proposal for Updating OECD Guideline 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (October 22, 2004)
Deviations from current test guideline:	Yes OECD 201 guideline (2011) The inoculum was approximately 1 x104 cells/mL, more than the recommended 2-5 x 103 cells/mL
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In a 72-hour toxicity study, cultures of *Desmodesmus subspicatus* were exposed to spiroxamine at nominal test concentrations of 9.53, 30.5, 97.7, 313 and 1000 µg a.s./L under static conditions.

The growth rate NOE_rC and E_rC_{50} values were <9.53 and 175 µg a.s./L, respectively. The percent inhibition of average specific growth rate in the treated algal cultures compared to the controls ranged from 16.9 to 68.1% after 72 hours exposure.

I. Materials and Methods

A. Materials

Test Material	Spiroxamine (KWG 4168)
Lot/Batch #:	EDTH004293
Purity:	97.5%
Description:	Light brown, clear, oily liquid
Stability of test compound:	Not reported

Reanalysis/Expiry date:	09 December 2006
Density:	Not reported
Treatments	
Test rates:	9.53, 30.5, 97.7, 313 and 1000 µg a.s./L
Solvent/vehicle:	None
Analysis of test concentrations:	Yes, measured concentrations 93 – 108% of nominal (mean 103%) on day 0 and 95 – 104% of nominal (mean 99.0%) on day 3
Test organisms	
Species:	*Desmodesmus subspicatus*
Source:	Collection of Algal Cultures, University of Göttingen, 37077 Göttingen, Germany
Test design	
Test vessel:	300-mL Erlenmeyer flasks containing 150 mL test medium
Test medium:	Prepared according to OECD 201 (2004)
Replication:	Three per test vessel, six per control
Initial cell density:	10,000 cells/mL
Duration of test:	72 hours
Environmental test conditions	
Temperature:	22.2 – 23.3°C
pH:	8.0 – 8.5
Photoperiod:	Under continuous illumination at 6990 – 5620 lux

B. Study Design

This study was conducted in order to assess the effects of exposure to spiroxamine on the green alga *Desmodesmus subspicatus* in a static test over 72 hours.

Test vessels were 300-mL Erlenmeyer flasks containing 150 mL test medium. These were placed on a tablet rotating at 100 rpm to prevent sedimentation of the cells while preventing further aeration. The test media were prepared to the OECD 201 guideline, sterilised by membrane filtration and aerated with sterile air. Test media were inoculated with approximately 10,000 cells/mL, from an exponentially-growing pre-culture prepared four days before the start of the test and cultivated under the same conditions as used in the test.

Nominal concentrations were 9.53, 30.5, 97.7, 313 and 1000 µg a.s./L, along with a control and solvent control, with three replicates per test condition and six replicates per control.

Morphological examination of cells were made over the exposure period on each study day by a microscope. Cell numbers per volume were estimated photometrically as a surrogate for biomass per volume.

Temperature was determined by one continuous measurement of an additional glass vessel filled with an equivalent amount of de-ionised water as in the test vessels. The pH was measured daily in all test levels and the control. Samples were analysed for spiroxamine concentration at test start and end.

Analytical method

Samples of water were analysed using the validated analytical method 00623, report reference M-031628-01-1 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the OECD 201 guideline were met.

- Biomass of control cultures to have increased exponentially by a factor of at least 16 (actual: increased by a factor of 42.4)
- Mean coefficient of variation for section-by-section specific growth rates in the control cultures to not exceed 35% (actual: 26.4%)
- The coefficient of variation of average specific growth rates over the whole test period between the replicate control cultures should not exceed 7% (actual: 2.4%)

The mean recoveries of treated, cell-free test vessels at test start ranged from 93 to 108% of nominal, with an overall mean recovery of 103%. A measured concentration 173% of nominal was observed in the 9.53 µg a.s./L test concentration, however this was deemed unrealistic and likely due to handling errors, and therefore excluded as an outlier. At test end, mean recoveries ranged from 95 to 104% of nominal, with an overall mean recovery of 99.0%. The results of this study have therefore been presented based on nominal test concentrations.

Table CA 8.2.6.1/03-1 Nominal and measured concentrations in treated, cell-free vessels at day 0

Nominal concentration (µg a.s./L)	Mean measured concentration (µg a.s./L)	% of nominal
Control	15.7*	-
Solvent control	8.00*	-
9.53	16.5	173†
30.5	32.9	108
97.7	91.2	93
313	327	104
1000	1060	106
-	Mean:	103

Limit of Quatification (LOQ): 1.25 µg/L

* Measured concentrations in the control are considered as not being evident in the exposure vessels and were most likely caused by handling errors since comparable amounts of spiroxamine were not found in 3-day samples of the same vessels

† For the same reasons this value is deemed unrealistic and therefore excluded as an outlier

Table CA 8.2.6.1/03-2 Nominal and measured concentrations in treated, cell-free vessels at day 3

Nominal concentration (µg a.s./L)	Mean measured concentration (µg a.s./L)	% of nominal
Control	<LOQ	-
Solvent control	<LOQ	-
9.53	9.10	95
30.5	29.7	97
97.7	93.5	96
313	324	104
1000	1033	103
-	Mean:	99.0

Limit of Quatification (LOQ): 1.25 µg/L

The following table details the effects of exposure on biomass (cell density):

Table CA 8.2.6.1/03-3 Cell density during exposure to spiroxamine

Nominal concentration (µg a.s./L)	Number of cells (x10^4/mL)[1] by time ± SD		
	24 h	48 h	72 h
Control	5.0 ± 0.844	13.6 ± 0.806	42.4 ± 2.087
Solvent control	4.7 ± 0.516	14.7 ± 0.505	38.9 ± 3.994
Pooled controls	4.9 ± 0.695	14.2 ± 0.850	40.7 ± 3.546
9.53	4.9 ± 0.330	12.7 ± 0.540	21.7 ± 0.563
30.5	4.3 ± 0.446	10.4 ± 0.478	14.4 ± 1.213
97.7	4.0 ± 0.535	6.8 ± 0.514	7.2 ± 0.802
313	1.3 ± 0.214	5.5 ± 0.333	4.8 ± 0.330
1000	2.0 ± 0.201	2.6 ± 0.206	3.3 ± 0.209

[1] Mean of three replicates (six replicates for the control) ± standard deviation

Statistically significant inhibition of the 0-24 hour growth rate was observed at test concentrations 97.7 and 1000 µg a.s./L compared to the pooled controls. For the 0-48 hour growth rate, significant inhibition to the controls could be observed at test concentrations 9.53, 30.5, 313 and 1000 µg a.s./L. Significant inhibition to the controls for the 0-72 hour growth rate could be observed in all test concentrations.

Table CA 8.2.6.1/03-4 Growth rates and inhibition of treated cultures

Nominal concentration (µg a.s./L)	0 - 24 h		0 - 48 h		0 - 72 h	
	Growth rate	% inhibition	Growth rate	% inhibition	Growth rate	% inhibition
Pooled controls	1.576	-	1.325	-	1.234	-
9.53	1.587	-0.7	1.272*	4.0	1.025*	16.9
30.5	1.444	8.4	1.171*	11.6	0.888*	28.1
97.7	1.381*	13.0	0.957	27.8	0.655*	46.9
313	1.463	7.2	0.856*	35.4	0.524*	57.5
1000	0.706*	55.2	0.485*	63.4	0.394*	68.1

* Statistically significantly different to the control (Welch-t test for Inhomogeneous Variances with Bonferroni Adjustment, α=0.05, one-sided smaller)

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

Table CA 8.2.6.1/03-5 Summary of results after 72-hour exposure to spiroxamine

Nominal concentration (µg a.s./L)	Cell number per mL after 72 h	0-72 h average specific growth rate per day	Inhibition of average specific growth rate (%)	Doubling time (days)
Control	424,320	-	-	-
Solvent control	389,290	-	-	-
Pooled controls	406,800	1.234	-	0.562
9.53	216,670	1.025	16.9	0.676
30.5	143,800	0.888	28.1	0.781
97.7	71,680	0.655	46.9	1.06
313	48,240	0.524	57.5	1.32
1000	32,640	0.394	68.1	1.76

A summary of the relevant endpoints is presented in the table below:

Table CA 8.2.6.1/03-6 Summary of derived endpoints

Growth rate	
E_rC_{50} (95% CI):	175 µg a.s./L (118 to 273 µg a.s./L)
E_rC_{20} (95% CI):	11.4 µg a.s./L (4.31 to 21.0 µg a.s./L)
E_rC_{10} (95% CI):	<9.53 µg a.s./L
LOE_rC:	<9.53 µg a.s./L
NOE_rC:	<9.53 µg a.s./L

III. Conclusion

In a 72-hour toxicity study, cultures of *Desmodesmus subspicatus* were exposed to spiroxamine at nominal test concentrations of 9.53, 30.5, 97.7, 313 and 1000 µg a.s./L under static conditions.

The growth rate NOE_rC and E_rC_{50} values were <9.53 and 175 µg a.s./L, respectively. The percent inhibition of average specific growth rate in the treated algal cultures compared to the controls ranged from 16.9 to 68.1% after 72 hours exposure.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 201 (2004), the current version of which is the OECD 201 "Freshwater alga and cyanobacteria, growth inhibition test", adopted 28 July 2011.

Validity criteria according to the current OECD 201 guideline (2011) were met.

- Biomass of control cultures to have increased exponentially by a factor of at least 16 (actual: increased by a factor of 42.4)
- Mean coefficient of variation for section-by-section specific growth rates in the control cultures to not exceed 35% (actual: 26.4%)
- The coefficient of variation of average specific growth rates over the whole test period between the replicate control cultures should not exceed 7% (actual: 2.4%)

It is noted that the report has based the validity criteria on the pooled control and solvent control data. Therefore the validity criteria have been re-assessed for the control data only and presented below:

- Biomass of control cultures to have increased exponentially by a factor of at least 16 (actual: increased by a factor of 42.4)
- Mean coefficient of variation for section-by-section specific growth rates in the control cultures to not exceed 35% (actual: 26.7%)
- The coefficient of variation of average specific growth rates over the whole test period between the replicate control cultures should not exceed 7% (actual: 1.36%)

All validity criteria have been met therefore the study is considered acceptable.

The E_rC_{50} value was determined to be 175 µg a.s./L.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.2.6.1/12
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10, EC20 and EC50 values for Desmodesmus subspicatus with KWG 4168 in an algal growth inhibition test
Report No:	0471836-ECO30
Document No:	M-761457-01-1
Guideline(s) followed in study:	Noone
Deviations from current test guideline:	None
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 M-273962-01-1 on the effects of exposure to KWG 4168 on the growth of algae (*Desmodesmus subspicatus*) did not provide estimates of EC_{10} or EC_{20} values based on yield. Therefore, these values have been calculated alongside the EC_{50} in accordance with the Annex to Com. Reg. 283/2013.

The resulting EC_{50} value was 10.52 µg a.s./L. EC_{10} and EC_{20} values could not be determined for yield due to values being beyond the tested concentrations.

I. Methods

The statistical evaluation was performed with statistical software ToxRatPro v3.3.0.

Effect concentrations with 10, 20 and 50% from the test item treatment when compared to the control were determined for yield after 72 hours exposure. A linear Probit regression was performed in order to determine EC_x values, with confidence limits estimated according to Fieller's theorem.

II. Results and Discussion

Yield at 72 hours

Regarding the calculation of the EC_{50} value for yield, a statistically significant concentration/response was found ($p(F) < 0.001$) for this parameter.

The resulting EC_{50} value and confidence intervals are represented in the following table below.

Table CA 8.2.6.1/12-1 Results of the Probit analysis of yield at 72 h: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Yield [µg a.s./L]		
	EC_{10} (95 % confidence interval)	EC_{20} (95 % confidence interval)	EC_{50} (95 % confidence interval)
Effect on yield at 72 h	n.d.	n.d.	10.52 (8.83 – 13.31)

n.d.: not determined since value is beyond the tested concentrations

The resulting EC_{50} value of 10.52 (95%CL: 8.83 – 13.31) µg a.s./L meets the goodness of fit criteria showing a significant concentration/response relationship, and therefore the estimated EC_{50} value is considered reliable. The resulting EC_{50} value was 10.52 µg a.s./L. EC_{10} and EC_{20} values could not be determined for yield due to values being beyond the tested concentrations.

III. Conclusion

The resulting EC_{50} values for yield at 72-hours was determined to be 10.52 µg a.s./L.

Assessment and conclusion by applicant:

EC_{10}, EC_{20} and EC_{50} values for growth rate were calculated in the study report therefore only yield endpoints have been determined in this statistical report.

The statistical re-evaluation of the data has determined a reliable EC_{50} value for yield. Reliable EC_{10} and EC_{20} values for yield could not be calculated.

The E_rC_{50} determined in the original study report of 175 µg a.s./L shall remain as the critical endpoint determined from this study.

The values determined in the re-evaluation work are considered to be fully valid.

Data Point:	KCA 8.2.6.1/04
Report Author:	[redacted]
Report Year:	1995
Report Title:	Influence of KWG 4168 on the growth of the green alga, Selenastrum capricornutum
Report No:	AJO/129595
Document No:	M-006518-01-1
Guideline(s) followed in study:	EEC Directive 79/831/E, Annex V, C.3, Algal Inhibition Test, Revised Version No. L383 A/179 (1992) EPA Guideline 540/9-86-134, Growth and Reproduction of Aquatic Plants, Tiers 1 and 2 (1986) ISO Guideline 8692: 1989 (E) "Water Quality - Fresh Water Algal Growth Inhibition Test with Scenedesmus subspicatus and Selenastrum capricornutum" (1989) OECD Guideline 201 "Alga Growth Inhibition Test" (1984)
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RARC (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

In a 120-hour toxicity study, cultures of *Selenastrum capricornutum* were exposed to KWG 4168 at nominal test concentrations of 0.18, 0.32, 0.56, 1.00, 1.80, 3.20, 5.60, 10.0, 18.0 and 32.0 µg a.s./L under static conditions.

The NOE_bC and E_bC_{50} values were 0.32 and 5.42 µg a.s./L, respectively. The percent inhibition of biomass in the treated algal culture as compared to the control ranged from -4.6 to 99.5% after 120 hours exposure. The NOE_rC and E_rC_{50} values were 1.80 and 19.43 µg a.s./L, respectively. The percent inhibition of growth rate in the treated algal culture as compared to the control ranged from -0.8 to 100% after 120 hours exposure.

I. Materials and Methods

A. Materials

Test Material KWG 4168

Lot/Batch #: 898114002

Purity: 96.40%

Description: Colourless liquid

Stability of test compound:	Not reported
Reanalysis/Expiry date:	07 August 1995
Density:	Not reported
Treatments	
Test rates:	Nominal: 0.18, 0.32, 0.56, 1.00, 1.80, 3.20, 5.60, 10.0, 18.0 and 32.0 µg a.s./L
Solvent/vehicle:	None
Analysis of test concentrations:	Yes, mean measured concentrations 121 and 96% of nominal at test start and end, respectively.
Test organisms	
Species:	Green alga *Selenastrum capricornutum* (now *Raphidocelis subcapitata*), strain 61.81
Source:	Collection of Algal Cultures, Universität Göttingen, Göttingen, Germany
Test design	
Test vessel:	300 mL Erlenmeyer flasks containing 150 mL solution
Test medium:	Nutrient solution prepared with slight modification to that described in OECD 201
Replication:	None reported
Initial cell density:	3 x 10^3 cells/mL
Duration of test:	120 hours
Environmental test conditions	
Temperature:	23 ± 2°C
pH:	7.99 – 9.25
Photoperiod:	Continuous lighting at 8000 lux

B. Study Design

This study was conducted in order to assess the growth of the green alga *Selenastrum capricornutum* when exposed to KWG 4168 over a duration of 120 hours.

Test concentrations were prepared from a stock solution and 150 mL of the solution was used in 300-mL Erlenmeyer flasks during the test. Nominal test concentrations were 0.18, 0.32, 0.56, 1.00, 1.80, 3.20, 5.60, 10.0, 18.0 and 32.0 µg a.s./L. Mean measured concentrations were 121 and 96% of nominal at test start and test end, respectively.

Test media were inoculated with enough 2 or 3-day old pre-culture to give a density of 3 x 10^3 cells/mL.

Incubation was at 23 ± 2°C and under continuous light at 8000 lux. Sedimentation of the cells or test substance was prevented by intermittent turning of the pole on which test flasks were suspended.

Cell numbers were determined microscopically on each day of the test using a Thoma counting chamber.

Analytical method

Samples of water were analysed using the validated analytical method 00252 M001, report reference M-008490-02-2 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the OECD 201 guideline in place at the time of study conduct were achieved because the cell density in the control cultures increased by a factor of at least 16 by the end of the test.

The mean recoveries of treated, cell-free test vessels at test start ranged from 77 to 157% of nominal, with an overall mean recovery of 121%. At test end, mean recoveries ranged from 40 to 132% of nominal, with an overall mean recovery of 96%. Results have been presented based on nominal concentrations.

Table CA 8.2.6.1/04-1 Nominal and measured concentrations in treated, cell-free vessels at day 0

Nominal concentration (µg a.s./L)	Mean measured concentration (µg a.s./L)	% of nominal
Control	-	-
0.18	0.231	133
0.32	0.381	124
0.56	0.661	122
1.00	1.12	116
1.80	2.20	122
3.20	3.87	125
5.60	4.16	77
10.0	10.9	113
18.0	27.2	157
32.0	36.4	118
-	Mean:	121

Limit of Quatification (LOQ): 0.09 µg/L

Table CA 8.2.6.1/04-2 Nominal and measured concentrations in treated, cell-free vessels at day 5

Nominal concentration (µg a.s./L)	Mean measured concentration (µg a.s./L)	% of nominal
Control	-	-
0.18	0.125	72
0.32	0.123	40
0.56	0.635	118
1.00	1.09	113
1.80	2.20	127
3.20	3.89	126
5.60	3.2	58
10.0	7.8	81
18.0	15.4	89
32.0	40.8	132
	Mean	96

Limit of Quatification (LOQ): 0.09 µg/L

Cell densities determined during the study are presented in the table below. In the 0.56 µg a.s./L test concentration, some cells were slightly swollen after 48, 72 and 96 hours. In the 1.00 µg a.s./L test concentration, some cells were slightly swollen after 48 hours and some cells were smaller than normal after 72 and 96 hours. In the 1.80 µg a.s./L test concentration, some cells were cylindrical in form after 48, 72, 96 and 120 hours. In the 3.20, 5.60, 10.0 and 18.0 µg a.s./L test concentrations, some cells were deformed after 24 hours, and after 48, 72, 96 and 120 hours exposure cells were cylindrical in form and

were swollen. At the highest test concentration, 32.0 µg a.s./L, all cells were deformed after 24 hours, and after 48, 72, 96 and 120 hours exposure cells were cylindrical in form and were swollen.

Table CA 8.2.6.1/04-3 Cell density during exposure to KWG 4168

Nominal concentration (µg a.s./L)	Number of cells (x10^4/mL)[1] by time (h)				
	24	48	72	96	120
Control	1.42 ± 0.58	12.83 ± 0.93	84.42 ± 7.51	240.33 ± 61.52	491.33 ± 26.22
0.18	2.50 ± 0.50	11.50 ± 1.00	87.33 ± 13.70	250.67 ± 40.46	518.67 ± 27.23
0.32	1.50 ± 1.00	11.67 ± 2.25	83.33 ± 9.02	262.00 ± 4.00	486.67 ± 12.22
0.56	1.50 ± 1.00	9.50 ± 1.00	62.00 ± 11.76	190.00 ± 11.14	485.33 ± 12.86
1.00	1.83 ± 0.29	9.67 ± 2.52	56.50 ± 3.91	244.67 ± 20.43	476.00 ± 4.00
1.80	1.33 ± 0.58	7.17 ± 1.76	60.50 ± 3.12	222.67 ± 18.58	521.33 ± 30.92
3.20	1.67 ± 0.58	10.50 ± 1.00	45.50 ± 2.65	158.67 ± 10.67	413.33 ± 37.17
5.60	1.33 ± 1.26	7.83 ± 0.76	44.33 ± 0.76	140.00 ± 18.03	364.00 ± 32.74
10.0	2.00 ± 0.50	6.33 ± 1.61	21.83 ± 1.04	68.83 ± 4.48	200.67 ± 11.34
18.0	1.50 ± 0.50	2.50 ± 0.50	4.50 ± 0.87	3.83 ± 0.76	8.50 ± 0.50
32.0	0.83 ± 0.58	1.50 ± 0.50	1.17 ± 0.76	0.50 ± 0.50	0.00 ± 0.00

[1] Mean of two samples of three replicates (six replicates for the control) ± standard deviation

Statistically significant inhibition of area under the growth curve (biomass) could be observed after 96 hours at test concentrations 3.20 µg a.s./L and above. After 120 hours, significant inhibition could be observed at test concentrations 0.56 µg a.s./L, and additionally at 3.20 µg a.s./L and above.

Table CA 8.2.6.1/04-4 Area under the growth curve and inhibition of treated cultures

Nominal concentration (µg a.s./L)	24 h		48 h		72 h		96 h		120 h	
	Area	% inhibition	Area	% inhibition	Area	% inhibition	Area	% inhibition	Area	% inhibition
Control	13	-	177		1337	-	5227		14000	-
0.18	26	-97.0	187	-5.6	1366	-2.2	5415	-3.6	14640	-4.6
0.32	13	-0.5	165	6.8	1298	2.9	5435	-4.0	14412	-2.9
0.56	14	-7.5	139	21.4	1026	23.3	4079	22.0	12176	13.0*
1.00	18	-37.3	149	15.8	936	30.0	4543	13.1	13184	5.8
1.80	12	7.5	107	39.5	912	31.8	4303	17.7	13224	5.5
3.20	16	-22.4	155	12.4	820	38.7	3263	37.6*	10120	27.7*
5.60	12	7.5	118	33.0	734	45.1	2939	43.8*	8980	35.9*
10.0	20	-52.2	113	36.1	444	66.8	1525	70.8*	4752	66.1*
18.0	14	-7.5	55	68.8	132	90.1	225	95.7*	366	97.4*
32.0	6	55.2	27	84.7	52	96.0	65	98.8*	64	99.5*

* Statistically significantly different to the control (Dunnett's test, p=0.05, one-sided)

Statistically significant inhibition of growth rate could be observed after 24 hours in all test concentrations except for the 1.80 µg a.s./L test concentration. After 48 hours exposure, significant inhibition was observed in all test concentrations. After 72 hours exposure, statistically significant inhibition could be observed at test concentrations 0.56 µg a.s./L and above. Statistically significant inhibition in growth rate was observed after 96 hours exposure at test concentrations 0.32 and 0.56 µg a.s./L, and additionally at concentrations of 3.20 µg a.s./L and above. After 120 hours exposure significant inhibition could be observed in test concentrations 3.20 µg a.s./L and above.

Table CA 8.2.6.1/04-5 Growth rate and inhibition of treated cultures

Nominal concentration (µg a.s./L)	24 h		48 h		72 h		96 h		120 h	
	Rate	% inhibition	Rate	% inhibition	Rate	% inhibition	Rate	% inhibition	Rate	% inhibition
Control	1.45	-	1.88	-	1.88	-	1.66	-	1.48	-
0.18	2.11	-44.8*	1.82	2.9*	1.89	-0.5	1.68	-1.1	1.49	-0.7
0.32	1.41	2.8*	1.82	2.8*	1.87	0.2	1.69	-1.9*	1.48	0.1
0.56	1.41	2.8*	1.73	8.1*	1.79	4.8*	1.61	6.0*	1.48	0.2

Nominal concentration (µg a.s./L)	24 h		48 h		72 h		96 h		120 h	
	Rate	% inhibition	Rate	% inhibition	Rate	% inhibition	Rate	% inhibition	Rate	% inhibition
1.00	1.80	-23.8*	1.72	8.1*	1.75	7.1*	1.68	-0.8	1.47	0.4
1.80	1.44	1.3	1.58	16.0*	1.77	5.9*	1.65	0.6	1.49	-0.8
3.20	1.67	-14.5*	1.78	5.4*	1.67	10.9*	1.57	5.7*	1.45	2.4*
5.60	1.24	14.5*	1.63	13.2*	1.67	11.4*	1.53	7.6*	1.42	4.1*
10.0	1.88	-28.9*	1.51	19.4*	1.43	23.9*	1.36	18.2*	1.30	12.1*
18.0	1.57	-7.9*	1.05	43.9*	0.90	52.2*	0.63	61.9*	0.67	54.8*
32.0	0.88	39.7*	0.79	58.2*	0.40	78.6*	0.14	91.4*	0.00	100.0*

* Statistically significantly different to the control (Dunnett's test, p=0.05, one-sided)

The endpoints derived from the results of this study have been summarised below.

Table CA 8.2.6.1/04-6 Summary of derived endpoints

Biomass		**Growth rate**	
E_bC_{50} (95% CI)	5.42 µg a.s./L (2.265 to 11.87 µg a.s./L)	E_rC_{50}	19.43 µg a.s./L
LOE_bC	0.56 µg a.s./L	LOE_rC	3.20 µg a.s./L
NOE_bC	0.32 µg a.s./L	NOE_rC	1.80 µg a.s./L

III. Conclusion

In a 120-hour toxicity study, cultures of *Selenastrum capricornutum* were exposed to KWG 4168 at nominal test concentrations of 0.18, 0.32, 0.56, 1.00, 1.80, 3.20, 5.60, 10.0, 18.0 and 32.0 µg a.s./L under static conditions.

The NOE_bC and E_bC_{50} values were 0.32 and 5.42 µg a.s./L, respectively. The percent inhibition of biomass in the treated algal culture as compared to the control ranged from -4.6 to 99.5% after 120 hours exposure. The NOE_rC and E_rC_{50} values were 1.80 and 19.43 µg a.s./L, respectively. The percent inhibition of growth rate in the treated algal culture as compared to the control ranged from -0.8 to 100% after 120 hours exposure.

Assessment and conclusion by applicant:

Validity criteria according to the OECD 201 guideline (2011) have been re-assessed and the outcome presented below.

1) The cell density increase in the control culture to be at least a factor of 16 (actual 281 after 72 hours and 1638 after 120 hours);
2) The mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, and 2-3) in the control culture to be ≤35% (actual 23.9% after 72 hours and 48.3% after 120 hours);
3) The coefficient of variation of the average specific growth rates over the whole test period should be ≤7% (actual 1.54% after 72 hours and 0.72% after 120 hours).

Using the 72-hour data, the validity criteria according to the current test guideline have all been achieved. However, when the 120-hour data are considered, not all of the criteria are achieved with the mean coefficient of variation being >35%.

On this basis the study is considered to be supporting information only.

The E_rC_{50} value was determined to be 19.43 µg a.s./L.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.2.6.1/13
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10, EC20 and EC50 values for Selenastrum capricornutum with KWG 4168 in an algal growth inhibition test
Report No:	0471836-ECO25
Document No:	M-761402-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
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 M-006518-01-1 on the effects of exposure to KWG 4168 on the growth of algae (*Selenastrum capricornutum*) did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values as well as EC_{50} values have been calculated in accordance with the Annex to Com. Reg. 283/2013 for yield and growth rate.

The resulting EC_{10}, EC_{20} and EC_{50} values for yield at 120 h were 3.60, 4.73 and 7.99 µg a.s./L, respectively. For growth rate after 120 h, the EC_{10}, EC_{20} and EC_{50} values were 9.20, 10.94 and 15.24 µg a.s./L, respectively.

I. Methods

The statistical evaluation was performed with statistical software ToxRatPro v3.3.0.

Effect concentrations with 10, 20 and 50% from the test item treatment when compared to the control were determined for yield and growth rate after 120 hours exposure. A Probit regression was performed for both parameters with confidence limits estimated according to Fieller's theorem.

II. Results and Discussion

Yield at 120 hours

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for yield at 120 h, a statistically significant concentration/response was found ($p(t) < 0.001$) for this parameter.

The resulting EC_{10}, EC_{20} and EC_{50} values and the respective confidence intervals are represented in the following table below.

Table CA 8.2.6.1/13-1 Results of the Probit analysis of yield at 120 h: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Yield		
	EC_{10} (95 % confidence interval) [µg a.s./L]	**EC_{20} (95 % confidence interval) [µg a.s./L]**	**EC_{50} (95 % confidence interval) [µg a.s./L]**
Effect on yield at 120 h	3.60 (3.03 – 4.09)	4.73 (4.17 – 5.22)	7.99 (7.43 – 8.59)

The resulting EC_{10}, EC_{20} and EC_{50} values of 3.60 (95%CL: 3.03 – 4.09), 4.73 (95%CL: 4.17 – 5.22) and 7.99 (95%CL: 7.43 – 8.59) µg a.s./L, respectively, meet the goodness of fit criteria and therefore the estimated ECx values are considered reliable.

Growth rate at 120 hours

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for growth rate at 120 h, a statistically significant concentration/response was found (p(F) <0.001) for this parameter.

The resulting EC_{10}, EC_{20} and EC_{50} values and the respective confidence intervals are represented in the following table below.

Table CA 8.2.6.1/13-2 Results of the Probit analysis of growth rate at 120 h: Selected effective concentrations (EC_x) of the test item and their 95% confidence limits

Parameter	Growth rate		
	EC_{10} (95 % confidence interval) [µg a.s./L]	**EC_{20} (95 % confidence interval) [µg a.s./L]**	**EC_{50} (95 % confidence interval) [µg a.s./L]**
Effect on growth rate at 120 h	9.20 (8.75 – 9.62)	10.94 (10.54 – 11.32)	15.24 (14.90 – 15.59)

The resulting EC_{10}, EC_{20} and EC_{50} values of 9.20 (95%CL: 8.75 – 9.62), 10.94 (95%CL: 10.54 – 11.32) and 15.24 (95%CL: 14.90 – 15.59) µg a.s./L, respectively, meet the goodness of fit criteria and therefore the estimated ECx values are considered reliable.

III. Conclusion

The resulting EC_{10}, EC_{20} and EC_{50} values for yield at 120-hours were determined to be 3.60, 4.73 and 7.99 µg a.s./L, respectively. The resulting EC_{10}, EC_{20} and EC_{50} values for growth rate at 120-hours were determined to be 9.20, 10.94 and 15.24 µg a.s./L.

Assessment and conclusion by applicant:

The statistical re-evaluation of the data has determined reliable EC_{10}, EC_{20} and EC_{50} values for both growth rate and yield. The E_rC_{50} determined in this re-evaluation work of 15.2 µg a.s./L is slightly lower than the E_rC_{50} determined in the original study report of 19.4 µg a.s./L therefore the E_rC_{50} of 15.2 µg a.s./L shall be taken as the critical endpoint determined from this study.

The values determined in the re-evaluation work are considered to be fully valid.

Data Point:	KCA 8.2.6.1/05
Report Author:	[redacted]
Report Year:	1995
Report Title:	Growth of Selenastrum capriornutum cells in nutrient medium containing high concentrations of KWG 4168
Report No:	AJO/131995
Document No:	M-006206-01-1
Guideline(s) followed in study:	EEC Directive 79/831/E, Annex V, C.3, Algal Inhibition Test, Revised Version No. L383 A/179 (1992) ISO Guideline 8692: 1989 (E) "Water Quality - Fresh Water Algal Growth Inhibition Test with Scenedesmus subspicatus and Selenastrum capricornutum" (1989) OECD Guideline 201 "Alga Growth Inhibition Test" (1984)
Deviations from current test guideline:	Yes OECD 201 (2011) Validity criteria could not be assessed
Previous evaluation:	yes, evaluated and classified DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

This study was conducted in order to assess the growth and recovery of the green alga *Selenastrum capricornutum* during and after exposure to KWG 4168 for 120 hours.

The E_rC_{50}, NOE_rC and LOE_rC values for *S. capricornutum* exposed to KWG 4168 for 120 hours are 19.3, 1.8 and 3.2 µg a.s./L, respectively.

To determine if the test item had long-term effects on the growth of the algal cells, cultures of *S. capricornutum* that had been exposed to the test item for 120 hours were transferred into fresh test media and incubated for 528 hours.

In cultures treated with 1.0 and 1.8 µg a.s./L, there were no significant differences in cell numbers between treated and controls after 96 h. In cultures treated with 3.2, 5.5 or 10 µg a.s./L, significant differences had disappeared after 168 h. In the cultures treated with 18 and 32 µg a.s./L, growth was initially slow, but after 528 h cell numbers in the treated and control cultures were the same. When cells from 528 h old cultures were inoculated into fresh, KWG 4168-free nutrient solution, and cells counted after 72 h, there were fewer cells in the 18 and 32 µg a.s./L cultures than in the controls. Differences between treated and control cultures were no longer evident at the time of the second counting (192 h).

I. Materials and Methods

A. Materials

Test Material KWG 4168

Lot/Batch #: 898114002

Purity: 96.40%

Description: Colourless liquid

Stability of test compound: Not reported

Reanalysis/Expiry date: 07 August 1995

Density: Not reported

Treatments	
Test rates:	0.18, 0.32, 0.56, 1.0, 1.8, 3.2, 5.6, 10.0, 18.0 and 32.0 µg a.s./L
Solvent/vehicle:	None
Analysis of test concentrations:	Yes (reported in [redacted] (1995). M-006518-01-1)
Test organisms	
Species:	Green alga *Selenastrum capricornutum* (now *Raphidocelis subcapitata*), strain 61.81
Source:	Collection of Algal Cultures, Universität Göttingen, Göttingen, Germany
Test design	
Test vessel:	Not reported
Test medium:	Nutrient solution corresponding to media described in OECD 201
Replication:	None reported
Initial density:	3 x 10^3 cells/mL
Duration of test:	120 / 526 hours
Environmental test conditions	
Temperature:	23 ± 2°C
pH:	Not reported
Photoperiod:	Continuous lighting at 8000 lux

B. Study Design

This study was conducted in order to assess the growth and recovery of the green alga *Selenastrum capricornutum* during and after exposure to KWG 4168 for 120 hours.

Test concentrations were prepared from a stock solution. Test media were inoculated with enough 3-day-old pre-culture to give a density of 3 x 10^4 cells/mL.

Incubation was at 23 ± 2°C and under continuous light at 8000 lux. Sedimentation of the cells or test substance was prevented by intermittent turning of the pole on which test flasks were suspended.

Cell numbers were determined microscopically using a Thoma counting chamber.

Routine growth inhibition test

The 120-hour E_rC_{50}, LOE_rC and NOE_rC values determined after exposure to test concentrations 0.18, 0.32, 0.56, 1.0, 1.8, 3.2, 5.6, 10.0, 18.0 and 32.0 µg a.s./L, along with a control. Cell counts were conducted after 24, 48, 72, 96 and 120 hours.

Growth recovery test

To follow the growth of cells in cultures that had been inhibited by KWG 4168 during the routine growth inhibition test, incubation was continued up to 528 hours. Additional cell counts were made after 168, 264m 336 and 456 hours. For control cultures and those treated with 18.0 or 32.0 µg a.s./L, further counts were made after 528 hours.

Growth of cells from treated cultures in fresh media

To determine if incubation with high concentrations of KWG 4168 impaired the capacity of *S. capricornutum* to grow in fresh media, cells from the 528-h controls and 18.0 and 32.0 µg a.s./L groups were inoculated at 3 x 10^3 cells/mL into 150 mL portions of fresh, untreated nutrient solution. The number of cells that had formed in each culture were counted after 72 and 192 hours.

II. Results and Discussion

Routine toxicity test

The E_rC_{50}, NOE_rC and LOE_rC values for *S. capricornutum* exposed to KWG 4168 are 19.3, 1.8 and 3.2 µg a.s./L, respectively.

Growth recovery test

After 168 hours of incubation, there was not statistically significant difference between the numbers of cells in cultures treated with 1.0, 1.8, 3.2, 5.6 and 10.0 µg a.s./L and the controls. In cultures treated with 18 and 32 µg a.s./L, growth recovery was notably slower, however once growth started it progressed rapidly and after 528 hours there were no statistically significant differences from the controls.

Table CA 8.2.6.1/05-1 Cell density during the toxicity phase and the growth recovery phase

Time (h)	Number of cells (x10^3/mL)[1] by concentration (µg a.s./L)							
	Control	**1.0**	**1.8**	**3.2**	**5.6**	**10.0**	**18.0**	**32.0**
24	14.2 ± 5.8	18.3 ± 2.9	23.3 ± 5.8	16.7 ± 3.8	13.3 ± 12.6	20 ± 5.0	15 ± 5.0	8.3 ± 5.8
48	128.3 ± 9.3	96.7 ± 25.2*	71.1 ± 17.6*	105 ± 10.0*	78.3 ± 7.6*	63.3 ± 16.1*	25 ± 5.0*	15 ± 5.0*
72	844.2 ± 7.5	565 ± 39.1*	605 ± 31.2*	445 ± 26.5*	443 ± 7.6*	218.3 ± 10.4*	45 ± 8.7*	11.7 ± 7.6*
96	2403 ± 615.2	24467 ± 204.3	2226.7 ± 185.8	1586.7 ± 109.7	1400 ± 180.3	688.3 ± 44.8*	38.3 ± 7.6*	5.0 ± 5.0*
120	4913 ± 262.2	4760 ± 40.0	5213.3 ± 300.2	4133.3 ± 371.7*	3640 ± 327.4*	2006.7 ± 113.7*	85 ± 5.0*	0.0 ± 0.0*
168	5650 ± 478.0	6070 ± 486.4	5890 ± 304.1	5240 ± 422.6	5510 ± 255.1	5250 ± 517.6	350 ± 115.7*	7 ± 5.2*
264	5490 ± 447.8	6370 ± 532.3	5930 ± 464.9	6680 ± 454.7	6470 ± 440.1	5280 ± 404.8	3290 ± 141.8*	70 ± 20.0*
336	5450 ± 439.3	6030 ± 326.6	6000 ± 401.6	5760 ± 281.7	5480 ± 567.4	5290 ± 369.8	4040 ± 641.5*	1000 ± 172.2*
456	5540 ± 575.0	5670 ± 397.2	6250 ± 442.3	5090 ± 330.5	5010 ± 314.6	5070 ± 363.7	3400 ± 236.0*	3990 ± 260.1*
528	4930 ± 398.5	-	-	-	-	-	4250 ± 260.1	4320 ± 368.3

[1] Mean of three replicates (six for the control)
* Statistically significantly different to the controls (T-test)

Growth of cells from treated cultures in fresh media

Seventy-two hours after inoculation into fresh nutrient media, algal cells from the treated cultures had grown but at an initially slower rate than that of cells from the untreated control cultures. After 192 hours, there was no statistically significant difference in the number of cells in cultures grown using inoculum from control or treated cultures.

Table CA 8.2.6.1/05-2 Cell density of cells transferred to fresh media

Time (h)	Number of cells (x10^3/mL)[1] by concentration (µg a.s./L)		
	Control	**18**	**32**
0	3.3 ± 0.3	2.8 ± 0.2	2.9 ± 0.2
72	1420 ± 285	913 ± 95*	497 ± 49

Time (h)	Number of cells (x10^3/mL)[1] by concentration (µg a.s./L)		
	Control	18	32
192	4993 ± 434	4840 ± 120	5293 ± 363

[1] Mean of three replicates (six for the control) ±SD (standard deviation)
* Statistically significantly different to the controls (T-test)

III. Conclusion

The E_rC_{50}, NOE_rC and LOE_rC values for *S. capricornutum* exposed to KWG 4168 for 120 hours are 19.3, 1.8 and 3.2 µg a.s./L, respectively.

To determine if the test item had long-term effects on the growth of the algal cells, cultures of *S. capricornutum* that had been exposed to the test item for 120 hours were transferred into fresh test media and incubated for 528 hours.

In cultures treated with 1.0 and 1.8 µg a.s./L, there were no significant differences in cell numbers between treated and controls after 96 h. In cultures treated with 3.2, 5.6 or 10 µg a.s./L, significant differences had disappeared after 168 h. In the cultures treated with 18 and 32 µg a.s./L, growth was initially slow, but after 528 h cell numbers in the treated and control cultures were the same. When cells from 528 h old cultures were inoculated into fresh, KWG 4168-free nutrient solution, and cells counted after 72 h, there were fewer cells in the 18 and 32 µg a.s./L cultures than in the controls. Differences between treated and control cultures were no longer evident at the time of the second counting (192 h).

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 201 (1984), the current version of which is the OECD 201 "Freshwater alga and cyanobacteria, growth inhibition test", adopted 28 July 2011.

This study was a recovery test which is not considered to be appropriate for use in the risk assessment. As such the validity criteria has not been re-assessed against the current test guideline.

The E_rC_{50} value was determined to be 19.3 µg a.s./L.

The study is considered to be supporting information only.

Data Point:	KCA 8.2.6.1/06
Report Author:	[redacted]; [redacted]
Report Year:	2000
Report Title:	Growth of the green alga, Scenedesmus subspicatus, during and after exposure with spiroxamine (KWG 4168)
Report No:	DOM 20055
Document No:	M-028201-01-1
Guideline(s) followed in study:	OECD Guideline 201 "Alga Growth Inhibition Test" (1984)
Deviations from current test guideline:	Yes OECD 201 (2011) Test was run for 22 days
Previous evaluation:	yes, evaluated and classified RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

In a 22-day toxicity study, cultures of *Scenedesmus subspicatus* were exposed to spiroxamine (KWG 4168) at nominal test concentrations of 1.00, 1.80, 3.20, 5.60, 10.0, 18.0 and 32.0 µg test item/L under static conditions.

Cells from the 18.0 and 32.0 µg test item/L concentrations were then incubated for a further 7 days in freshly prepared, uncontaminated media to study the ability to recover.

There were no significant differences in cell numbers between the treatment concentrations 1.0 and 5.6 µg/L and the controls during the entire exposure period. In cultures treated with 10.0, 18.0 and 32.0 µg test item/L growth was initially slower and cells showed some visible effects after 5 days. Cultures treated with 18.0 and 32.0 µg test item/L showed some visible effects after 8 days. After 19 days, only cultures treated with 32.0 µg test item/L were still significantly reduced in growth and the algal cells showed some visible effects.

Twenty-four hours after transfer into fresh, uncontaminated test media, no significant differences between the control and both previously treated cultures were observed. Algal cells from previous exposure levels up to at least 32.0 µg spiroxamine/L are able to recover after elimination of the compound.

I. Materials and Methods

A. Materials

Test Material	Spiroxamine (M 12713) (KWG 4168)
Lot/Batch #:	05488753
Purity:	97.8%
Description:	Yellowish liquid
Stability of test compound:	Not reported
Reanalysis/Expiry date:	August 2001
Density:	Not reported
Treatments	
Test rates:	Nominal: 1.00, 1.80, 3.20, 5.60, 10.0, 18.0 and 32.0 µg test item/L
Solvent/vehicle:	None reported
Analysis of test concentrations:	Yes in the highest two concentrations and the stock solution (mean measured recoveries 60 - 63% of nominal)
Test organisms	
Species:	Green alga *Scenedesmus subspicatus*, strain 86.61 ESP
Source:	Collection of Algal Cultures, University of Göttingen, Germany
Test design	
Test vessel:	300 mL Erlenmeyer flasks containing 150 mL test medium
Test medium:	Media prepared (with slight modifications) to OECD 201
Replication:	Not reported
Initial cell density:	1×10^4 cells/mL
Duration of test:	22 days
Environmental test conditions	
Temperature:	22.4 – 24.4°C
pH:	7.88 – 9.11

Photoperiod: Continuous illumination at mean 6875 lux

B. Study Design

This study was conducted in order to assess the effects of exposure to spiroxamine (KWG 4168) on the green alga *Scenedesmus subspicatus* in a static test over 22 days.

Test vessels were 300-mL Erlenmeyer flasks containing 150 mL test medium, sealed with cotton wool and/or cellulose plugs. These were suspended on a pole, the intermittent turning of which prevented sedimentation of the cells. The test media were prepared with slight modification to the OECD 201 guideline. Media were then inoculated with approximately 10,000 cells/mL, from a pre-culture prepared five days before the start of the test and cultivated under equivalent conditions.

Nominal test concentrations were 1.00, 1.80, 3.20, 5.60, 10.0, 18.0 and 32.0 µg test item/L.

After 22 days of exposure, the cells from the control and the 18.0 and 32.0 µg/L treatment levels were transferred into new untreated nutrient medium. For this purpose the cells were filtered and incubated for another 24 hours in freshly prepared medium (lag-phase). Then approximately 1 x 10^4 cells/mL were inoculated into fresh, spiroxamine-free nutrient medium to reach a well comparable start situation for growth. These cultures were counted after 3, 4 and 6 days of growth.

Cell numbers were determined microscopically at a magnification of 400 times using a Thoma counting chamber.

Quantitative analysis of spiroxamine in samples of the nutrient medium was conducted at test start.

Analytical method

Samples of water were analysed using the validated analytical method 00623, report reference M-031628-01-1 (**see Doc MCA Section 4**).

II. Results and Discussion

The study was not conducted to any guidelines and validity criteria can therefore not be assessed.

The quantities of spiroxamine (KWG 4168) found at the beginning of the test were 60 to 63% (average 62%) of the nominal concentrations. All reported results are based on nominal concentrations.

Table CA 8.2.6.1/06-1 Nominal and measured concentrations at test start

Nominal concentration (µg test item (a.s.)/L)	Mean measured concentration (µg a.s./L)	% of nominal
5.60 (5.48)	3.51	63
32.0 (31.3)	19.3	60
10000 (9780)	6100	61
-	Mean	62

[1] Stock solution

There were no significant differences in cell numbers between the treatment concentrations 1.0 and 5.6 µg/L and the controls during the entire exposure period. In cultures treated with 10.0, 18.0 and 32.0 µg test item/L growth was initially slower and cells showed some visible effects after 5 days. Cultures treated with 18.0 and 32.0 µg test item/L showed some visible effects after 8 days. After 19 days, only cultures treated with 32.0 µg test item/L were still significantly reduced in growth and the algal cells showed some visible effects.

Twenty-four hours after transfer into fresh, uncontaminated test media, no significant differences between the control and both previously treated cultures were observed.

The following table details the effects of exposure on biomass (cell density) during the exposure phase:

Table CA 8.2.6.1/06-2 Cell density during the exposure period to spiroxamine

Nominal concentration (µg/L)	Number of cells ($x10^4$/mL)[1] by time ± SD			
	2 days	5 days	8 days	19 days
Control	8.17 ± 2.50	209 ± 15.89	311 ± 18.58	394.67 ± 32.66
1.00	9.17 ± 3.88	219.33 ± 13.01	324.67 ± 9.87	405.33 ± 68.86
1.80	10.83 ± 4.73	215.33 ± 15.14	313.33 ± 20.03	376 ± 39.4
3.20	8.00 ± 1.80	200.00 ± 13.11	320.67 ± 27.74	397.33 ± 38.02
5.60	8.67 ± 3.69	212.00 ± 17.09	290.00 ± 36.06	386.67 ± 4.62
10.0	5.67 ± 1.44	156.00 ± 9.17	284.00 ± 18.00	428.00 ± 20.00
18.0	7.17 ± 2.84	125.67 ± 24.99	238.00 ± 15.62	390.67 ± 34.02
32.0	3.33 ± 0.29	100.33 ± 10.41	139.00 ± 12.12	159.67 ± 13.58

[1] Mean of two counts of three replicates (six replicates for the control) ± standard deviation

The following table details the effects of exposure on biomass (cell density) during the recovery phase:

Table CA 8.2.6.1/06-3 Cell density during the exposure period to spiroxamine

Nominal concentration (µg/L)	Number of cells ($x10^4$/mL)[1] by time ± SD		
	3 days	4 days	6 days
Control	18.08 ± 3.68	28.83 ± 4.75	30.42 ± 3.18
18.0	20.17 ± 8.02	29.00 ± 4.09	34.17 ± 3.75
32.0	19.33 ± 7.97	30.17 ± 4.19	30.67 ± 6.11

[1] Mean of two counts of three replicates (six replicates for the control) ± standard deviation

III. Conclusion

In a 22-day toxicity study, cultures of *Scenedesmus subspicatus* were exposed to spiroxamine (KWG 4168) at nominal test concentrations of 1.00, 1.80, 3.20, 5.60, 10.0, 18.0 and 32.0 µg test item/L under static conditions.

Cells from the 18.0 and 32.0 µg test item/L concentrations were then incubated for a further 7 days in freshly prepared, uncontaminated media to study the ability to recover.

There were no significant differences in cell numbers between the treatment concentrations 1.0 and 5.6 µg/L and the controls during the entire exposure period. In cultures treated with 10.0, 18.0 and 32.0 µg test item/L growth was initially slower and cells showed some visible effects after 5 days. Cultures treated with 18.0 and 32.0 µg test item/L showed some visible effects after 8 days. After 19 days, only cultures treated with 32.0 µg test item/L were still significantly reduced in growth and the algal cells showed some visible effects.

Twenty-four hours after transfer into fresh, uncontaminated test media, no significant differences between the control and both previously treated cultures were observed. Algal cells from previous exposure levels up to at least 32.0 µg spiroxamine/L are able to recover after elimination of the compound.

Assessment and conclusion by applicant:

The study was based on the OECD 201 test guideline but was a non-standard test as it was conducted for a 22-day period followed by a recovery period. It is therefore not considered necessary or appropriate to re-assess the data against the validity criteria for the current OECD 201 test guideline.

Concentrations of the test item in the solution was determined only for the two highest test concentrations and the stock solution, none of which had recoveries within ±20% of nominal. Analytical verification is therefore insufficient to adequately describe test concentrations.

The study is therefore considered as supporting information only.

Metabolites

Data Point:	KCA 8.2.6.1/07
Report Author:	[redacted]
Report Year:	2007
Report Title:	Desmodesmus subspicatus growth inhibition test with spiroxamine - desethyl
Report No:	EBKWX080
Document No:	M-288232-01-1
Guideline(s) followed in study:	OECD Guideline 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (March 23, 2006)
Deviations from current test guideline:	Yes OECD 201 (2011) The inoculum was approximately 1 x 104 cells/mL, more than the recommended 2-5 x103 cells/mL
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In a 72-hour toxicity study, cultures of *Desmodesmus subspicatus* were exposed to spiroxamine-desethyl at nominal test concentrations of 0.0763, 0.244, 0.781, 2.50 and 8.00 mg/L under static conditions.

The growth rate NOE_rC and E_rC_{50} values were <0.0763 and 0.737 mg/L, respectively. The percent inhibition of average specific growth rate in the treated algal cultures compared to the controls ranged from 26.2 to 78.6% after 72 hours exposure.

I. Materials and Methods

A. Materials

Test Material Spiroxamine-desethyl

Lot/Batch #:	921103ELB02
Purity:	98%
Description:	Clear brown oily liquid
Stability of test compound:	Not reported
Reanalysis/Expiry date:	07 December 2009
Density:	Not reported

Treatments

Test rates:	0.0763, 0.244, 0.781, 2.50 and 8.00 mg/L
Solvent/vehicle:	None
Analysis of test concentrations:	Yes, measured concentrations 88 – 103% of nominal (mean 96.6%) on day 0 and 80 – 104% of nominal (mean 90.0%) on day 3

Test organisms

Species:	*Desmodesmus subspicatus*
Source:	Collection of Algal Cultures, University of Göttingen, 37077 Göttingen, Germany

Test design

Test vessel:	300-mL Erlenmeyer flasks containing 150 mL test medium
Test medium:	Prepared according to OECD 201 (2006)
Replication:	Three per test vessel, six per control
Initial cell density:	10,000 cells/mL
Duration of test:	72 hours
Environmental test conditions	
Temperature:	21.6 – 22.1°C
pH:	7.9 – 8.2
Photoperiod:	Under continuous illumination at 6110 – 7870 lux

B. Study Design

This study was conducted in order to assess the effects of exposure to spiroxamine-desethyl on the green alga *Desmodesmus subspicatus* in a static test over 72 hours.

Test vessels were 300-mL Erlenmeyer flasks containing 150 mL test medium. These were placed on a tablet rotating at 100 rpm to prevent sedimentation of the cells while preventing further aeration, and were sealed with cellulose plugs. The test media were prepared to the OECD 201 guideline, sterilised by membrane filtration and aerated with sterile air. Media were then inoculated with approximately 10,000 cells/mL, from an exponentially-growing pre-culture prepared four days before the start of the test and cultivated under equivalent conditions.

Nominal concentrations were 0.0763, 0.244, 0.781, 2.50 and 8.00 mg/L, along with a control and solvent control, with three replicates per test concentration and six replicates per control. Mean measured concentrations ranged from 88 to 103% of nominal at test start and from 80 to 104% at test end, therefore results are based on nominal concentrations.

Morphological examination of cells were made over the exposure period on each study day by a microscope. Cell numbers per volume were estimated photometrically as a surrogate for biomass per volume.

Temperature was determined by one continuous measurement of an additional glass vessel filled with an equivalent amount of de-ionised water as in the test vessels. The pH was measured daily in all test levels and the control. Samples were analysed for spiroxamine-desethyl concentration at test start and end.

Analytical method

Samples of water were analysed using the validated analytical method 01046, report reference M-287479-01-1 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the OECD 201 guideline were met.

- Biomass of control cultures to have increased exponentially by a factor of at least 16 (actual: increased by a factor of 35)
- Mean coefficient of variation for section-by-section specific growth rates in the control cultures to not exceed 35% (actual: 32.8%)
- The coefficient of variation of average specific growth rates over the whole test period between the replicate control cultures should not exceed 7% (actual: 1.0%)

- The pH of the control medium to not increase by more than 1.5 units during the test (actual: control pH ranged from 7.9 to 8.2)

The mean recoveries of treated, cell-free test vessels at test start ranged from 88 to 103% of nominal, with an overall mean recovery of 96.6%. At test end, mean recoveries ranged from 80 to 104% of nominal, with an overall mean recovery of 90.0%. The results of the study have therefore been presented in terms of nominal test concentrations.

Table CA 8.2.6.1/07-1 Nominal and measured concentrations in treated, cell-free vessels at day 0

Nominal concentration (mg/L)	Mean measured concentration (mg/L)	% of nominal
Control	<0.00508	-
Solvent control	<0.00508	-
0.0763	0.0739	97
0.244	0.233	95
0.781	0.687	88
2.50	2.500	100
8.00	8.219	103
-	Mean:	96.6

Table CA 8.2.6.1/07-2 Nominal and measured concentrations in treated, cell-free vessels at day 3

Nominal concentration (mg/L)	Mean measured concentration (mg/L)	% of nominal
Control	<0.00508	-
Solvent control	<0.00508	-
0.0763	0.0647	85
0.244	0.207	85
0.781	0.627	80
2.50	2.403	96
8.00	8.294	104
-	Mean:	90.0

The following table details the effects of exposure on biomass (cell density):

Table CA 8.2.6.1/07-3 Cell density during exposure to spiroxamine-desethyl

Nominal concentration (mg/L)	Number of cells ($x10^4$/mL)[1] by time ± SD		
	24 h	48 h	72 h
Control	2.4 ± 0.63	10.7 ± 1.01	35.2 ± 4.32
Solvent control	2.3 ± 0.60	9.4 ± 1.09	29.2 ± 0.94
0.0763	2.3 ± 0.10	7.6 ± 0.41	12.1 ± 0.65
0.244	1.9 ± 0.21	6.4 ± 0.29	8.2 ± 0.20
0.781	1.8 ± 0.00	4.4 ± 0.38	5.5 ± 0.55
2.50	1.6 ± 0.44	3.3 ± 0.53	3.7 ± 0.37
8.00	0.3 ± 0.00	1.2 ± 0.26	2.1 ± 0.21

[1] Mean of three replicates (six replicates for the control) ± standard deviation

Statistically significant inhibition of the 0-48 hour and 0-72 hour growth rates to the solvent control could be observed at all test concentrations.

Table CA 8.2.6.1/07-4 Growth rates and inhibition of treated cultures

Nominal concentration (mg/L)	0 - 24 h		0 - 48 h		0 - 72 h	
	Growth rate	% inhibition	Growth rate	% inhibition	Growth rate	% inhibition
Solvent control	0.802	-	1.119	-	1.125	-

Nominal concentration (mg/L)	0 - 24 h		0 - 48 h		0 - 72 h	
	Growth rate	% inhibition	Growth rate	% inhibition	Growth rate	% inhibition
0.0763	0.836	-4.3	1.013*	9.4	0.830*	26.2
0.244	0.664	17.1	0.930*	16.8	0.703*	37.5
0.781	0.605	24.6	0.740*	33.9	0.566*	49.7
2.50	0.441	45.0	0.599*	46.4	0.431*	61.7
8.00	-1.217	251.8	0.078*	93.0	0.241*	78.6

* Statistically significantly different to the control (Williams Multiple Sequential t-test, α=0.05, one-sided smaller)

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

Table CA 8.2.6.1/07-5 Summary of results after 72-hour exposure to spiroxamine-desethyl

Nominal concentration (mg/L)	Cell number per mL after 72 h	0-72 h average specific growth rate per day	Inhibition of average specific growth rate (%)	Doubling time (days)
Solvent control	292,000	1.125		0.616
0.0763	121,000	0.830	26.2	0.835
0.244	82,000	0.703	37.5	0.986
0.781	55,000	0.566	49.7	1.22
2.50	37,000	0.431	61.7	1.61
8.00	21,000	0.241	78.6	2.88

A summary of the relevant endpoints is presented in the table below:

Table CA 8.2.6.1/07-6 Summary of derived endpoints

Growth rate	
E_rC_{50} (95% CI):	0.737 mg/L (0.543 to 0.997 mg a.s./L)
E_rC_{20} (95% CI):	0.0429 mg/L (0.0196 to 0.0742 mg a.s./L)
E_rC_{10} (95% CI):	Not determinable
LOE_rC:	<0.0763 mg/L
NOE_rC:	<0.0763 mg/L

III. Conclusion

In a 72-hour toxicity study, cultures of *Desmodesmus subspicatus* were exposed to spiroxamine-desethyl at nominal test concentrations of 0.0763, 0.244, 0.781, 2.50 and 8.00 mg/L under static conditions.

The growth rate NOE_rC and E_rC_{50} values were ≤0.0763 and 0.737 mg/L, respectively. The percent inhibition of average specific growth rate in the treated algal cultures compared to the controls ranged from 26.2 to 78.6% after 72 hours exposure.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 201 (2006), the current version of which is the OECD 201 "Freshwater alga and cyanobacteria, growth inhibition test", adopted 28 July 2011.

Validity criteria according to the OECD 201 guideline (2011) are the same as those in the version in force at the time this study was conducted.

- Biomass of control cultures to have increased exponentially by a factor of at least 16 (actual: increased by a factor of 35)
- Mean coefficient of variation for section-by-section specific growth rates in the control cultures to not exceed 35% (actual: 32.8%)
- The coefficient of variation of average specific growth rates over the whole test period between the replicate control cultures should not exceed 7% (actual: 1.0%)

- The pH of the control medium to not increase by more than 1.5 units during the test (actual: control pH ranged from 7.9 to 8.2)

It is noted that the study report assessed the validity criteria of the solvent control data only therefore the validity criteria for the control data have also been assessed below.

- Biomass of control cultures to have increased exponentially by a factor of at least 16 (actual: increased by a factor of 35)
- Mean coefficient of variation for section-by-section specific growth rates in the control cultures to not exceed 35% (actual: 30.4%)
- The coefficient of variation of average specific growth rates over the whole test period between the replicate control cultures should not exceed 7% (actual: 3.58%)

The validity criteria have been met therefore the study is considered to be acceptable.

The E_rC_{50} value was determined to be 0.737 mg/L.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.2.6.1/14
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10, EC20 and EC50 values for Desmodesmus subspicatus with KWG 4168- desethyl in an algal growth inhibition test
Report No:	0471836-ECO32
Document No:	M-761465-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
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 M-288232-01-1 on the effects of exposure to KWG 4168-desethyl on the growth of algae (*Desmodesmus subspicatus*) did not provide estimates of EC_{10}, EC_{20} or EC_{50} values for yield. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013.

The resulting EC_{50} value was 30.59 µg a.s./L. EC_{10} and EC_{20} values could not be calculated due to values being beyond the tested concentrations.

I. Methods

The statistical evaluation was performed with statistical software ToxRatPro v3.3.0.

Effect concentrations with 10, 20 and 50% effect from the test item treatment when compared to the control were determined for yield after 72 hours exposure. A linear Probit regression was performed, with confidence limits for the EC_x estimated according to Fieller's theorem

II. Results and Discussion

Yield at 72 hours

Regarding the calculation of the EC_{50} value for yield at 72 d, since p(F) = <0.001, there is no significant lack of fit.

The resulting EC_{50} value and confidence intervals are represented in the following table below.

Table CA 8.2.6.1/14-1 Results of the Probit analysis with yield at 72 h: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Yield [µg a.s./L]		
	EC_{10} (95 % confidence interval)	EC_{20} (95 % confidence interval)	EC_{50} (95 % confidence interval)
Effect on yield at 72 h	n.d.	n.d.	30.59 (24.94 – 36.54)

n.d.: not determined since value is beyond the tested concentrations

The resulting EC_{50} value of 30.59 (95%CL: 24.94 – 36.54) µg a.s./L meets the goodness of fit criteria showing a significant concentration/response relationship, and therefore is considered reliable. EC_{10} and EC_{20} values could not be calculated.

III. Conclusion

The resulting EC_{50} value for yield at 72-hours were determined to be 30.59 µg a.s./L.

Assessment and conclusion by applicant:

EC_{10}, EC_{20} and EC_{50} values for growth rate were calculated in the study report therefore only yield endpoints have been determined in this statistical report.

The statistical re-evaluation of the data has determined a reliable EC_{50} value for yield. Reliable EC_{10} and EC_{20} values for yield could not be calculated.

The E_rC_{50} determined in the original study report of 737 µg a.s./L shall remain as the critical endpoint determined from this study.

The values determined in the re-evaluation work are considered to be fully valid.

KWG 4168-despropyl (M02)

Data Point:	KCA 8.2.6.1/15
Report Author:	[redacted]
Report Year:	2020
Report Title:	KWG4168-despropyl: Toxicity to Pseudokirchnella subcapitata in an algal growth inhibition test
Report No:	14307120
Document No:	M-680695-01-2
Guideline(s) followed in study:	Regulation 1107/2009 (Europe) OECD No. 201
Deviations from current test guideline:	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 purpose of this test was to determine the inhibitory effect of the test item KWG 4168-despropyl on the growth of the freshwater green algae *Pseudokirchneriella subcapitata.*

In a 72-hour algae inhibition test, triplicate cultures of *Pseudokirchneriella subcapitata* were exposed to KWG 4168-despropyl at nominal test concentrations of 0.038, 0.122, 0.391, 1.25 and 4.00 mg/L under static conditions.

The 72-hour E_yC_{50} was calculated to be 0.0425 mg pure metabolite/L and the E_rC_{50} 0.383 mg pure metabolite/L. The 72-hour E_yC_{10} could not be determined and the E_rC_{10} was determined to be 0.0203 mg pure metabolite/L. The 72-hour NOE_yC was determined to be <0.0293 mg pure metabolite/L and the associated 72-hour LOE_yC of 0.0293 mg pure metabolite/L. The 72-hour NOE_rC was determined to be <0.0293 mg pure metabolite/L and the associated 72-hour LOE_rC is 0.0293 mg pure metabolite/L.

I. Materials and Methods

A. Materials

Test Material	KWG 4168-despropyl
Lot/Batch #:	AE 1344303-PU-01
Purity:	99.1% w/w
Description:	Clear colourless oily liquid
Stability of test compound:	Sufficient based on expiration date
Expiry date:	13 May 2022
Density:	Not reported
Treatments	
Test rates:	Nominal: 0.038, 0.122, 0.391, 1.25 and 4.00 mg test item/L Initial mean measured: 0.0293, 0.0972, 0.293, 0.962 and 3.10 mg pure metabolite/L
Solvent/vehicle:	None
Analysis of test concentrations:	Yes, initial mean measured concentrations 75.4 - 80.4% of nominal
Test organisms	
Species:	*Pseudokirchneriella subcapitata*, Strain No. 61.81 SAG formerly known as *Selenastrum capricornutum*, and recently renamed as *Raphidocelis subcapitata* (KORSHIKOV).
Source:	Cultivated in the laboratories of ibacon; original source: "Sammlung von Algenkulturen, Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Universität Göttingen", 37073 Göttingen, Germany.
Test design	
Test vessel:	Erlenmeyer flasks of 50 mL volume with approximately 50 mL of test medium covered with air permeable glass dishes, stoppers or caps.
Test medium:	OECD medium
Replication:	Three replicates per test concentration and six replicates in the control
Initial cell density:	5000 cells/mL
Duration of test:	72 hours
Environmental test conditions	
Temperature:	22.4 to 23.3°C
pH:	8.1 to 9.2
Photoperiod:	Continuous illumination at 4470 to 5070 lux

B. Study Design

The purpose of this test was to determine the inhibitory effect of the test item KWG 4168-despropyl on the growth of the freshwater green algae *Pseudokirchneriella subcapitata.*

A stock solution of 10 mg test item/L was prepared by dissolving 20.2 mg test item into 2020 mL test water by intense stirring for 24 hours. Adequate volumes of this stock solution were diluted with test water to prepare the test media of the desired nominal test concentrations; 0.038, 0.122, 0.391, 1.25 and 4.00 mg test item/L. The test media were prepared just before introduction of the algae (= start of the test).

Pseudokirchneriella subcapitata, strain no. 61.81 SAG, formerly known as *Selenastrum capricornutum*, and recently renamed as *Raphidocelis subcapitata*. The algae were cultivated in the laboratories of ibacon under standardised conditions according to the test guidelines. The test was started (0 hours) by inoculation of a biomass of nominal 5000 algal cells per mL test medium. These cells were taken from an exponentially growing pre-culture, which was set up 4 days prior to the test start under the same conditions as in the test.

The test was performed with three replicates per test concentration and six replicates in the control. Test units were 50 mL Erlenmeyer flasks with approximately 50 mL volume of test medium covered with a permeable glass dishes, stoppers or caps.

The cell density on each observation time was determined by spectrophotometric measurement. Therefore, defined volumes of the algal suspensions from all replicates and from the blanks were sampled after 24, 48 and 72 hours of exposure, and were not replaced. The algal cell densities were calculated by subtracting the absorption of the blanks, from each of the measured absorption of the test media (with algae).

Based on the counted cell densities and the absorption from an algal suspension and its dilutions, a linear regression was performed for the calculation of the cell densities of the replicates during the test.

Incubation was at 22.4 to 23.3°C and under continuous illumination at 4470 to 5070 lux. The pH of the test and control media ranged from 8.1 to 9.4.

Analytical method

Samples of water were analysed using the validated analytical method 01046 adapted, report reference M-680695-01-2 **(see Doc MCA Section 4)**.

II. Results and Discussion

Validity criteria according to the OECD 201 guideline (2011) were met:

- Cell density of control cultures to increase by at least 16x (actual: 178.7x)
- Mean coefficient of variation for section-by-section specific growth rates in control cultures to be ≤35% (actual: 18.3%)
- Coefficient of variation of average specific growth rates in control cultures over the test period to be ≤7% (actual: 2.0%)

At the start of the test, 78% of the nominal test concentrations were found (average of all test concentrations). After 72 hours test duration, 76% of the nominal value was determined (average of all test concentrations). During the test, the algae were exposed to a mean of 77% of nominal. Initial mean measured concentrations ranged from 75.4 to 80.4% of nominal.

Table CA 8.2.6.1/15-1 Summary of analytical results

Nominal concentrations		Fresh (0 hours)[1]		Aged (72 hours)[1]		Initial mean measured concentrations[2]	
[mg test item/L]	[mg pure metabolite/L]	% of nominal	RSD [%]	% of nominal	RSD [%]	[mg test item/L]	[mg pure metabolite/L]
Control	0	-	-	-	-	-	-
0.038	0.0377	78	0	69	2	0.0296	0.0293
0.122	0.121	80	1	77	0	0.0981	0.0972
0.391	0.387	76	4	78	1	0.295	0.293
1.25	1.24	78	2	78	0	0.971	0.962
4.00	3.96	78	1	79	0	3.13	3.10

[1]Mean value of all measured samples per treatment group
[2]Tabulated results represented results rounded to three significant digits
RSD: relative standard deviation per treatment group, number of analysed samples = 2 per group

Table CA 8.2.6.1/15-2 Mean algal cell densities during the test period of 72 hours

Initial measured concentration [mg pure metabolite/L]	Cell density (cells/mL)		
	24 hours	48 hours	72 hours
Control	2.748	21.408	89.338
0.0293	2.788	17.398	55.512
0.0972	2.947	11.523	22.718
0.293	2.470	6.202	6.044
0.962	1.835	3.820	3.344
3.10	0.644	2.232	1.438

Table CA 8.2.6.1/15-3 Mean algal yield during the test period of 72 hours

Initial measured concentration [mg pure metabolite/L]	Mean yield (cells/mL) and % inhibition after					
	24 hours		48 hours		72 hours	
	Yield	% inhib.	Yield	% inhib.	Yield	% inhib.
Control	2.248		20.908		88.838	-
0.0293	2.288	-1.8	16.898	19.2*	55.012	38.1*
0.0972	2.447	-8.8	11.023	47.3*	22.218	75.0*
0.293	0.970	56.8*	5.702	72.7*	5.544	93.8*
0.962	1.335	40.6*	3.320	84.1*	2.844	96.8*
3.10	0.144	93.6*	1.732	91.7*	0.938	98.9*

*Mean value significantly different from the control

Table CA 8.2.6.1/15-4 Growth rate during the test period of 72 hours

Initial measured concentration [mg pure metabolite/L]	Mean growth rates per day					
	0 - 24 hours		0 - 48 hours		0 - 72 hours	
	Growth rate	% inhib.	Growth rate	% inhib.	Growth rate	% inhib.
Control	1.700		1.874	-	1.727	-
0.0293	1.718	-1.0	1.775	5.3*	1.570	9.1*
0.0972	1.772	-4.2	1.568	16.3*	1.271	26.4*
0.293	1.598	6.0	1.259	32.8*	0.831	51.9*
0.962	1.298	23.6*	1.015	45.8*	0.631	63.5*
3.10	0.239	85.9*	0.746	60.2*	0.351	79.7*

*Mean value significantly different from the control

The determined endpoints have been summarised in the table below:

Table CA 8.2.6.1/15-5 Endpoints determined in the 72-hour test

Parameter	Yield [mg pure metabolite/L]	Growth rate [mg pure metabolite/L]
72 hour EC_{50}	0.0425 (0.0400 – 0.0451)	0.383 (0.323 – 0.456)
72 hour EC_{20}	0.0148* (0.0131* - 0.0165*)	0.0557 (0.0402 – 0.0735)
72 hour EC_{10}	n.d.	0.0203* (0.0129* - 0.0291*)
72 hour NOEC	< 0.0293	< 0.0293
72 hour LOEC	0.0293	0.0293

95 % confidence intervals reported in parentheses
* Values are extrapolated

III. Conclusion

The influence of KWG 4168-despropyl on the growth of the freshwater green algae *Pseudokirchneriella subcapitata* was assessed in a static concentration-response test.

The 72-hour E_yC_{50} was calculated to be 0.0425 mg pure metabolite/L and the 72-hour E_rC_{50} value was calculated to be 0.383 mg pure metabolite/L. The 72-hour NOE_yC was determined to be <0.0293 mg pure metabolite/L and the associated 72-hour LOE_yC was 0.0293 mg pure metabolite/L. The 72-hour NOE_rC was determined to be <0.0293 mg pure metabolite/L and the associated 72-hour LOE_rC was 0.0293 mg pure metabolite/L.

Assessment and conclusion by applicant:

This is a new study that has not been previously submitted or evaluated.

Validity criteria according to the OECD 201 guideline (2011) were met:

- Cell density of control cultures to increase by at least 16x (actual: 178.7x)
- Mean coefficient of variation for section-by-section specific growth rates in control cultures to be ≤35% (actual: 18.3%)
- Coefficient of variation of average specific growth rates in control cultures over the test period to be ≤7% (actual: 2.0%)

The study is therefore considered acceptable.

The 72-hour E_rC_{50} value was determined to be 0.383 mg pure metabolite/L.

KWG 4168-N-oxide (M03)

Data Point:	KCA 8.2.6.1/08
Report Author:	[redacted]
Report Year:	2007
Report Title:	Desmodesmus subspicatus growth inhibition test with spiroxamine - N - oxid
Report No:	EBKWX081
Document No:	M-288235-01-1
Guideline(s) followed in study:	OECD Guideline 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (March 23, 2006)
Deviations from current test guideline:	Yes OECD 201 (2011) The inoculum was approximately 1 x 104 cells/mL, more than the recommended 2-5 x103 cells/mL The concentration series did not cover the preferable range of growth inhibition of 5 – 75%
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In a 72-hour toxicity study, cultures of *Desmodesmus subspicatus* were exposed to spiroxamine-N-oxid at nominal test concentrations of 0.286, 0.916, 2.93, 9.38 and 30.0 mg p.m./L under static conditions.

The growth rate NOE$_r$C and E$_r$C$_{50}$ values were 0.286 and 31.7 mg p.m./L, respectively. The percent inhibition of average specific growth rate in the treated algal cultures compared to the controls ranged from 0.8 to 46.7% after 72 hours exposure.

I. Materials and Methods

A. Materials

Test Material Spiroxamine-N-oxid

Lot/Batch #: KTS 10324-1-2

Purity: 86.6%

Description: Colourless viscous oil

Stability of test compound: Not reported

Reanalysis/Expiry date: 07 March 2007

Density: Not reported

Treatments

Test rates: 0.286, 0.916, 2.93, 9.38 and 30.0 mg p.m./L

Solvent/vehicle: None

Analysis of test concentrations: Yes, measured concentrations 82 – 102% of nominal (mean 93%) on day 0 and 90 – 101% of nominal (mean 96.4%) on day 3

Test organisms

Species: *Desmodesmus subspicatus*, strain SAG 86.61 ESP

Source:	Collection of Algal Cultures, University of Göttingen, 37077 Göttingen, Germany
Test design	
Test vessel:	300-mL Erlenmeyer flasks containing 150 mL test medium
Test medium:	Prepared according to OECD 201 (2006)
Replication:	Three per test vessel, six per control
Initial cell density:	10,000 cells/mL
Duration of test:	72 hours
Environmental test conditions	
Temperature:	21.5 – 22.2°C
pH:	8.0 – 8.2
Photoperiod:	Under continuous illumination at 6130 – 7850 lux

B. Study Design

This study was conducted in order to assess the effects of exposure to spiroxamine-N-oxid on the green alga *Desmodesmus subspicatus* in a static test over 72 hours.

Test vessels were 300-mL Erlenmeyer flasks containing 150 mL test medium. These were placed on a tablet rotating at 100 rpm to prevent sedimentation of the cells while preventing further aeration, and were sealed with cellulose plugs. The test media were prepared to the OECD 201 guideline, sterilised by membrane filtration and aerated with sterile air. Media were then inoculated with approximately 10,000 cells/mL, from an exponentially-growing pre-culture prepared four days before the start of the test and cultivated under equivalent conditions.

Nominal concentrations were 0.286, 0.916, 2.93, 9.38 and 30.0 mg pure metabolite (p.m.)/L, along with a control and solvent control, with three replicates per test concentration and six replicates per control. Mean measured concentrations ranged from 82 to 102% of nominal at test start and from 90 to 101% at test end, therefore results are based on nominal concentrations.

Morphological examination of cells were made over the exposure period on each study day by a microscope. Cell numbers per volume were estimated photometrically as a surrogate for biomass per volume.

Temperature was determined by one continuous measurement of an additional glass vessel filled with an equivalent amount of de-ionised water as in the test vessels. The pH was measured daily in all test levels and the control. Samples were analysed for spiroxamine-N-oxid concentration at test start and end.

Analytical method

Samples of water were analysed using the validated analytical method 01046, report reference M-287479-01-1 **(see Doc MCA Section 4)**.

II. Results and Discussion

Validity criteria according to the OECD 201 guideline (2011) were met.

- Biomass of control cultures to have increased exponentially by a factor of at least 16 (actual: increased by a factor of 25 and 23 in the control and solvent control, respectively)
- Mean coefficient of variation for section-by-section specific growth rates in the control cultures to not exceed 35% (actual: 34.8%)

- The coefficient of variation of average specific growth rates over the whole test period between the replicate control cultures should not exceed 7% (actual: 3.1%)
- The pH of the control medium to not increase by more than 1.5 units during the test (actual: control pH ranged from 8.0 to 8.2)

The mean recoveries of treated, cell-free test vessels at test start ranged from 82 to 102% of nominal, with an overall mean recovery of 93.0%. At test end, mean recoveries ranged from 90 to 101% of nominal, with an overall mean recovery of 96.4%. The results of the study have therefore been based on nominal test concentrations.

Table CA 8.2.6.1/08-1 Nominal and measured concentrations in treated, cell-free vessels at day 0

Nominal concentration (mg p.m./L)	Mean measured concentration (mg p.m./L)	% of nominal
Control	<0.005180	-
Solvent control	<0.005180	-
0.286	0.270	94
0.916	0.755	82
2.93	2.982	102
9.38	8.592	92
30.0	28.483	95
-	Mean:	93

Table CA 8.2.6.1/08-2 Nominal and measured concentrations in treated, cell-free vessels at day 3

Nominal concentration (mg p.m./L)	Mean measured concentration (mg p.m./L)	% of nominal
Control	<0.005180	-
Solvent control	<0.005180	-
0.286	0.273	95
0.916	0.828	90
2.93	2.853	97
9.38	9.510	101
30.0	29.766	99
-	Mean:	96.4

The following table details the effects of exposure on biomass (cell density):

Table CA 8.2.6.1/08-3 Cell density during exposure to spiroxamine-N-oxid

Nominal concentration (mg p.m./L)	Number of cells ($x10^4$/mL)[1] by time ± SD		
	24 h	48 h	72 h
Control	2.2 ± 0.23	9.7 ± 0.77	24.5 ± 1.54
Solvent control	2.3 ± 0.64	9.5 ± 0.69	23.3 ± 2.69
Pooled controls	2.3 ± 0.47	9.6 ± 0.71	23.9 ± 2.19
0.286	2.2 ± 0.10	9.7 ± 0.30	23.2 ± 0.32
0.916	2.2 ± 0.42	9.9 ± 0.30	17.0 ± 1.19
2.93	1.9 ± 0.27	7.9 ± 0.34	10.4 ± 0.64
9.38	2.1 ± 0.10	7.5 ± 0.56	7.7 ± 0.19
30.0	2.1 ± 0.27	5.7 ± 0.00	5.4 ± 0.11

[1] Mean of three replicates (six replicates for the control) ± standard deviation

Statistically significant inhibition of growth rates to the pooled controls could be observed at test concentrations 2.93, 9.38 and 30.0 mg p.m./L after 48 hours exposure, and at test concentrations 0.916, 2.93, 9.38 and 30.0 mg p.m./L after 72 hours exposure.

Table CA 8.2.6.1/08-4 Growth rates and inhibition of treated cultures

Nominal concentration (mg p.m./L)	0 - 24 h		0 - 48 h		0 - 72 h	
	Growth rate	% inhibition	Growth rate	% inhibition	Growth rate	% inhibition
Pooled controls	0.813	-	1.129	-	1.057	-
0.286	0.809	0.5	1.135	-0.5	1.049	0.8
0.916	0.800	1.6	1.145*	-1.4	0.943*	10.8
2.93	0.630	22.5	1.030*	8.8	0.779*	26.3
9.38	0.755	7.2	1.009*	10.7	0.679*	35.8
30.0	0.721	11.3	0.873*	22.7	0.563*	46.7

* Statistically significantly different to the control (Williams' Multiple Sequential t-test, α=0.05, one-sided smaller)

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

Table CA 8.2.6.1/08-5 Summary of results after 72-hour exposure to spiroxamine-N-oxid

Nominal concentration (mg p.m./L)	Cell number per mL after 72 h	0-72 h average specific growth rate per day	Inhibition of average specific growth rate (%)	Doubling time (days)
Pooled controls	239,000	1.057	-	0.656
0.286	232,000	1.049	0.8	0.661
0.916	170,000	0.943	10.8	0.735
2.93	104,000	0.779	26.3	0.890
9.38	77,000	0.679	35.8	1.02
30.0	54,000	0.563	46.7	1.23

A summary of the derived endpoints is presented in the table below:

Table CA 8.2.6.1/08-6 Summary of derived endpoints

Growth rate	
E_rC_{50} (95% CI):	31.7 mg p.m./L (15.3 to 169 mg p.m./L)
E_rC_{20} (95% CI):	2.50 mg p.m./L (0.541 to 5.03 mg p.m./L)
E_rC_{10} (95% CI):	0.658 mg p.m./L (0.041 to 1.81 mg p.m./L)
LOE_rC:	0.916 mg p.m./L
NOE_rC:	0.286 mg p.m./L

III. Conclusion

In a 72-hour toxicity study, cultures of *Desmodesmus subspicatus* were exposed to spiroxamine-N-oxid at nominal test concentrations of 0.286, 0.916, 2.93, 9.38 and 30.0 mg p.m./L under static conditions.

The growth rate NOE_rC and E_rC_{50} values were 0.286 and 31.7 mg p.m./L, respectively. The percent inhibition of average specific growth rate in the treated algal cultures compared to the controls ranged from 0.8 to 46.7% after 72 hours exposure.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 201 (2006), the current version of which is the OECD 201 "Freshwater alga and cyanobacteria, growth inhibition test", adopted 28 July 2011.

Validity criteria according to the OECD 201 guideline (2011) are the same as those in the guideline in force at the time this study was conducted. It is noted that the report has assessed the validity criteria on the pooled control and solvent control data therefore the control data only has been re-assessed against the validity criteria and the results presented below:

- Biomass of control cultures to have increased exponentially by a factor of at least 16 (actual: increased by a factor of 25)
- Mean coefficient of variation for section-by-section specific growth rates in the control cultures to not exceed 35% (actual: 33.3%)
- The coefficient of variation of average specific growth rates over the whole test period between the replicate control cultures should not exceed 7% (actual: 1.96%)

The validity criteria have all been met therefore the study is considered acceptable.

The E_rC_{50} value was determined to be 31.7 mg p.m./L.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.2.6.1/16
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10, EC20 and EC50 values for Desmodesmus subspicatus with KWG 4168-Noxide in an algal growth inhibition test
Report No:	0471836-ECO33
Document No:	M-761467-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
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 M-288235-01-1 on the effects of exposure to KWG 4168-N-oxide on the growth of algae (*Desmodesmus subspicatus*) did not provide estimates of EC_i, EC_{20} or EC_{50} values. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013.

The resulting EC_{10}, EC_{20} and EC_{50} values for yield at 72 h were 217.92, 525.75 and 2834.80 µg p.m./L, respectively.

I. Methods

The statistical evaluation was performed with statistical software ToxRatPro v3.3.0.

Effect concentrations with 10, 20 and 50% from the test item treatment when compared to the control were determined for yield after 72 hours exposure. A linear Probit regression was performed, with confidence limits for the EC values estimated according to Fieller's theorem.

II. Results and Discussion

Yield at 72 hours

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for yield at 72 h, a statistically significant concentration/response was found (p(F) <0.001) for this parameter.

The resulting EC_{10}, EC_{20} and EC_{50} values and the respective confidence intervals are represented in the following table below.

Table CA 8.2.6.1/16-1 Results of the Probit analysis with yield at 72 h: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Yield [µg p.m./L]		
	EC_{10} (95 % confidence interval)	EC_{20} (95 % confidence interval)	EC_{50} (95 % confidence interval)
Effect on yield at 72 h	217.92 (99.94 – 368.02)	525.75 (299.54 – 780.08)	2834.80 (2136.28 – 3759.14)

The resulting EC_{10}, EC_{20} and EC_{50} values of 217.92 (95% CL: 99.94 – 368.02), 525.75 (95% CL: 299.54 – 780.08) and 2834.80 (95%CL: 2136.28 – 3759.14) µg p.m./L, respectively, meet the goodness of fit criteria showing a significant concentration/response relationship, and therefore the estimated EC_x values are considered reliable.

III. Conclusion

The resulting EC_{10}, EC_{20} and EC_{50} value for yield at 72-hours were determined to be 217.92, 525.75 and 2834.80 µg p.m./L, respectively.

Assessment and conclusion by applicant:

EC_{10}, EC_{20} and EC_{50} values for growth rate were calculated in the study report therefore only yield endpoints have been determined in this statistical report.

The statistical re-evaluation of the data has determined a reliable EC_{10}, EC_{20} and EC_{50} value for yield.

The E_rC_{50} determined in the original study report of 31,700 µg p.m./L shall remain as the critical endpoint determined from this study.

The values determined in the re-evaluation work are considered to be fully valid.

KWG 4168-carboxylic acid (M06)

Data Point:	KCA 8.2.6.1/09
Report Author:	[redacted]
Report Year:	2008
Report Title:	Desmodesmus subspicatus growth inhibition test with spiroxamine - carbocylic acid
Report No:	EBKWL018
Document No:	M-309818-01-1
Guideline(s) followed in study:	OECD Guideline 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (March 23, 2006)
Deviations from current test guideline:	Yes OECD 201 (2011): • The inoculum was approximately 1 x10^4 cells/mL, more than the recommended 2-5 x10^3 cells/mL • The concentration series did not cover the preferable range of growth inhibition of 5 - 75%
Previous evaluation:	Yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In a 72-hour toxicity study, cultures of *Desmodesmus subspicatus* were exposed to spiroxamine-carboxylic acid at nominal test concentrations of 9.54, 30.5, 97.7, 313, 1000 and 3200 µg p.m./L under static conditions.

The growth rate NOE_rC and E_rC_{50} values were 1000 and >3200 µg p.m./L, respectively. The percent inhibition of average specific growth rate in the treated algal cultures compared to the controls ranged from -2.8 to 7.1% after 72 hours exposure.

I. Materials and Methods

A. Materials

Test Material	Spiroxamine-carbocylic acid
Lot/Batch #:	SES 10277-2-1
Purity:	90.6% w/w
Description:	Light yellow oil
Stability of test compound:	
Reanalysis/Expiry date:	18 January 2009
Density:	
Treatments	
Test rates:	9.54, 30.5, 97.7, 313, 1000 and 3200 µg p.m./L
Solvent/vehicle:	None
Analysis of test concentrations:	Yes, measured concentrations 98 – 103% of nominal (mean 100%) on day 0 and 98 – 110% of nominal (mean 103%) on day 3
Test organisms	
Species:	*Desmodesmus subspicatus*, strain SAG 86.61 ESP
Source:	Collection of Algal Cultures, University of Göttingen, 37077 Göttingen, Germany
Test design	
Test vessel:	300-mL Erlenmeyer flasks containing 150 mL test medium
Test medium:	Prepared according to OECD 201 (2006)
Replication:	Three per test vessel, six per control
Initial cell density:	10,000 cells/mL
Duration of test:	72 hours
Environmental test conditions	
Temperature:	21.4 – 22.2°C
pH:	7.9 – 8.1
Photoperiod:	Under continuous illumination at 7220 – 7580 lux

B. Study Design

This study was conducted in order to assess the effects of exposure to spiroxamine-carbocylic acid on the green alga *Desmodesmus subspicatus* in a static test over 72 hours.

Test vessels were 300-mL Erlenmeyer flasks containing 150 mL test medium. These were placed on a tablet rotating at 100 rpm to prevent sedimentation of the cells while preventing further aeration, and were sealed with cellulose plugs. The test media were prepared to the OECD 201 guideline, sterilised by membrane filtration and aerated with sterile air. Media were then inoculated with approximately 10,000 cells/mL, from an exponentially-growing pre-culture prepared four days before the start of the test and cultivated under equivalent conditions.

Nominal test concentrations were 9.54, 30.5, 97.7, 313, 1000 and 3200 µg pure metabolite (p.m.)/L, along with a control, with three replicates per test concentration and six replicates per control. Mean measured concentrations ranged from 82 to 102% of nominal at test start and from 90 to 101% at test end, therefore results are based on nominal concentrations.

Morphological examination of cells were made over the exposure period on each study day by a microscope. Cell numbers per volume were estimated photometrically as a surrogate for biomass per volume.

Temperature was determined by one continuous measurement of an additional glass vessel filled with an equivalent amount of de-ionised water as in the test vessels. The pH was measured daily in all test levels and the control. Samples were analysed for spiroxamine-carbocylic acid concentration at test start and end.

Analytical method

Samples of water were analysed using the validated analytical method 01122, report reference M-308346-01-1 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the OECD 201 guideline were met:

- Biomass of control cultures to have increased exponentially by a factor of at least 16 (actual: increased by a factor of 21)
- Mean coefficient of variation for section-by-section specific growth rates in the control cultures to not exceed 35% (actual: 26.1%)
- The coefficient of variation of average specific growth rates over the whole test period between the replicate control cultures should not exceed 7% (actual: 5.6%)
- The pH of the control medium to not increase by more than 1.5 units during the test (actual: control pH ranged from 7.9 to 8.0)

The mean recoveries of treated, cell-free test vessels at test start ranged from 98 to 103% of nominal, with an overall mean recovery of 100%. At test end, mean recoveries ranged from 98 to 110% of nominal, with an overall mean recovery of 103%.

Table CA 8.2.6.1/09-1 Nominal and measured concentrations in treated, cell-free vessels at day 0

Nominal concentration (µg p.m./L)	Mean measured concentration (µg p.m./L)	% of nominal
Control	<1.32	-
9.54	9.44	99
30.5	30.5	100
97.7	101	103
313	313	100
1000	995	100

Nominal concentration (µg p.m./L)	Mean measured concentration (µg p.m./L)	% of nominal
3200	3144	98
-	Mean:	100

Table CA 8.2.6.1/09-2 Nominal and measured concentrations in treated, cell-free vessels at day 3

Nominal concentration (µg p.m./L)	Mean measured concentration (µg p.m./L)	% of nominal
Control	<1.32	-
9.54	9.35	98
30.5	33.5	110
97.7	98.5	101
313	331	106
1000	1042	104
3200	3166	99
-	Mean:	103

The results of the study have been presented based on nominal concentrations.

The following table details the effects of exposure on biomass (cell density):

Table CA 8.2.6.1/09-3 Cell density during exposure to spiroxamine-carbocylic acid

Nominal concentration (µg p.m./L)	Number of cells ($x10^4$/mL)[1] by time ± SD		
	24 h	48 h	72 h
Control	2.5 ± 0.55	7.5 ± 2.00	21.3 ± 3.53
9.54	2.7 ± 1.15	6.8 ± 2.08	22.0 ± 3.97
30.5	1.7 ± 0.76	8.8 ± 3.69	23.2 ± 4.75
97.7	2.7 ± 0.76	9.2 ± 4.19	17.8 ± 0.29
313	1.3 ± 0.58	6.7 ± 2.52	17.0 ± 2.18
1000	2.0 ± 1.32	9.7 ± 3.21	19.0 ± 1.80
3200	1.3 ± 1.26	6.0 ± 2.18	17.2 ± 1.76

[1] Mean of two counts of three replicates (six replicates for the control) ± standard deviation

Statistically significant inhibition of growth rates to the control was observed only at the test concentrations 3200 µg p.m./L after 72 hours exposure. No significant inhibition was observed after 48 hours exposure.

Table CA 8.2.6.1/09-4 Growth rates and inhibition of treated cultures

Nominal concentration (µg p.m./L)	0 - 24 h		0 - 48 h		0 - 72 h	
	Growth rate	% inhibition	Growth rate	% inhibition	Growth rate	% inhibition
Control	0.898	-	0.992	-	1.015	-
9.54	0.924	-2.9	0.943	4.9	1.026	-1.1
30.5	0.441	50.9	1.062	-7.0	1.043	-2.8
97.7	0.954	-6.2	1.076	-8.5	0.960	5.4
313	0.231	74.3	0.922	7.1	0.942	7.1
1000	0.441	50.9	1.112	-12.1	0.980	3.4
3200	-2.63*	393	0.876	11.8	0.946*	6.7

* Statistically significantly different to the control (Williams Multiple Sequential t-test, α=0.05, one-sided smaller or Welch-t test for inhomogeneous variances with Bonferroni adjustment)

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

Table CA 8.2.6.1/09-5 Summary of results after 72-hour exposure to spiroxamine-carbocylic acid

Nominal concentration (µg p.m./L)	Cell number per mL after 72 h	0-72 h average specific growth rate per day	Inhibition of average specific growth rate (%)	Doubling time (days)
Control	213,000	1.015	-	0.683
9.54	220,000	1.026	-1.1	0.676
30.5	232,000	1.043	-2.8	0.665
97.7	178,000	0.960	5.4	0.722
313	170,000	0.942	7.1	0.736
1000	190,000	0.980	3.4	0.707
3200	172,000	0.946	6.7	0.733

A summary of the endpoints determined in the study are presented in the table below.

Table CA 8.2.6.1/09-6 Summary of derived endpoints

Growth rate	
E_rC_{50}:	>3200 µg p.m./L
E_rC_{20}:	>3200 µg p.m./L
E_rC_{10}:	>3200 µg p.m./L
LOE_rC:	3200 µg p.m./L
NOE_rC:	1000 µg p.m./L

III. Conclusion

In a 72-hour toxicity study, cultures of *Desmodesmus subspicatus* were exposed to spiroxamine-carboxylic acid at nominal test concentrations of 9.54, 30.5, 97.7, 313, 1000 and 3200 µg p.m./L under static conditions.

The growth rate NOE_rC and E_rC_{50} values were 1000 and >3200 µg p.m./L, respectively. The percent inhibition of average specific growth rate in the treated algal cultures compared to the controls ranged from -2.8 to 7.1% after 72 hours exposure.

Assessment and conclusion by applicant:

The study was conducted to the OECD test guideline 201 (2006), the current version of which is the OECD 201 "Freshwater alga and cyanobacteria, growth inhibition test", adopted 28 July 2011.

Validity criteria according to the current 2011 version of the OECD 201 guideline are the same as that used in the guideline in force at the time of study conduct and therefore remain the same. The validity criteria have been met:

- Biomass of control cultures to have increased exponentially by a factor of at least 16 (actual: increased by a factor of 21)
- Mean coefficient of variation for section-by-section specific growth rates in the control cultures to not exceed 35% (actual: 26.1%)
- The coefficient of variation of average specific growth rates over the whole test period between the replicate control cultures should not exceed 7% (actual: 5.56%)

The study is therefore considered acceptable.

The E_rC_{50} value was determined to be >3200 µg p.m./L.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.2.6.1/17
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10, EC20 and EC50 values for Desmodesmus subspicatus with KWG 4168- carbocylic acid in an algal growth inhibition test
Report No:	0471836-ECO34
Document No:	M-761469-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
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 M-309818-01-1 on the effects of exposure to KWG 4168-carbocylic acid on the growth of algae (*Desmodesmus subspicatus*) did not provide estimates of EC_{10}, EC_{20} and EC_{50} values for yield.

For the determination of EC_X values, no reliable fit could be achieved, therefore EC_x values could not be calculated.

I. Methods

The statistical evaluation was performed with statistical software ToxRatPro v3.3.0.

Due to a lack of statistically significant concentration/response, EC_x values could not be reliably calculated.

II. Results and Discussion

Yield at 72 hours

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for yield at 72 h, the Probit analysis could not produce reliable EC_x values due to lack of a statistically significant concentration/response.

III. Conclusion

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for yield at 72 h, the Probit analysis could not produce reliable EC_x values due to lack of a statistically significant concentration/response.

Assessment and conclusion by applicant:

EC_{10}, EC_{20} and EC_{50} values for growth rate were calculated in the study report therefore only yield endpoints have been determined in this statistical report.

The statistical re-evaluation of the data could not determine reliable EC_{10}, EC_{20} and EC_{50} values for yield due to a lack of a dose-response. These values are therefore considered to be >3,200 µg p.m./L.

The E_rC_{50} determined in the original study report of >3,200 µg p.m./L shall remain as the critical endpoint determined from this study.

The values determined in the re-evaluation work are considered to be fully valid.

For procedural reasons studies listed in the Table 8.2.6.1-1 below are included in the current dossier as available data or information previously submitted but not necessarily evaluated. However, these reports have been fully superseded by newer studies. Consequently, no summaries of the reports have been included in the dossier.

Table CA 8.2.6.1-1: Studies previously submitted and not relied upon for the risk assessment

Data Point	Document No.	Date	Title
KCA 8.2.6.1/09	M-309818-01-1	2008	Desmodesmus subspicatus growth inhibition test with spiroxamine - carbocylic acid

CA 8.2.6.2 Effects on growth of an additional algal species

Data Point:	KCA 8.2.6.2/01
Report Author:	[redacted]
Report Year:	1998
Report Title:	Toxicity of 14C-KWG 4168 to the marine diatom Skeletonema costatum
Report No:	107927
Document No:	M-006512-01-1
Guideline(s) followed in study:	No EU Guideline followed
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Spiroxamine is not an herbicide or plant growth regulator (PGR), nor does it have herbicidal activity therefore studies with additional algal species from a different taxonomic group to green algae are not specifically required. However, data are available with several other algal species which have been summarised below.

Executive Summary

In a 96-hour toxicity study, cultures of *Skeletonema costatum* were exposed to ^{14}C-KWG 4168 at nominal test concentrations of 0.63, 1.25, 2.5, 5.0 and 10 µg a.s./L under static conditions.

The 96-hour growth data was analysed as cell density, growth rate and cumulative biomass (as represented by the area under the growth curve). For each endpoint the data were analysed using ANOVA followed by the Dunnett's test to determine the lowest observed effect concentration (LOEC) and the no-observed-effect-concentration (NOEC). The 96-hour LOEC was 1.29 µg a.s./L and the 96-hour NOEC was 0.63 µg a.s./L for all endpoints.

The EC_{50} based on cell density was determined to be 1.3 µg a.s./L. The EC_{50} based on growth rate was determined to be 6.3 µg a.s./L. The EC_{50} based on biomass was determined to be 1.3 µg a.s./L.

I. Materials and Methods

A. Materials

Test Material ^{14}C-KWG 4168

Lot/Batch #: C-681B

Purity: 98.2% a.s.

Description: Not reported

Stability of test compound: Not reported

Reanalysis/Expiry date:	Not reported
Density:	Not reported
Treatments	
Test rates:	Nominal: 0.63, 1.25, 2.5, 5.0 and 10 µg a.s./L
Solvent/vehicle:	Acetonitrile
Analysis of test concentrations:	Yes, mean measured concentrations 97-107% of nominal
Test organisms	
Species:	Marine diatom *Skeletonema costatum*
Source:	The Starr Collection, University of Texas, Austin, Texas
Test design	
Test vessel:	Sterile, 250-mL borosilicate glass culture flasks filled with approximately 100 mL of test solution and capped with sterile glass closures
Test medium:	Enriched saltwater media was filter sterilized (0.45 µm). The batch of nutrient media used to prepare the test solutions was prepared at pH 8.0 and did not require pH adjustment
Replication:	Three replicate vessels were prepared for each concentration and used to determine daily cell density. The highest test concentration had five replicates: 3 replicates for cell density determinations and two additional replicates that were only used to provide a sufficient volume of solution for Day 4 measured concentration analysis
Initial cell density:	1 x 10^4 cells/mL
Duration of test:	96 hours
Environmental test conditions	
Temperature:	19.8 - 20.8°C
Salinity:	25‰
pH:	8.0 - 9.0
Photoperiod:	16 hour light : 8 hours darkness, light intensity at 4300 lux

B. Study Design

This study was conducted in order to assess the toxicity of the marine diatom *Skeletonema costatum* when exposed to ^{14}C-KWG 4168 over 72 hours.

Test concentrations were prepared from a stock solution, and 100 mL of the solution was used in 250-mL borosilicate glass culture flasks filled during the test. Nominal test concentrations were 0.63, 1.29, 2.46, 5.35 and 10.36 µg a.s./L.

Test media were inoculated with enough 3-day-old pre-culture to give a density of 1 x10^4 cells/mL.

Incubation was at 19.8 to 20.8°C under a photoperiod of 16 hours light to 8 hours dark at 4300 lux. Sedimentation of the cells and test substance was avoided by housing the vessels on a shaker table, which was operated at 100 revolutions per minute.

Each day, density was determined in three replicates at each test concentration using a light microscope and an Improved Neubauer haemocytometer.

II. Results and Discussion

The study was conducted to the American Society for Testing and Materials (ASTM). 1990. Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae. ASTM Standard El 2 1 8, Philadelphia, PA. No specific validity criteria were included in the report.

The initial measured concentrations of ^{14}C-KWG 4168 were 0.63, 1.29 2.46, 5.35 and 10.36 µg a.s./L which represents 98 to 107% of the nominal test concentrations. The ^{14}C-KWG 4168 was not stable in the test system as determined by the 72-hour radio-TLC analysis. Due to this breakdown of the parent compound in the test system, the statistics were based upon the initial measured concentration of the test solutions (EPA, 1994). No undissolved test substance was visually observed in the test vessels throughout the test period.

Table CA 8.2.6.2/01-1 Measured test concentrations of ^{14}C-KWG 4168 based upon liquid scintillation counting during the exposure of *Skeletonema costatum*

Nominal concentration (µg/L)	Day 0[a] (µg/L)	Percent of nominal	Day 4[b] (µg/L)	Percent of nominal
Control[1]	<0.08	-	<0.08	-
Solvent control[1]	<0.08	-	<0.08	-
0.63	0.63	100	0.63	100
1.25	1.29	103	1.26	101
2.5	2.46	98	2.46	99
5	5.35	107	5.26	105
10	10.36	104	9.85	99
Lab recovery[2]	1.94	97	1.93	97

[a] These values represent the total amount of radioactivity in the test solutions. Percent parent analysis on Day 0 determined that 92% of the total radioactivity was present as KWG 4168 technical

[b] These values represent the total amount of radioactivity in the test solutions. Percent parent analysis on Day 4 determined that 20% of the total radioactivity was present as KWG 4168 technical. The compound was not stable under test conditions after 4 days.

[1] Not detected at or above the validated limit of detection (0.08 µg/L)

[2] Lab recovery for Day 0 and Day 4 based upon a nominal lab concentration of 2.0 µg/L

The growth curves clearly show decreased growth in the 1.29, 2.46, 5.35 and 10.36 µg a.s./L test levels as compared to the control.

Table CA 8.2.6.2/01-2 Cell density during the toxicity phase and the growth recovery phase

Initial measured concentration (mg/L)	Mean cell density (cells/mL) x 10^4			
	Day 1	Day 2	Day 3	Day 4
Control	3.95	21	72.6	167.2
Solvent control	3.83	22.2	72.4	173.4
0.63	3.89	21.8	72.2	168.4
1.29	3.78	17.3	43.2	72.5
2.46	2.34	4.39	13.8	33.3
5.35	2.82	3.02	4.04	29.3
10.36	2.16	0.96	0.84	1.49

A summary of the endpoints derived from the data is presented in the table below:

Table CA 8.2.6.2/01-3 Summary of derived endpoints

Cell density	
EC_{25} (95% CI)	0.7 µg a.s./L (95% C.I. 0.4 - 1.2 µg a.s./L)
EC_{50} (95% CI)	1.3 µg a.s./L (95% C.I. 0.9 - 2.0 µg a.s./L)

Growth rate	
E_rC_{25} (95% CI)	5.3 µg a.s./L (95% C.I. 3.4 – 8.2 µg a.s./L)
E_rC_{50} (95% CI)	6.3 µg a.s./L (95% C.I. 4.4 – 8.9 µg a.s./L)
Area under the growth curve (biomass)	
E_bC_{25} (95% CI)	0.7 µg a.s./L (95% C.I. 0.5 -1.2 µg a.s./L)
E_bC_{50} (95% CI)	1.3 µg a.s./L (95% C.I. 1.0 - 1.9 µg a.s./L)

III. Conclusion

In a 96-hour toxicity study, cultures of *Skeletonema costatum* were exposed to ^{14}C-KWG 4168 at nominal test concentrations of 0.63, 1.25, 2.5, 5.0 and 10 µg a.s./L under static conditions.

The 96-hour growth data was analysed as cell density, growth rate and cumulative biomass (as represented by the area under the growth curve). For each endpoint the data were analysed using ANOVA followed by the Dunnett's test to determine the lowest observed effect concentration (LOEC) and the no-observed-effect-concentration (NOEC). The 96-hour LOEC was 1.29 µg a.s./L and the 96-hour NOEC was 0.63 µg a.s./L for all endpoints.

The EC_{50} based on cell density was determined to be 1.3 µg a.s./L. The EC_{50} based on growth rate was determined to be 6.3 µg a.s./L. The EC_{50} based on biomass was determined to be 1.3 µg a.s./L.

Assessment and conclusion by applicant:

The study was conducted to the American Society for Testing and Materials (ASTM). 1999. Standard Guide for Conducting Static 96-h Toxicity Tests with Microalgae. ASTM Standard El 2 1 8, Philadelphia, PA. The control data have therefore been re-assessed against the validity criteria according to the current OECD 201 test guideline (2011).

Validity criteria according to OECD 201 (2011) were met:

- Cell density of control cultures to increase by at least 16x (actual: 170)
- Mean coefficient of variation for section-by-section specific growth rates in control cultures to be ≤35% (actual: 29.4%)
- Coefficient of variation of average specific growth rates in control cultures over the test period to be ≤10% (actual: 0.96%)

The validity criteria according to the OECD 201 test guideline have been met therefore the study is considered acceptable. It should be noted that the results have been based on initial measured concentrations when they would preferably have been based on mean measured concentrations which took the Day 4 measurements in to account. However, based on total radioactivity the total residues at Day 4 were consistent with the nominal concentrations therefore it is believed to be acceptable to use the initial measured concentrations here.

The E_rC_{50} based on growth rate was determined to be 6.3 µg a.s./L.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.2.6.2/05
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10, EC20 and EC50 values for Skeletonema costatum with 14C-KWG 4168 in an algal growth inhibition test
Report No:	0471836-ECO26
Document No:	M-761414-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
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 M-006512-01-1 on the effects of exposure to ^{14}C-KWG 4168 on the growth of algae (*Skeletonema costatum*) did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values as well as EC_{50} values have been calculated in accordance with the Annex to Com. Reg. 283/2013.

The resulting EC_{50} value for yield at 96 h was 1.29 µg a.s./L. The resulting EC_{50} value for growth rate at 96 h was 6.33 µg a.s./L.

I. Methods

The statistical evaluation was performed with statistical software ToxRatPro v3.3.0.

Effect concentrations with 10, 20 and 50% from the test item treatment when compared to the pooled control were calculated for yield and growth rate after 96 hours exposure but due to the steep curve EC_{10} and EC_{20} values could not be considered reliable for yield and growth rate. A Probit analysis was conducted on the yield data with confidence limits based on Fieller's theorem, while a non-linear regression 3-parameter Logistic (weighted) with confident limits estimated by Monte-Carlo simulation.

II. Results and Discussion

Yield at 96 hours

Regarding the EC_{50} calculation for yield at 96 h, a statistically significant concentration/response was found (p(F) =0.001) for this parameter.

The resulting EC_{50} value and the respective confidence interval is presented in the following table below.

Table CA 8.2.6.2/05-1 Results of the Probit analysis of yield at 96 h: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Yield
	EC_{50} (95 % confidence interval) [µg a.s./L]
Effect on yield at 96 h	1.29 (0.99 – 1.66)

The resulting EC_{50} value of 1.29 (95%CL: 0.99 – 1.66) µg a.s./L, met the goodness of fit criteria and therefore the estimated EC_{50} value is considered reliable. Reliable EC_{10} and EC_{20} values could not be determined.

Growth rate at 96 hours

Regarding the EC_{50} calculation for yield at 96 h, a statistically significant concentration/response was found (p(F) <0.001) for this parameter.

The resulting EC_{50} and the respective confidence interval is presented in the following table below.

Table CA 8.2.6.2/05-2 Results of the 3-param. Logistic analysis of growth rate at 96 h: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Growth rate
	EC_{50} (95% confidence interval) [µg a.s./L]
Effect on growth rate at 96 h	6.33 (4.51 – 8.20)

The resulting EC_{50} value of 6.33 (95%CL: 4.51 – 8.20) µg a.s./L, respectively, met the goodness of fit criteria and therefore the estimated EC_{50} value is considered reliable. Reliable EC_{10} and EC_{20} values could not be determined.

III. Conclusion

The resulting EC_{50} value for yield at 96 hours was determined to be 1.29 µg a.s./L. The resulting EC_{50} value for growth rate at 96-hours was determined to be 6.33 µg a.s./L. Reliable EC_{10} and EC_{20} values could not be determined.

Assessment and conclusion by applicant:

The statistical re-evaluation of the data could not determine reliable EC_{10} and EC_{20} values for yield and growth rate.

The E_rC_{50} determined in this re-evaluation work of 6.33 µg a.s./L is considered to be the same as the growth rate EC_{50} value of 6.3 µg a.s./L from the original study report. The E_rC_{50} of 6.3 µg a.s./L shall be taken as the critical endpoint determined from this study.

The values determined in the re-evaluation work are considered to be fully valid.

Data Point:	KCA 8.2.6.2/02
Report Author:	[redacted]
Report Year:	1997
Report Title:	Toxicity of KWG 4168 technical to the blue-green alga Anabaena flos-aquae
Report No:	107706
Document No:	M-006537-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	Yes OECD 201 (2011) Mean coefficient of variation for section-by-section specific growth rates in control cultures to be ≤35% (actual: 44.9%)
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017) Inspite of the high coefficient of variation the mean of the sectional growth rates (37.19 %,day to day) after three days the test is acceptable.
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

In a 96-hour toxicity study, triplicate cultures of *Anabaena flos-aquae* were exposed to KWG 4168 Technical at a mean measured test concentration of 0.99 mg a.s./L under static conditions.

Since there was no adverse effect on the *Anabaena flos-aquae* at the limit test concentration of 0.99 mg a.s./L, a 4-Day EC_{50} was established as >0.99 mg a.s./L.

I. Materials and Methods

A. Materials

Test Material	KWG 4168 technical	^{14}C-KWG 4168
Lot/Batch #:	09216	Vial number: C-681A
Purity:	96.3% a.s.	99.1% a.s. (specific activity 47.9 µCi/mL)
Description:	Not reported	Not reported
Stability of test compound:	Not reported	Not reported
Reanalysis/Expiry date:	Not reported	Not reported
Density:	Not reported	Not reported

Treatments

Test rates:	Nominal: 1.00 mg a.s./L Mean measured: 0.99 mg a.s./L
Solvent/vehicle:	Methanol
Analysis of test concentrations:	Yes, measured concentrations 98 – 99% of nominal

Test organisms

Species:	Blue-green alga, *Anabaena flos-aquae*
Source:	The Starr Collection, Univ. of Texas at Austin

Test design

Test vessel:	250-mL borosilicate glass culture flasks filled with approximately 100 mL of test solution and capped with sterile glass closures
Test medium:	Freshwater Nutrient Media (ASTM, 1990)
Replication:	Three replicate vessels
Initial cell density:	1 x 10^4 cells/mL
Duration of test:	96 hours
Environmental test conditions	
Temperature:	23.9 – 24.2°C
Conductivity:	91 – 97 µmhos/cm
pH:	7.5 – 9.2
Photoperiod:	Continuous lighting at ~2200 lux

B. Study Design

The objective of the study was to determine the growth effects of KWG 4168 technical to the blue-green alga, *Anabaena flos-aquae*.

Test concentrations were prepared from a stock solution and 100 mL of the solution was added to each 250 mL Erlenmeyer flask at the start of the test. The nominal test concentration was 1.0 mg a.s./L. The corresponding measured test concentration was 0.99 mg a.s./L.

The *Anabaena flos-aquae* used for this study was obtained from an in-house culture (AF-28). The algae culture was maintained in the laboratory since July 26, 1996. The culture was originally obtained from The Starr Collection Univ. of Texas at Austin. The algae used in the definitive test was taken from a three day old batch culture of *Anabaena flos-aquae* (in log phase growth). Test media were inoculated with enough 3-day old pre-culture to give a density of 1 x 10^4 cells/mL.

The exposure of *Anabaena flos-aquae* to KWG 4168 was conducted under static conditions. Test vessels were sterile, 250 mL borosilicate glass culture flasks filled with approximately 100 mL of test solution and capped with sterile glass closures. Testing was conducted in an environmental chamber. The position of the test vessels was re-randomized daily. The shaker table was operated at 100 revolutions per minute. An array of cool white fluorescent lights produced a 24-hour light photoperiod and a light intensity of approximately 2200 lux.

Each day, density was determined in three replicates at each test concentration using a light microscope and an Improved Neubauer haemocytometer. Samples of KWG 4168 test solutions, including controls, were taken on day zero and day four to measure actual exposure concentrations.

Incubation was at 23.9 to 24.2°C and under continuous light at ~2200 lux.

II. Results and Discussion

The study was conducted to an older EPA test guideline and no specific validity criteria were included in the report.

The recoveries of treated test vessels during the study was 98 to 99% of nominal. The study result has been based on the mean measured test concentration.

Table CA 8.2.6.2/02-1 Measured test concentrations based upon LSC during the exposure of *Anabaena flos-aquae*

Nominal (mg a.s./L)	Measured concentration[1] (mg a.s./L)			
	Day 0	Day 4	Mean ± SD	Percent of nominal
Control	ND	ND	-	-
Solvent control	ND	ND	-	-
1.0	0.98	0.99	0.99 ± 0.01	99
Lab recovery[2]	0.52	0.52	0.52 ± 0.00	104

ND Not detected at or above the validated limit of detection (0.1 mg/L)
[1] Measured concentration based upon liquid scintillation counting
[2] Lab recovery based upon a lab spike of 0.5 mg/L

The growth curves clearly show similar growth through Day 4. Since there was no adverse effect on the *Anabaena flos-aquae* at the limit test concentration of 0.99 mg a.s./L, a 4-day EC_{50} was established as >0.99 mg a.s./L.

Table CA 8.2.6.2/02-2 Day 4 cell growth during the exposure of *Anabaena flos-aquae* to KWG 4168 Technical

Mean measured concentration (mg a.s./L)	Rep.	Cell density (cells/mL) x 10^4		Percent of pooled control growth
		Day 4	Day 4 mean	
Control	A	134.50	137.50	100
	B	135.20		
	C	143.00		
Solvent control	A	141.00	138.11	100
	B	147.50		
	C	125.83		
Pooled control		-	137.84	
0.99	A	132.50	129.17	94
	B	124.50		
	C	130.50		

III. Conclusion

In a 96-hour toxicity study, triplicate cultures of *Anabaena flos-aquae* were exposed to KWG 4168 Technical at a mean measured test concentration of 0.99 mg a.s./L under static conditions.

Since there was no adverse effect on the *Anabaena flos-aquae* at the limit test concentration of 0.99 mg a.s./L, a 4-Day EC_{50} was established as >0.99 mg a.s./L.

Assessment and conclusion by applicant:

The study was conducted to the FIFRA Guideline 123-2 Growth and Reproduction of Aquatic Plants (Tier 2). The study has therefore been re-assessed against the validity criteria according to the current OECD 201 test guideline (2011).

The below validity criteria according to OECD 201 (2011) were met:

- Cell density of control cultures to increase by at least 16x (actual: 138)
- Coefficient of variation of average specific growth rates in control cultures over the test period to be ≤10% (actual: 1.13)

The criterion relating to section-by-section specific growth rates was not met:

- Mean coefficient of variation for section-by-section specific growth rates in control cultures to be ≤35% (actual: 44.9%)

Not all of the validity criteria have been met and the study results clearly show that this was an insensitive species to spiroxamine. The study is therefore considered to be supporting information only.

As this was a limit test, the data are not suitable for statistical re-evaluation for ECx values. The E_rC_{50} and E_yC_{50} can both be considered to be >0.99 mg a.s./L.

Data Point:	KCA 8.2.6.2/03
Report Author:	[redacted]
Report Year:	1997
Report Title:	Toxicity of 14C-KWG 4168 to the freshwater diatom Navicula pelliculosa
Report No:	107837
Document No:	M-006542-01-1
Guideline(s) followed in study:	ASTM (1990) guideline "Standard Guide for Conducting Static 96 h Toxicity Tests with Microalgae"
Deviations from current test guideline:	Yes OECD 201 (2011) Mean coefficient of variation for section-by-section specific growth rates in the control cultures to not exceed 35% (actual: 54.1%)
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

In a 96-hour toxicity study, cultures of *Navicula pelliculosa* were exposed to ^{14}C-KWG 4168 at measured test concentrations of 2.64, 4.36, 7.70, 14.35 and 23.95 a.s./L under static conditions.

The growth rate NOE_rC and E_rC_{50} values were 7.70 and 11.85 µg a.s./L, respectively. The percent inhibition of average specific growth rate in the treated algal cultures compared to the pooled controls ranged from -3.9 to 97.4% after 96 hours exposure.

I. Materials and Methods

A. Materials

Test Material	KWG 4168	^{14}C-KWG 4168
Lot/Batch #:	93-R-008-2	Vial C-681A
Purity:	96.3%	99.1%
Description:	Not reported	Not reported
Stability of test compound:	Not reported	Not reported
Reanalysis/Expiry date:	Not reported	Not reported
Density:	Not reported	Not reported

Treatments

Test rates:	Nominal:	3.2, 5.4, 9.0, 15.0 and 25.0 µg a.s./L
	Measured:	2.64, 4.36, 7.70, 14.35 and 23.95 a.s./L

Solvent/vehicle:	Methanol
Analysis of test concentrations:	Yes, mean measured concentrations 81 – 96% of nominal
Test organisms	
Species:	Freshwater diatom *Navicula pelliculosa*
Source:	In-house culture
Test design	
Test vessel:	250 mL borosilicate glass flasks containing 50 mL test solution, capped with sterile 150 mL beakers
Test medium:	Sterile freshwater media (ASTM, 1990)
Replication:	Four replicates
Initial cell density:	10,000 cells/mL
Duration of test:	96 hours
Environmental test conditions	
Temperature:	24.0 – 24.7°C
pH:	7.5 – 8.9
Photoperiod:	Continuous illumination at approx. 4300 lux

B. Study Design

This study was conducted in order to assess the effects of exposure to ^{14}C-KWG 4168 to the freshwater diatom *Navicula pelliculosa* in a 96-hour static test. Test concentrations were selected based on the results of preliminary testing.

Test concentrations were prepared using volumes of a stock solution.

Nominal test concentrations were 3.2, 5.4, 9.0, 15.0 and 25.0 µg a.s./L, with corresponding measured concentrations of 2.64, 4.36, 7.70, 14.35 and 23.95 a.s./L. A control and solvent control were also used.

The freshwater media was prepared according to ASTM (1990), and was sterilised using a 0.45 µm filter, and was adjusted to pH 7.5 with 0.1 N HCl.

Media were inoculated with 10,000 cells/mL, from a 3-day old batch culture in log phase growth. Four replicates were used for each test concentration, and three additional replicates were prepared for the 25 µg/L test concentrations to ensure an adequate volume of test solution for analytical measurements. Replicate test vessels were randomly positioned in an environmental chamber on a 100 rpm shaker table. Vessels were held under continuous illumination at approximately 4300 lux.

Test vessels were sterile, 250-mL borosilicate glass culture flasks filled with approximately 50 mL test solution and capped with sterile 150-mL beakers.

Cell density was determined daily using a light microscope and a haemocytometer.

The temperature of the test system was automatically determined hourly, and measured manually daily. Measurements of pH were taken at test start and test end in the control and 3.2, 9.0 and 25.0 µg a.s./L test concentrations.

II. Results and Discussion

The study was conducted to an older EPA test guideline and no specific validity criteria were included in the report.

The mean recoveries of ^{14}C-KWG 4168 in the test solutions after 96 hours exposure were 81 to 96% of nominal. The results of the study have been presented based on mean measured test concentrations.

Table CA 8.2.6.2/03-1 Nominal and measured concentrations of ^{14}C-KWG 4168 over the exposure period

Nominal concentration (µg a.s./L)	Measured concentration (µg a.s./L)			
	Day 0	Day 4	Mean ± SD	% of nominal
Control	<LOD	<LOD	-	-
Solvent control	<LOD	<LOD	-	-
3.2	3.11	2.17	2.64 ± 0.67	83
5.4	4.23	4.48	4.36 ± 0.13	81
9.0	8.32	7.08	7.70 ± 0.62	85
15.0	14.2	14.5	14.35 ± 0.15	96
25.0	24.2	23.7	23.95 ± 0.25	96

LOD Limit of Detection: 0.5 µg/L

Significant differences in growth were observed at the 14.35 and 23.95 µg a.s./L test concentrations compared to the pooled controls after 96 hours exposure.

Table CA 8.2.6.2/03-2 Mean algal density and growth inhibition of treated cultures

Mean measured concentration (µg a.s./L)	24-hour mean cell density (x10^4)	48-hour mean cell density (x10^4)	76-hour mean cell density (x10^4)	96-hour mean cell density (x10^4)	96-hour inhibition (%)
Control	2.78	20.26	118.94	227.81	-
Solvent control	1.96	18.73	131.56	249.56	-
Pooled controls	-	-	-	238.69	-
2.64	1.99	16.15	105.63	213.75	10.4
4.36	2.25	20.73	121.13	248.06	-3.9
7.70	2.34	12.38	98.56	225.00	5.7
14.35	1.42[a]	1.98[a]	5.41[a]	63.41*	73.4
23.95	1.00[a]	1.04[a]	1.63[a]	6.15*	97.4

[a] Means are estimates due to some replicates <LOD (1 x10^4 cells/mL)
* Statistically significantly different to the pooled controls (p<0.05)

A summary of the endpoints derived in the study is presented below:

Table CA 8.2.6.2/03-3 Summary of derived endpoints

Growth rate	
E_rC_{50} (95% CI):	11.85 µg a.s./L (10.02 to 14.03 µg a.s./L)
NOE_rC:	7.70 µg a.s./L

III. Conclusion

In a 96-hour toxicity study, cultures of *Navicula pelliculosa* were exposed to ^{14}C-KWG 4168 at measured test concentrations of 2.64, 4.36, 7.70, 14.35 and 23.95 a.s./L under static conditions.

The growth rate NOE_rC and E_rC_{50} values were 7.70 and 11.85 µg a.s./L, respectively. The percent inhibition of average specific growth rate in the treated algal cultures compared to the pooled controls ranged from -3.9 to 97.4% after 96 hours exposure.

Assessment and conclusion by applicant:

The study was conducted to the ASTM (1990) guideline "Standard Guide for Conducting Static 96-hour Toxicity Tests with Microalgae". Validity criteria have therefore been re- assessed according to the OECD 201 guideline (2011):

After 96 hours exposure, the following criteria were met:

- Biomass of control cultures to have increased exponentially by a factor of at least 16 (actual: increased by a factor of 239)
- The coefficient of variation of average specific growth rates over the whole test period between the replicate control cultures should not exceed 10% (actual: 2.84%)

After 96 hours exposure, the following criteria were not met:

- Mean coefficient of variation for section-by-section specific growth rates in the control cultures to not exceed 35% (actual: 54.1%)

Not all of the validity criteria have been met therefore the study is considered to be supporting information only.

The E_rC_{50} value was determined to be 11.85 µg a.s./L.

A supplemental statistical re-analysis report (M-280532-01-1) was submitted in order to calculate 72-hour endpoints. This has been summarised below.

Data Point:	KCA 8.2.6.2/04
Report Author:	[redacted]
Report Year:	2006
Report Title:	Non-GLP recalculation report Navicula pelliculosa growth inhibition test with 14 C - KWG 4168
Report No:	DOM 26021
Document No:	M-280532-01-1
Guideline(s) followed in study:	OECD 201 (March 23, 2006)
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

This non-GLP recalculation report was conducted to provide a 0-72 hour growth rate EC_{50} for the freshwater diatom *Navicula pelliculosa* exposed to ^{14}C-KWG 4168 over 96 hours under static conditions in the study M-006542-01-1.

The growth rate $NOEC_r$ and E_rC_{50} values were 7.70 and 11.9 µg a.s./L, respectively. The percent inhibition of average specific growth rate in the treated algal cultures compared to the pooled controls ranged from 0.5 to 91.5% after 72 hours exposure.

I. Materials and Methods

A. Materials

Refer to study [redacted], 2006 (M-006542-01-1).

B. Study Design

This non-GLP recalculation report was conducted to provide a 0-72 hour growth rate EC_{50} for the freshwater diatom *Navicula pelliculosa* exposed to ^{14}C-KWG 4168 over 96 hours under static conditions in study [redacted], 2006 (M-006542-01-1).

Recalculation was done using the program ToxRat Professional v.2.09.

II. Results and Discussion

Table CA 8.2.6.2/04-1 Summary of results after 72-hour exposure to ^{14}C-KWG 4168

Nominal concentration (µg a.s./L)	Cell number per mL after 72 h	Cell number per mL after 72 h	0-72 h average growth rate	Inhibition of average specific growth rate (%)
Pooled controls	10,000	1,056,000	1.605	-
2.64	10,000	1,211,000	1.546	3.7
4.36	10,000	1,986,000	1.597	0.5
7.70	10,000	986,000	1.528	4.8
14.35	10,000	54,000	0.386	76.0
23.95	10,000	16,000	0.136	91.5

III. Conclusion

A summary of the recalculated endpoints after 72-hour exposure is presented below:

Table CA 8.2.6.2/04-2 Summary of derived endpoints

Growth rate	
E_rC_{50} (95% CI):	11.9 µg a.s./L (8.73 to 13.6 µg a.s./L)
E_rC_{20} (95% CI):	9.44 µg a.s./L (4.68 to 11.2 µg a.s./L)
E_rC_{10} (95% CI):	8.36 µg a.s./L (3.32 to 10.4 µg a.s./L)
LOE_rC:	14.35 µg a.s./L
NOE_rC:	7.70 µg a.s./L

Assessment and conclusion by applicant:

The study is a non-GLP recalculation report for study M-006542-01-1 and is considered to be acceptable. Further statistical re-evaluation has been conducted and has been presented below.

Data Points:	KCA 8.2.6.2/06
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of LC10, EC20 and EC50 values for Navicula pelliculosa with 14C-KWG 4168 in an algal growth inhibition test
Report No:	047836-ECO31
Document No:	M-761458-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
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 M-006542-01-1 on the effects of exposure to ^{14}C-KWG 4168 on the growth of algae (*Navicula pelliculosa*) did not provide estimates of 72-h EC_{10}, EC_{20} or EC_{50} values based on yield. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013.

The resulting EC_{10}, EC_{20} and EC_{50} values for yield at 72 hours were 6.83, 7.60 and 9.32 µg a.s./L, respectively.

I. Methods

The statistical evaluation was performed with statistical software ToxRatPro v3.3.0.

Effect concentrations with 10, 20 and 50% from the test item treatment when compared to the pooled control were determined for yield after 72 hours exposure. A Probit regression was performed to determine EC_x values, with confidence limits determined according to Fieller's theorem.

II. Results and Discussion

Yield at 72 hours

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for yield at 72 h, a statistically significant concentration/response was found (p(F) <0.002) for this parameter.

The resulting EC_{10}, EC_{20} and EC_{50} values and the respective confidence intervals are represented in the following table below.

Table CA 8.2.6.2/06-1 Results of the Probit analysis of yield at 72 h: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Yield		
	EC_{10} (95 % confidence interval) [µg a.s./L]	**EC_{20} (95 % confidence interval) [µg a.s./L]**	**EC_{50} (95 % confidence interval) [µg a.s./L]**
Effect on yield at 72 h	6.83 (4.73 – 7.64)	7.60 (6.20 – 8.50)	9.32 (8.35 – 13.01)

The resulting EC_{10}, EC_{20} and EC_{50} values of 6.83 (95%CL: 4.73 – 7.64), 7.60 (95%CL: 6.20 – 8.50) and 9.32 (95%CL: 8.35 – 13.01) µg a.s./L, respectively, meet the goodness of fit criteria by showing a significant concentration/response relationship, and therefore the estimated ECx values are considered reliable.

III. Conclusion

The resulting EC_{10}, EC_{20} and EC_{50} values for yield at 72 hours were determined to be 6.83, 7.60 and 9.32 µg a.s./L, respectively.

Assessment and conclusion by applicant:

The statistical re-evaluation of the data was conducted for yield only because growth rate values have already been determined in the re-assessement report M-280532-01-1.

The E_rC_{50} determined in the re-assessement report M-280532-01-1 of 11.9 µg a.s./L shall be taken as the critical endpoint determined from this algal study.

The values determined in the re-evaluation work are considered to be fully valid.

CA 8.2.7 Effects on aquatic macrophytes

Spiroxamine is not an herbicide or plant growth regulator (PGR), nor does it have herbicidal activity therefore studies with aquatic macrophytes are not specifically required. However, studies with *Lemna* are available and have been summarised below.

Data Point:	KCA 8.2.7/01
Report Author:	[redacted]
Report Year:	1996
Report Title:	KWG 4168 - toxicity (14 days) to Lemna gibba G3
Report No:	DOM 96013
Document No:	M-006497-01-1
Guideline(s) followed in study:	FIFRA Guideline 123-2 Growth and Reproduction of Aquatic Plants (Tier 2)
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

This study was conducted in order to evaluate the toxicity of KWG 4168 to Duckweed (*Lemna gibba* G3). The test objective was to determine the 14-day EC_{25} and EC_{50} values as well as the NOAEC and the LOAEC values of KWG 4168 for the test species. EC_{25} and EC_{50} were calculated in two ways: based on the number of fronds on day 14 and based on growth rate (μ) from day 0 to day 14.

In a 14-day toxicity study, Duckweed (*Lemna gibba* G3) were exposed to KWG 4168 at mean measured test concentrations of 0.12, 0.24, 0.45, 0.87, 2.22, 6.14 and 13.1 mg a.s./L under static conditions. The 14-day NOEC and EC_{50} values based on growth rate were 0.24 and 2.65 mg a.s./L, respectively. The percent growth inhibition (frond number) in the treated culture as compared to the control ranged from 0 to 95.5%. The EC_{50} values based on frond number was 1.91 mg a.s./L.

Chlorosis was recorded on day 9, day 12 and day 14 in the concentrations from 2.22 to 13.1 mg a.s./L.

I. Materials and Methods

A. Materials

Test Material KWG 4168

Lot/Batch #: 17002/90

Purity: 95.3%

Description: Clear brown liquid

Stability of test compound: Not reported

Reanalysis/Expiry date: Certified until July 9 1996

Density: None reported

Treatments

Test rates: Nominal: 0.24, 0.48, 0.95, 1.91, 3.81, 7.62 and 15.2 mg a.s./L
Measured: 0.12, 0.24, 0.45, 0.87, 2.22, 6.14 and 13.1 mg a.s./L

Solvent/vehicle: Acetone

Test organisms

Species: Duckweed (*Lemna gibba* G3)

Source:	Dr. Janet Slovin, Horticulture Crops Quality Laboratory, U.S. Department of Agriculture, Beltsville, MD, U.S.A
Acclimatisation period:	*Lemna gibba* G3 taken from <14 day old stock culture at test initiation
Test design	
Test vessel:	400 mL glass dishes: 10 cm diameter x 6 cm height
Test medium:	Hoaglands E
Replication:	Three test vessels
No. animals/vessel:	Four plants per test vessel
Duration of test:	14 days
Environmental test conditions	
Temperature:	Test vessels incubated at 25 ± 2°C
pH:	Test initiation: 4.6 to 4.9 Test termination: 4.8 to 5.2
Photoperiod:	Continuous illumination of 4842 lux provided by overhead cool white lights

B. Study Design

Duckweed was exposed for 14 days under static conditions to mean measured concentrations of 0.12, 0.24, 0.45, 0.87, 2.22, 6.14 and 13.1 mg a.s./L. Test vessels were incubated at 25 ± 2°C and continuously illuminated at 4842 lux by overhead cool-white lights for the duration of the study. Four plants, consisting of four fronds each, were aseptically added to each test vessel using a sterile inoculum hook. Hoaglands E medium was used in the test.

Frond counts were made using a lighted magnifying lens on study days 2, 5, 7, 9, 12 and 14 (fronds visible projecting beyond the edge of the parent frond were counted) to determine growth. Samples were analysed on day 0 and 14 for the actual concentration of test substance present in the test medium at each treatment level and in the no-treatment controls. The fronds were removed from the vessels at test termination, the contents of all replicate vessels were combined and the pH was measured. All test solutions were then submitted for day-14 analysis.

Growth data were used to conduct statistical analysis; t-test to determine if controls can be pooled, chi-square test to determine the normality of the data set and Bartlett's test for homogeneity of variances. The non-parametric Kruskal-Wallis' and Dunn's Multiple Comparison test were used to determine significant differences between the control and treatment groups for data which did not fit a normal distribution.

Analytical method

Samples of water were analysed using the validated analytical method 00252 M001, report reference M-008490-02-2 (**see Doc MCA Section 4**).

II. Results and Discussion

The study was conducted to an older EPA test guideline and no specific validity criteria were included in the report.

All results of this study are based upon the mean measured test concentrations of KWG 4168.

Table CA 8.2.7/01-1 Nominal and measured concentrations of KWG 4168

Nominal concentration (mg a.s./L)	Measured concentrations in mg/L		
	Day 0 average	Day 14 average	Mean day 0 to day 14
Control	<0.025	<0.025	<0.025
Solvent control	<0.025	<0.025	<0.025
0.24	0.17	0.0642 *)	0.12
0.48	0.27	0.21	0.24
0.95	0.43	0.47	0.45
1.91	0.90	0.85	0.87
3.81	2.70	1.73	2.22
7.62	6.14	6.14	6.14
15.2	13.8	12.4	13.1

Limit of quantification for KWG 4168: 0.025 mg/L
*): Probably microbiological contamination on day 14, visible by a slight turbidity of medium.

Percent inhibition of growth relative to the solvent control was calculated for each concentration based upon the mean frond counts of each concentration on day 14. On day 14, mean frond count was 244, 240, 236, 202, 222, 114, 19 and 11 at solvent control, 0.12, 0.24, 0.45, 0.87, 2.22, 6.14 and 13.1 mg a.s./L, respectively.

Table CA 8.2.7/01-2 Percent inhibition of growth (fronds) on day 14

Mean measured concentration (mg a.s./L)	Mean frond counts on day 14	Percent inhibition on day 14
Solvent control	244	0
0.12	240	1.6
0.24	236	3.0
0.45	202	17.0
0.87	222	9.0
2.22	114	53.2
6.14	19	92.1
13.1	11	95.5

The percent inhibition of growth rate relative to the solvent control was calculated, for each concentration, based upon the growth rate of each concentration from day 0 until day 14. The percent inhibition of growth rate from day 0 until day 14 was 0.61, 1.22, 6.93, 3.47, 27.93, 93.69 and 113.75% at 0.12, 0.24, 0.45, 0.87, 2.22, 6.14 and 13.1 mg a.s./L, respectively.

Table CA 8.2.7/01-3 Growth rate inhibition of day 0 until day 14

Mean measured concentration (mg a.s./L)	Growth rate (μ)	Percent inhibition of growth rate (day 0 – day 14 (Iμ)
Solvent control	0.19	---
0.12	0.19	0.61
0.24	0.19	1.22
0.45	0.18	6.93
0.87	0.19	3.47
2.22	0.14	27.93
6.14	0.01	93.69
13.1	-0.03	113.75

The 14-day EC_{25} and EC_{50} values for frond count were 0.91 and 1.91 mg a.s./L, respectively.

Table CA 8.2.7/01-4 Summary of frond counts at day 14

Endpoint	Effect concentration (mg a.s./L)
EC_{25}	0.91
EC_{50}	1.91

The 14-day EC_{25} and EC_{50} values for growth rate were 0.47 and 2.65 mg a.s./L, respectively.

Table CA 8.2.7/01-5 Summary of growth rate µ at day 14

Endpoint	Effect concentration (mg a.s./L)
EC_{25}	1.47
EC_{50}	2.65

The resulting NOEC and LOEC values after 14-days were 0.24 and 0.5 mg a.s./L, respectively.

Table CA 8.2.7/01-6 NOEC AND LOEC values

Endpoint	Effect concentration (mg a.s./L)
NOEC	0.24
LOEC	0.45

III. Conclusion

In a 14-day toxicity study, Duckweed (*Lemna gibba* G3) were exposed to KWG 4168 at mean measured test concentrations of 0.12, 0.24, 0.45, 0.87, 2.22, 6.14 and 13.1 mg a.s./L under static conditions. The 14-day NOEC and EC_{50} values based on growth rate were 0.24 and 2.65 mg a.s./L, respectively. The percent growth inhibition (frond number) in the treated culture as compared to the control ranged from 0 to 95.5%. The EC_{50} values based on frond number was 1.91 mg a.s./L.

Chlorosis was recorded on day 9, day 12 and day 14 in the concentrations from 2.22 to 13.1 mg a.s./L.

Assessment and conclusion by applicant:

Validity criteria according to the OECD 221 (2006) guideline have been assessed as part of the non-GLP recalculation report presented in the subsequent study. This study was 14-days in duration therefore the data have been re-assessed in order to determine 7-day EC_{50} values based on growth rate and yield.

The study is considered acceptable. The EC_{50} values based on frond number was 1.91 mg a.s./L.

A supplemental statistical re-analysis report (M-303423-01-1) was submitted in order to calculate 7-day endpoints. This has been summarised below.

Data Point:	KCA 8.2.7/02
Report Author:	[redacted]
Report Year:	2008
Report Title:	Non-GLP recalculation report: KWG 4168 - toxicity (14 days) to Lemna gibba G3
Report No:	DOM 28002
Document No:	M-303421-01-1
Guideline(s) followed in study:	Originally reported under US-EPA FIFRA § 123-2, Tier 2 Non-target Aquatic Plant Toxicity Recent recalculation is based on OECD 221 Lemna sp. Growth Inhibition Test (March 23, 2006)
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The aim of this non-GLP recalculation report was to fulfil the OECD 221 requirements at least partly, which ask for the (0-7 day)-EC_{50} for growth rate of frond number. An additional EC_{50} calculation for the second endpoint (total frond area of plants or dry weight of plants) according to the new OECD 221 (2006) was not possible, because no such raw data exist for this study.

The 7-day E_rC_{50} of spiroxamine to *Lemna gibba* G3 is 6.78 mg a.s./L, based on mean measured concentrations.

I. Materials and Methods

A. Materials

Refer to M-008497-01-1 for methods of the biological test.

B. Study Design

The EC_{50} for growth rate of frond number was calculated by the applicant. Recalculation was done using the commercial program ToxRat Professional.

II. Results and Discussion

Validity criteria according to the OECD 221 (2006) guideline were met.

- The doubling time in the control must be less than 2.5 days (60 hours) corresponding to approximately a seven-fold increase in seven days and an average specific growth rate of 0.275 d^{-1} (the doubling time in this test for Day 0-7 was 2.4 days).

At day 7, the final frond counts were 88, 97, 83, 72, 75, 66, 67, 60, 41 and 17 at control, solvent control, pooled controls, 0.12, 0.24, 0.45, 0.87, 2.22, 6.14 and 13.1 mg a.s./L, respectively. From day 0 to 7, the average growth rates for frond number were 0.285, 0.265, 0.275, 0.255, 0.261, 0.243, 0.245, 0.231, 0.174 and 0.045 at control, solvent control, pooled controls, 0.12, 0.24, 0.45, 0.87, 2.22, 6.14 and 13.1 mg a.s./L, respectively. Percent inhibition of average growth rate for frond number from day 0-7 values were 7.1, 5.0, 11.8, 10.8, 16.2, 36.7 and 83.5% at 0.12, 0.24, 0.45, 0.87, 2.22, 6.14 and 13.1 mg a.s./L, respectively.

Table CA 8.2.7/02-1 Frond numbers, average growth rates and % inhibition

Mean*) measured concentration (mg a.s./L)	Final frond no. (replicate means, day 7)	Average growth rates for frond no. (day 0-7) [1/day]	% inhibition of average (day 0-7) growth rate for frond no.
Control	88	0.285	---

Mean*) measured concentration (mg a.s./L)	Final frond no. (replicate means, day 7)	Average growth rates for frond no. (day 0-7) [1/day]	% inhibition of average (day 0-7) growth rate for frond no.
Solvent control	77	0.265	---
Pooled controls	83	0.275	---
0.12	72	0.255	7.1
0.24	75	0.261	5.0
0.45	66	0.243	11.8
0.87	67	0.245	10.8
2.22	60	0.231	16.2
6.14	41	0.174	36.7
13.1	17	0.045	83.5

*): Because of missing analytical data at day 7, mean measured data (day 0-7) were calculated based on initial (day 0) and final (day 14) data from the study, which is also supported by relative stable recoveries of the test item over the elapsed time of 14 days

The resulting NOEC and LOEC values at day 7 were 0.24 and 0.45 mg a.s./L, respectively. The E_rC_{50} value was 6.78 mg a.s./L, respectively, with a corresponding 95% confidence interval of 3.57 to 12.54 mg a.s./L.

Table CA 8.2.7/02-2 Results for the endpoint frond number based on growth rate

Endpoint (0-7 day)	Effect on frond no. [mg a.s./L]
E_rC_{10} (Cl 95%)	2.06 (0.06-3.79)
E_rC_{20} (Cl 95%)	3.11 (0.28-5.03)
E_rC_{50} (Cl 95%)	6.78 (3.57-12.54)
LOEC	0.45
NOEC	0.24

III. Conclusion

The 7-day E_rC_{50} of spiroxamine to *Lemna gibba* G3 is 6.78 mg a.s./L, based on mean measured concentrations.

Assessment and conclusion by applicant:

This recalculation report was produced in order to determine a 7-day EC_{50} based on growth rate.

Validity criteria according to the OECD 221 (2006) guideline were met.

- The doubling time in the control must be less than 2.5 days (60 hours) corresponding to approximately a seven-fold increase in seven days and an average specific growth rate of 0.275 d^{-1} (the doubling time in this test for Day 0-7 was 2.4 days and the growth rate was 0.285 d^{-1}).

The validity criterion was met therefore the endpoint derived here is considered to be acceptable. Further reanalyses have been conducted in order to determine EC_{10}, EC_{20} and EC_{50} values in terms of both growth rate and yield. The results of these reanalyses have been summarised below.

Data Point:	KCA 8.2.7/05
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10, EC20 and EC50 values for Lemna gibba with KWG 4168 in a Lemna sp. growth inhibition test
Report No:	0471836-ECO35
Document No:	M-760417-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
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 M-006497-01-1 on the effects of exposure to KWG 4168 on the growth of *Lemna gibba* did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values as well as EC_{50} values have been calculated in accordance with the Annex to Com. Reg. 283/2013.

The resulting EC_{10}, EC_{20} and EC_{50} values for yield at 7 d were 0.22 (95% CL: 0.08 – 0.40), 0.62 (95% CL: 0.31 – 0.95) and 3.02 (95%CL: 2.21 – 4.06) mg a.s./L, respectively.

The resulting EC_{10}, EC_{20} and EC_{50} values for yield at 14 d were 0.56 (95%CL: 0.33 – 0.76), 0.93 (95%CL: 0.67 – 1.15) and 1.99 (95%CL: 1.71 – 2.34) mg a.s./L, respectively. For growth rate after 14 d, the EC_{10}, EC_{20} and EC_{50} values were 1.26 (95%CL: 1.05 – 1.44), 1.82 (95%CL: 1.61 – 2.01) and 3.17 (95%CL: 2.94 – 3.43) mg a.s./L, respectively.

I. Methods

The statistical evaluation was performed with statistical software ToxRatPro v3.3.0.

Effect concentrations with 10, 20 and 50% from the test item treatment when compared to the controls were determined for yield after 7 and 14 days exposure and growth rate of frond number after 14 days exposure. A Weibull regression was performed with confidence limits for the EC_x values estimated according to Fieller's theorem. EC_x calculation for total frond area of plants or dry weight of plants was not possible, because no such raw data exist for this study.

II. Results and Discussion

Yield (frond number) at 7 days

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for yield at 7 d, a statistically significant concentration/response was found ($p(F) < 0.001$) for this parameter.

The resulting EC_{10}, EC_{20} and EC_{50} values and the respective confidence intervals are represented in the following table below.

Table CA 8.2.7/05-1 Results of the Weibull analysis of yield at 7 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Yield [mg a.s./L]		
	EC_{10} (95 % confidence interval)	**EC_{20} (95 % confidence interval)**	**EC_{50} (95 % confidence interval)**
Effect on yield at 7 d	0.22 (0.08 – 0.40)	0.62 (0.31 – 0.95)	3.02 (2.21 – 4.06)

The resulting EC_{10}, EC_{20} and EC_{50} values of 0.22 (95% CL: 0.08 – 0.40), 0.62 (95% CL: 0.31 – 0.95) and 3.02 (95%CL: 2.21 – 4.06) mg a.s./L, respectively, meet the goodness of fit criteria showing a significant concentration/response relationship, and therefore the estimated ECx values are considered reliable.

Yield (frond number) at 14 days

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for yield at 14 d, a statistically significant concentration/response was found (p(F) <0.001) for this parameter.

The resulting EC_{10}, EC_{20} and EC_{50} values and the respective confidences intervals are represented in the following table below.

Table CA 8.2.7/05-2 Results of the Weibull analysis of yield at 14 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Yield [mg a.s./L]		
	EC_{10} (95 % confidence interval)	EC_{20} (95 % confidence interval)	EC_{50} (95 % confidence interval)
Effect on yield at 14 d	0.56 (0.33 – 0.76)	0.93 (0.67 – 1.15)	1.99 (1.71 – 2.34)

The resulting EC_{10}, EC_{20} and EC_{50} values of 0.56 (95% CL: 0.33 – 0.76), 0.93 (95% CL: 0.67 – 1.15) and 1.99 (95%CL: 1.71 – 2.34) mg a.s./L, respectively, meet the goodness of fit criteria showing a significant concentration/response relationship, and therefore the estimated ECx values are considered reliable.

Growth rate (frond number) at 14 days

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for growth rate at 14 d, a statistically significant concentration/response was found (p(F) <0.001) for this parameter.

The resulting EC_{10}, EC_{20} and EC_{50} values and the respective confidence intervals are represented in the following table below.

Table CA 8.2.7/05-3 Results of the Weibull analysis of growth rate at 14 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Growth rate [mg a.s./L]		
	EC_{10} (95 % confidence interval)	EC_{20} (95 % confidence interval)	EC_{50} (95 % confidence interval)
Effect on growth rate at 14 d	1.26 (1.05 – 1.44)	1.82 (1.61 – 2.01)	3.17 (2.94 – 3.43)

The resulting EC_{10}, EC_{20} and EC_{50} values of 1.26 (95%CL: 1.05 – 1.44), 1.82 (95%CL: 1.61 – 2.01) and 3.17 (95%CL: 2.94 – 3.43) mg a.s./L, respectively, meet the goodness of fit criteria showing a significant concentration/response relationship, and therefore the estimated ECx values are considered reliable.

III. Conclusion

The resulting EC_{10}, EC_{20} and EC_{50} values for yield (frond number) at 7 days were determined to be 0.22, 0.62 and 3.02 mg a.s./L. The resulting EC_{10}, EC_{20} and EC_{50} values for yield (frond number) at 14 days were determined to be 0.56, 0.93 and 1.99 mg a.s./L. The resulting EC_{10}, EC_{20} and EC_{50} values for growth rate (frond number) at 14 days were determined to be 1.26, 1.82 and 3.17 mg a.s./L.

Assessment and conclusion by applicant:

The statistical re-evaluation of the data was conducted in order to complete the data set for EC_x values for yield and growth rate. 7-day EC_x values based on growth rate have already been calculated in the 2008 re-calculation report (M-303421-01-1) therefore calculation of these values was not repeated

here. 14-day EC_x values for growth rate and 7-day and 14-day EC_x values based on yield have been determined.

The lowest EC_{50} remains the 14-day value determined in the original study report of 1,910 µg a.s./L. The 7-day E_rC_{50} value was determined to be 6,780 µg a.s./L. In order to maintain a conservative risk assessment, the original EC_{50} of 1,910 µg a.s./L shall be taken as the critical endpoint determined from this algal study.

The values determined in the re-evaluation work are considered to be fully valid.

Data Point:	KCA 8.2.7/03
Report Author:	[redacted]
Report Year:	1997
Report Title:	14C-KWG 4168- toxicity (14 days) to Lemna gibba G3
Report No:	DOM 97017
Document No:	M-006540-01-1
Guideline(s) followed in study:	FIFRA Guideline 123-2 Growth and Reproduction of Aquatic Plants (Tier 2)
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

This study was conducted in order to evaluate the toxicity of ^{14}C-KWG 4168 to Duckweed (*Lemna gibba* G3). The test objective was to determine the 14-day EC_{25} and EC_{50} values as well as the NOAEC and LOAEC values of ^{14}C-KWG 4168 for the test species.

In a 14-day toxicity study, Duckweed (*Lemna gibba* G3) were exposed to ^{14}C-KWG 4168 at mean measured test concentrations of 0.37, 0.70, 1.38, 2.73, 5.28 or 10.42 mg a.s./L under static conditions. Growth was determined by counting frond numbers on day 2, 5, 7, 9, 12 and 14 and by determination of the biomass, based on the dry weights of the whole plant on day 14. The 14-day NOEAC and EC_{50} values based on frond number were 0.70 and 2.76 mg a.s./L respectively. The 14-day NOEAC and EC_{50} values based on biomass were 2.73 and 9.38 mg a.s./L respectively.

Frond count values at concentrations ≥ 1.38 mg/L on day 14 were significantly different from the pooled controls. Chlorosis was recorded from day 5 to day 14 in the two highest test levels and also from Day 12 to Day 14 at 2.73 mg/L.

I. Materials and Methods

A. Materials

Test Material ^{14}C-KWG 4168

Lot/Batch #: 10039/3

Purity: >98%

Description: Oily liquid

Reanalysis/Expiry date: Not reported

Density: Not reported

Treatments	
Test rates:	Measured: 0.37, 0.70, 1.38, 2.73, 5.28 and 10.42 mg a.s./L Nominal: 0.3, 0.6, 1.2, 2.4, 4.8 and 9.6 mg a.s./L
Solvent/vehicle:	Acetone
Analysis of test concentrations:	Yes, mean measured concentrations represent 109-123% of nominal
Test organisms	
Species:	Duckweed (*Lemna gibba* G3)
Source:	Dr. Janet Slovin, Horticulture Crops Quality Laboratory, U.S. Department of Agriculture, Beltsville, MD, U.S.A.
Acclimatisation period:	*Lemna gibba* G3 taken from ≤14 day old stock culture at test initiation
Test design	
Test vessel:	400 mL glass dishes: 10 cm diameter x 6 cm height
Test medium:	Hoaglands E
Replication:	Three test vessels
No. animals/vessel:	Five plants of three fronds per vessel (16 fronds per vessel)
Duration of test:	14 days
Environmental test conditions	
Temperatures:	Test vessels incubated at 25 ± 2°C
pH:	Test initiation: 5.0 to 5.3; Test termination: 5.2 to 5.7
Photoperiod:	Continuous illumination of 5122 lux provided by overhead cool white lights

B. Study Design

Duckweed was exposed for 14 days under static conditions to mean measured concentrations of 0.37, 0.70, 1.38, 2.73, 5.28 and 10.42 mg a.s./L. Test vessels were incubated at 25 ± 2°C and continuously illuminated at 5122 lux by overhead cool-white lights for the duration of the study. Five plants, consisting of three fronds each, were aseptically added to each test vessel using a sterile inoculum hook. Hoaglands E medium was used in the test.

Frond counts were made using a lighted magnifying lens on study days 2, 5, 7, 9, 12 and 14 (fronds visible projecting beyond the edge of the parent frond were counted) to determine growth.

Growth data expressed as frond counts and biomass (based on dry weights of plants) on day 14 were used to conduct statistical analysis; chi-square test to determine the normality of the data set and Bartlett's test for homogeneity of variances. The non-parametric Kruskal-Wallis' and Dunn's Multiple Comparison test were used to determine significant differences between the control and treatment groups for data which did not fit a normal distribution. In case of frond number, statistical analyses were performed in comparison to the pooled controls (solvent control and pure medium control). In case of biomass, statistical analyses were compared to the solvent control.

II. Results and Discussion

The study was conducted to an older EPA test guideline and no specific validity criteria were included in the report.

The measured concentrations determined during the test are summarised in the table below.

Table CA 8.2.7/03-1 Measured concentrations

Nominal concentration (mg a.s./L)	Measured concentration (mg a.s./L)		Mean measured concentration (mg a.s./L)	% of nominal
	Day 0 [a]	Day 14 [a]		
Control	<0.03*	<0.03*	<0.03*	-
Solvent control	<0.03*	<0.03*	<0.03*	-
0.3	0.37	0.37	0.37	123
0.6	0.69	0.70	0.70	117
1.2	1.37	1.38	1.38	115
2.4	2.70	2.75	2.73	114
4.8	5.15	5.41	5.28	110
9.6	10.18	10.66	10.42	109

*Limit of Detection (LOD) based on the threshold value of LSC measurement
[a] based on mean of 3 replicates

All results of this study are based upon the mean measured test concentrations of KWG 4168.

The percent inhibition of growth relative to the solvent control was calculated for each concentration based upon the mean frond counts and the mean dry weight (in mg) on day 14. On day 14, mean frond count was 278, 273, 234, 167, 16 and 10 at 0.37, 0.70, 1.38, 2.73, 5.28 and 10.42 mg a.s./L, respectively. On day 14, mean dry weight was 45.9, 46.5, 51.0, 43.5, 27.4 and 19.6 mg at 0.37, 0.70, 1.38, 2.73, 5.28 and 10.42 mg a.s./L, respectively.

Table CA 8.2.7/03-2 Percent inhibition of growth on day 14

Mean measured concentrations in mg/L	Mean frond counts on day 14	Percent inhibition (frond number) on day 14	Mean dry weight in mg on day 14	Percent inhibition (dry weight) on day 14
Pooled controls	277	---	---	---
Solvent control	---	---	39.4	---
0.37	278	-0.3→0*)	45.9	-17→0*)
0.70	273	1	46.5	-18→0*)
1.38	234	16	51.0	-29→0*)
2.73	167	40	43.5	-10→0*)
5.28	16	94	27.4	30
10.42	10	96	19.6	50

*): Negative values for percent inhibition indicates stimulation. Stimulation of growth as observed very often near the effect threshold is going unnoticed. Therefore such values are settled as zero before further calculations.

The 14-day EC_{25} and EC_{50} values for frond count were 1.82 and 2.76 mg a.s./L, respectively. The NOEC and LOEC values for frond count were 0.70 and 1.38 mg a.s./L, respectively. The 14-day EC_{25} and EC_{50} values for biomass were 5.92 and 9.38 mg a.s./L, respectively, the NOEC and LOEC values for biomass were 2.73 and 5.28 mg a.s./L, respectively.

Table CA 8.2.7/03-3 Summary of endpoints

Endpoint	Effect concentration (mg a.s./L)	
	Frond number	Biomass
EC_{25}	1.82	5.92
EC_{50}	2.76	9.38
NOEC	0.70	2.73
LOEC	1.38	5.28

III. Conclusion

In a 14-day toxicity study, Duckweed (*Lemna gibba* G3) were exposed to ^{14}C-KWG 4168 at mean measured test concentrations of 0.37, 0.70, 1.38, 2.73, 5.28 or 10.42 mg a.s./L under static conditions. Growth was determined by counting frond numbers on day 2, 5, 7, 9, 12 and 14 and by determination of the biomass, based on the dry weights of the whole plant on day 14. The 14-day NOEAC and EC_{50} values based on frond number were 0.70 and 2.76 mg a.s./L respectively. The 14-day NOEAC and EC_{50} values based on biomass were 2.73 and 9.38 mg a.s./L respectively.

Assessment and conclusion by applicant:

Validity criteria according to the OECD 221 (2006) guideline have been assessed as part of the non-GLP recalculation report presented in the subsequent study. This study was 14-days in duration therefore the data have been re-assessed in order to determine 7-day EC_{50} values based on growth rate and yield.

The study is considered acceptable. The 14-day EC_{50} value based on frond number was 2.76 mg a.s./L.

A supplemental statistical re-analysis report (M-303443-01-1) was submitted in order to calculate 7-day endpoints, where possible. This has been summarised below.

Data Point:	KCA 8.2.7/04
Report Author:	[redacted]
Report Year:	2008
Report Title:	Non-GLP recalculation report: 14C-KWG 4168- toxicity (14 days) to Lemna gibba G3
Report No:	DOM 28003
Document No:	M-303443-01-1
Guideline(s) followed in study:	Originally reported under US-EPA FIFRA § 123-2, Tier 2 Non-target Aquatic Plant Toxicity Recent recalculation is based on OECD 221 Lemna sp. Growth Inhibition Test (March 23, 2006)
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The aim of this non-GLP recalculation report was to fulfil the OECD 221 requirements, which ask for the (0-7 day) EC_{50} for growth rate of frond number and an additional EC_{50} growth rate calculation for the second endpoint (dry weight of plants), which was done for the total elapsed test period of 14 days.

Based on mean measured concentrations, the 7-day E_rC_{50} (frond number) of spiroxamine to *Lemna gibba* G3 is 5.60 mg a.s./L and the 14-day E_rC_{50} (dry weight) is 21.2 mg a.s./L.

I. Materials and Methods

A. Materials

Refer to M-006540-01-1 for methods of the biological test.

B. Study Design

The EC_{50} for growth rate of frond number and dry weight was calculated by the applicant. Recalculation was done using the commercial program ToxRat Professional.

II. Results and Discussion

Validity criteria according to the OECD 221 (2006) guideline were met.

- The doubling time in the control must be less than 2.5 days (60 hours) corresponding to approximately a seven-fold increase in seven days and an average specific growth rate of 0.275 d^{-1} (the doubling time in this test for Day 0-7 was 2.5 days).

At day 7, the percent inhibition of average growth rate for frond number for each concentration was -1.6, 0.1, -1.6, 7.8, 42.1 and 98.8% at 0.37, 0.70, 1.38, 2.73, 5.28 and 10.42 mg a.s./L, respectively.

Table CA 8.2.7/04-1 First endpoint: Frond numbers, average growth rates and % inhibition

Mean*) measured concentration [mg a.s./L]	Final frond no. (replicate means, day 7)	Average growth rates for frond no. (day 0-7) [1/day]	% inhibition of average (day 0-7) growth rate for frond no.**)
Control	84	0.278	---
Solvent control	82	0.273	---
Pooled controls	83	0.276	---
0.37	85	0.280	-1.6
0.70	83	0.275	0.1
1.38	85	0.280	-1.6
2.73	71	0.254	7.8

Mean*) measured concentration [mg a.s./L]	Final frond no. (replicate means, day 7)	Average growth rates for frond no. (day 0-7) [1/day]	% inhibition of average (day 0-7) growth rate for frond no.**)
5.28	37	0.160	42.1
10.42	12	0.003	98.8

*): Because of missing analytical data at day 7, mean measured data (day 0-7) were calculated based on initial (day 0) and final (day 14) data from the study, which is also supported by relative stable recoveries of the test item over the elapsed time of 14 days.
**): Negative values mean stimulation of growth relative to the controls

At day 7, the percent inhibition of average growth rate for dry weight of plants for each concentration was -1.3, -1.3, -4.9, 0.4, 15.2 and 25.9% at 0.37, 0.70, 1.38, 2.73, 5.28 and 10.42 mg a.s./L, respectively.

Table CA 8.2.7/04-2 Second endpoint: Dry weight of plants, average growth rates and % inhibition

Mean measured (day 0-14) concentration [mg a.s./L]	Final dry weight of plants (replicate means, day 14)	Average growth rates for dry weight of plants (day 0-14) [1/day]	% inhibition of average (day 0-14) growth rate for dry weight of plants*)
Control	50	0.232	---
Solvent control	39	0.216	---
Pooled controls	45	0.224	---
0.37	46	0.227	-1.3
0.70	47	0.227	-1.3
1.38	51	0.235	-4.9
2.73	44	0.223	0.4
5.28	27	0.190	15.2
10.42	20	0.166	25.9

*): Negative values mean stimulation of growth relative to the controls

The 7-day E_rC_{50} value for average growth rates for frond number was 5.60 mg a.s./L. The NOEC and LOEC values were 1.38 and 2.73 mg a.s./L, respectively.

Table CA 8.2.7/04-3 Results for the first endpoint (average growth rates for frond number)

Endpoint (0-7 day)	Effect on frond no. [mg a.s./L]
E_rC_{10} (CI 95%)	3.51 (2.44-4.08)
E_rC_{20} (CI 95%)	4.13 (3.23-4.60)
E_rC_{50} (CI 95%)	5.60 (5.15-6.19)
LOEC	2.73
NOEC	1.38

The 14-day E_rC_{50} value for average growth rates for dry weight of plants was 21.2 mg a.s./L. The NOEC and LOEC values were 2.73 and 5.28 mg a.s./L, respectively.

Table CA 8.2.7/04-4 Results for the second endpoint (average growth rates for dry weight of plants)

Endpoint (0-14 day)	Effect on dry weights of plants [mg a.s./L]
E_rC_{10} (CI 95%)	4.76 (2.85-6.03)
E_rC_{20} (CI 95%)	7.96 (6.36-10.0)
E_rC_{50} (CI 95%)	21.2 (14.9-51.8)
LOEC	5.28
NOEC	2.73

III. Conclusion

Based on mean measured concentrations, the 7-day E_rC_{50} (frond number) of spiroxamine to *Lemna gibba* G3 is 5.60 mg a.s./L and the 14-day E_rC_{50} (dry weight) is 21.2 mg a.s./L.

Assessment and conclusion by applicant:

This recalculation report was produced in order to determine a 7-day EC_{50} for frond number based on growth rate.

Validity criteria according to the OECD 221 (2006) guideline were met.

- The doubling time in the control must be less than 2.5 days (60 hours) corresponding to approximately a seven-fold increase in seven days and an average specific growth rate of 0.275 d^{-1} (the doubling time in this test for Day 0-7 was 2.5 days and the growth rate was 0.278 d^{-1}).

The validity criterion was met therefore the endpoints derived here are considered to be acceptable. Further reanalyses have been conducted in order to determine EC_{10}, EC_{20} and EC_{50} values in terms of both growth rate and yield. The results of these reanalyses have been summarised below.

Data Point:	KCA 8.2.7/06
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10, EC20 and EC50 values for Lemna gibba with 14C-KWG 4168 in a Lemna sp. growth inhibition test
Report No:	0471836-ECO36
Document No:	M-760416-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
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 M-006540-01-1 on the effects of exposure to ^{14}C-KWG 4168 on the growth of *Lemna gibba* did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values as well as EC_{50} values have been calculated in accordance with the Annex to Com. Reg. 283/2013.

The resulting EC_{10}, EC_{20} and EC_{50} values for yield at 7 d were 2.34, 2.86 and 4.23 mg a.s./L, respectively.

The resulting EC_{10}, EC_{20} and EC_{50} values for yield at 14 d were 1.29, 1.77 and 2.86 mg a.s./L, respectively. For growth rate after 14 d, the EC_{10}, EC_{20} and EC_{50} values were 2.53, 2.79 and 3.67 mg a.s./L respectively.

I. Methods

The statistical evaluation was performed with statistical software ToxRatPro v3.3.0.

Effect concentrations with 10, 20 and 50% from the test item treatment when compared to the control were determined for yield after 7 and 14 days exposure, and growth rate after 14 days. A Probit regression was performed, with confidence limits for the EC_x values estimated according to Fieller's theorem for growth rate at 7 and 14 days, while a Weibull regression was performed, with confidence limits for the EC_x values estimated according to Fieller's theorem, for yield at 14 days.

II. Results and Discussion

Yield (frond number) at 7 days

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for yield at 7 d, a statistically significant concentration/response was found (p(F) <0.001) for this parameter.

The resulting EC_{10}, EC_{20} and EC_{50} values and the respective confidence intervals are represented in the following table below.

Table CA 8.2.7/06-1 Results of the Probit analysis of yield at 7 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Yield [mg a.s./L]		
	EC_{10} (95 % confidence interval)	**EC_{20} (95 % confidence interval)**	**EC_{50} (95 % confidence interval)**
Effect on yield at 7 d	2.34 (2.02 – 2.60)	2.86 (2.57 – 3.11)	4.23 (3.98 – 4.49)

The resulting EC_{10}, EC_{20} and EC_{50} values of 2.34 (95% CL: 2.02 – 2.60), 2.86 (95% CL: 2.57 – 3.11) and 4.23 (95%CL: 3.98 – 4.49) mg a.s./L, respectively, meet the goodness of fit criteria showing a significant concentration/response relationship, and therefore the estimated ECx values are considered reliable.

Yield (frond number) at 14 days

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for yield at 14 d, a statistically significant concentration/response was found (p(F) <0.001) for this parameter.

The resulting EC_{10}, EC_{20} and EC_{50} values and the respective confidence intervals are represented in the following table.

Table CA 8.2.7/06-2 Results of the Weibull analysis of yield at 14 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Yield [mg a.s./L]		
	EC_{10} (95 % confidence interval)	**EC_{20} (95 % confidence interval)**	**EC_{50} (95 % confidence interval)**
Effect on yield at 14 d	1.29 (0.91 – 1.57)	1.77 (1.41 – 2.03)	2.86 (2.59 – 3.17)

The resulting EC_{10}, EC_{20} and EC_{50} values of 1.29 (95% CL: 0.91 – 1.57), 1.77 (95% CL: 1.41 – 2.03) and 2.86 (95%CL: 2.59 – 3.17) mg a.s./L, respectively, meet the goodness of fit criteria showing a significant concentration/response relationship, and therefore the estimated ECx values are considered reliable.

Growth rate (frond number) at 14 days

Regarding the calculation of EC_{10}, EC_{20} and EC_{50} values for growth rate at 14 d, a statistically significant concentration/response was found (p(F) <0.001) for this parameter.

The resulting EC_{10}, EC_{20} and EC_{50} values and the respective confidence intervals are represented in the following table.

Table CA 8.2.7/06-3 Results of the Probit analysis of growth rate at 14 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits

Parameter	Growth rate [mg a.s./L]		
	EC_{10} (95 % confidence interval)	EC_{20} (95 % confidence interval)	EC_{50} (95 % confidence interval)
Effect on growth rate at 14 d	2.53 (2.39 – 2.64)	2.79 (2.69 – 2.90)	3.67 (3.20 – 3.67)

The resulting EC_{10}, EC_{20} and EC_{50} values of 2.53 (95% CL: 2.39 – 2.64), 2.79 (95% CL: 2.69 – 2.90) and 3.67 (95%CL: 3.20 – 3.67) mg a.s./L, respectively, meet the goodness of fit criteria showing a significant concentration/response relationship, and therefore the estimated ECx values are considered reliable.

III. Conclusion

The resulting EC_{10}, EC_{20} and EC_{50} values for yield (frond number) at 7 days were determined to be 2.34, 2.86 and 4.23 mg a.s./L. The resulting EC_{10}, EC_{20} and EC_{50} values for yield (frond number) at 14 days were determined to be 1.29, 1.77 and 2.86 mg a.s./L. The resulting EC_{10}, EC_{20} and EC_{50} values for growth rate (frond number) at 14 days were determined to be 2.53, 2.79 and 3.67 mg a.s./L.

Assessment and conclusion by applicant:

The statistical re-evaluation of the data was conducted in order to complete the data set for EC_x values for yield and growth rate. 7-day EC_x values based on growth rate (frond number) and 14-day EC_x values based on growth rate (biomass) have already been calculated in the 2008 re-calculation report (M-303443-01-1) therefore calculation of these values was not repeated here.

The lowest E_rC_{50} determined was 3,670 µg a.s./L which is based on frond number after 14-days. The 7-day E_rC_{50} value based on frond number was determined to be 5,600 µg a.s./L. In order to maintain a conservative risk assessment, the 14-day E_rC_{50} of 3,670 µg a.s./L shall be taken as the critical endpoint determined from this algal study.

The values determined in the re-evaluation work are considered to be fully valid.

CA 8.2.8 Further testing on aquatic organisms

No further data with spiroxamine technical are available. The studies presented above are considered sufficient to address the data requirements therefore no additional studies are considered to be necessary.

Relevant literature on aquatic organisms

No relevant scientifically peer-reviewed open literature could be found on spiroxamine or its major metabolites, from an ecotoxicological perspective, on aquatic organisms. Details of the literature search undertaken can be found in the M-CA Section 9 of the current submission.

CA 8.3 Effect on arthropods

CA 8.3.1 Effects on bees

Studies have been conducted using spiroxamine technical which have been submitted and summarised here. Other studies are also available with the representative formulations which have been presented and summarised in M-CP Section 10.

The endpoints are summarised below.

Table CA 8.3.1 Summary of bee toxicity studies with spiroxamine

Organism	Test item	Test type	Endpoints		Reference
Adult honey bee (*Apis mellifera*)	Spiroxamine	Acute oral	48 h LD_{50} >100 µg a.s./bee	EU	M-008208-01-1

Organism	Test item	Test type	Endpoints		Reference
Adult bumble bee (*Bombus terrestris*)	Spiroxamine	Acute oral	48 h LD_{50} >50.9 µg a.s./bumblebee	NEW	M-688128-01-1
Adult honey bee (*Apis mellifera*)	Spiroxamine	Acute contact	48 h LD_{50} 4.2 µg a.s./bee	EU	M-008208-01-1
Adult bumble bee (*Bombus terrestris*)	Spiroxamine	Acute contact	48 h LD_{50} >100 µg a.s./bumblebee	NEW	M-510841-01-1
Honey bee larva (*Apis mellifera*)	Spiroxamine	Chronic larva (22 day emergence)	LD_{50} >33 µg a.s./larva NOED 33 µg a.s./larva	NEW	M-623462-01-1

EU: previously evaluated as part of the original EU review and listed in EFSA conclusion and DAR
NEW: new study or data generated since the previous EU review or previously not submitted

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:	1994
Report Title:	KWG 4168 - Acute toxicity to honey bees (Apis mellifera)
Report No:	BAY169(A)932259
Document No:	M-008208-01-1
Guideline(s) followed in study:	EPPO 170
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Honey bees (*Apis mellifera*) were exposed to KWG 4168 in a 48-hour acute oral and contact toxicity test.

In the oral toxicity study, KWG 4168 was administered in the feeding solution at an application rate of 100 µg a.s./bee. An untreated control group and a solvent control group was also tested. The oral LD_{50} value, with 95% confidence, was >100 µg a.s./bee.

In the contact toxicity study, KWG 4168 was administered to bees at concentrations of 0.625, 1.25, 2.5, 5.0 and 10 µg a.s./bee. The LD_{50} value after 48-hour contact with KWG 4168 was 4.2 µg a.s./bee, with 95% confidence limits of 3.2 to 5.4 µg a.s./bee.

The cumulative mortality for oral administration after 48 hours was 10 bees out of the original population of 60 bees. The total out of the control group was one bee out of the original 30 bees.

The cumulative mortality for contact administration after 48 hours was 26 bees out of the original population of 30 bees when exposed to maximum 10 µg a.s./bee of KWG 4168. The total out of the control group was one bee out of the original 30 bees.

I. Materials and Methods

A. Materials

Test Material	KWG 4168
Lot/Batch #:	PT 898114002
Purity:	97.8%
Description:	Pale yellow liquid
Stability of test compound:	Not reported
Reanalysis/Expiry date:	27 Jan 1994
Density:	Not reported
Treatments	
Test rates:	Contact: 0.625, 1.25, 2.5, 5.0 and 10 µg a.s./bee Oral: 100 µg a.s./bee
Solvent/vehicle:	Acetone
Analysis of test concentrations:	No
Test organisms	
Species:	Honey bees, *Apis mellifera*
Source:	Mr. R. Baker, 19 Abbotts Crescent, St Ives, Cambridgeshire, UK.
Acclimatisation period:	None. Bees were dosed 3 hours after removal from hive
Feeding:	Bees were not fed prior to exposure. For oral administration, the bees were fed 20% sucrose solution after exposure. No difference in consumption rate was noted to the test item.
Treatment for disease:	None reported
Test design	
Test vessel:	Cylindrical wire mesh cages 11.5 cm long x 4.0 cm in diameter.
Replication:	Oral: 6 replicates (10 bees per replicate) for test item, 3 replicates of 10 bees for both control and solvent control. Contact: 3 replicates of 10 bees for control, solvent control and the test item concentrations.
No. animals/vessel:	10
Duration of test:	48 hours
Environmental test conditions	
Temperature:	24 - 25°C

Photoperiod: Darkness, except for during procedures where subdued lighting was used.

B. Study Design

This study was conducted in order to assess the acute toxicity of KWG 4168 on honey bees (*Apis mellifera*) in oral and contact tests over 48 hours.

Sterile female honey bees (*Apis mellifera*) were collected from hives and dosed within 3 hours.

Ten bees were placed in each cylindrical wire mesh cages 11.5 cm long x 4.0 cm in diameter. For the contact administration the bees were anaesthetised with CO_2 one vessel at a time. A 1.0 µL droplet of the diluted test item was applied to the ventral thorax of each bee using a micrometer syringe.

For the oral administration a 50 µl aliquot of the stock solution was added to 950 µL of 20% sucrose solution. 0.2 mL of this solution was administered per test vessel. It was assumed that the bees in the test vessel received similar doses of 20 µL/bee of the test item.

For the oral test the test item was administered in the feeding solution at an application rate of 100 µg a.s./bee. Control concentrations containing 20% sucrose solution only were administered, as were solvent control concentrations of 1.0 µL acetone/bee.

Following oral administration of the test item the bees were fed a 20% sucrose solution and no deviations between feeding patterns of this solution and the test item solution were noted.

For the contact test the concentrations of KWG 4168 applied were 0.625, 1.25, 2.5, 5.0 and 10 µg a.s./bee to the ventral thorax at an application rate of 1.0 µL droplets (test substance dispersed in acetone). Toxic standard concentrations of 0.025, 0.050, 0.10, 0.20 and 0.40 µg a.s./bee of dimethoate were applied as were solvent control concentrations of 1.0 µL acetone/bee.

The test vessels were kept in darkness (except for during procedures) and at 24-25°C with a relative humidity of 61-66%.

Observations on mortality were made at 24 and 48 hours and were defined as the absence of response to physical stimulation.

II. Results and Discussion

Specific assessment of guideline validity criteria were not reported in the study report.

Cumulative mortality data are given below for the oral and contact routes of administration. All results are expressed in terms of the nominal concentration.

Table CA 8.3.1.1.1/01-1 Cumulative mortality data for honey bees exposed to KWG 4168 for 48 hours (oral administration)

Nominal concentration (µg a.s./bee)	Cumulative mortality by replicate (%)													
	24 hours							48 hours						
	1	2	3	4	5	6	Total	1	2	3	4	5	6	Total
Control	0	0	0	-	-	-	0	0	10	0	-	-	-	3.3
Solvent control	0	10	0	-	-	-	3.3	10	10	0	-	-	-	6.7
100	20	0	20	20	10	10	13.3	20	10	20	20	20	10	16.7

Initial population: 10 per replicate

Table CA 8.3.1.1.1/01-2 Cumulative mortality data for honey bees exposed to KWG 4168 for 48 hours (contact administration)

Nominal concentration (µg a.s./bee)	Cumulative mortality (%)							
	24 hours				48 hours			
	1	2	3	Total	1	2	3	Total
Control	0	0	0	0	0	10	0	3.3
Solvent control	0	0	0	0	10	0	0	3.3

Nominal concentration (µg a.s./bee)	Cumulative mortality (%)							
	24 hours				48 hours			
	1	2	3	Total	1	2	3	Total
0.625	0	10	0	3.3	0	10	0	3.3
1.25	20	10	0	10.0	20	10	0	10.0
2.5	40	20	60	40.0	40	30	60	43.3
5.0	40	30	40	36.7	50	50	40	46.7
10	80	70	60	70.0	90	90	80	86.7

Initial population: 10 per replicate

No marked reactions to exposure (other than death) were noted in any of the control or test animals throughout the duration of the study.

The 24-hour oral and contact LD_{50} values for honey bees after exposure to KWG 4168 were >100 µg a.s./bee and 5.5 µg a.s./bee, respectively.

The 48-hour oral and contact LD_{50} values for honey bees after exposure to KWG 4168 were >100 µg a.s./bee and 4.2 µg a.s./bee, respectively.

The 48-hour LD_{50} values with 95% confidence limits for the reference substance, dimethoate, were 0.11 µg/bee (0.085 – 0.13 µg/bee) for the oral test and 0.12 µg/bee (0.094 – 0.14 µg/bee) for the contact test, respectively.

III. Conclusion

Honey bees (*Apis mellifera*) were exposed to KWG 4168 in a 48-hour acute oral and contact toxicity test.

The 48-hour oral and contact LD_{50} values for honey bees after exposure to KWG 4168 were >100 µg a.s./bee and 4.2 µg a.s./bee, respectively.

The 24-hour LD_{50} values with 95% confidence limits for the reference substance, dimethoate, were 0.12 µg/bee (0.095 – 0.14 µg/bee) for the oral test and 0.14 µg/bee (0.11 – 0.17 µg/bee) for the contact test, respectively. The 48-hour LD_{50} values with 95% confidence limits for the reference substance, dimethoate, were 0.11 µg/bee (0.085 – 0.13 µg/bee) for the oral test and 0.12 µg/bee (0.094 – 0.14 µg/bee) for the contact test, respectively.

Assessment and conclusion by applicant:

Validity criteria according to the current OECD 213 (1998) and OECD 214 (1998) test guidelines has been assessed.

- Average mortality for the total number of controls must not exceed 10% at the end of the test (actual: max. 6.7% in the oral test and 3.3% in the contact test, respectively)
- The 24-hour LD_{50} of the toxic standards to be 0.10 to 0.35 µg/bee for oral toxicity and 0.10 to 0.30 µg/bee for contact toxicity (actual: 24-hour LD_{50} 0.12 µg/bee and 0.14 µg/bee in the oral and contact tests, respectively)

Validity criteria were met therefore the study is considered acceptable.

The 48-hour oral and contact LD_{50} values for honey bees after exposure to KWG 4168 were >100 µg a.s./bee and 4.2 µg a.s./bee, respectively.

Data Point:	KCA 8.3.1.1.1/02
Report Author:	[redacted]
Report Year:	2020
Report Title:	Spiroxamine tech.: Effects (acute oral) on bumblebees (Bombus terrestris L.) in the laboratory
Report No:	143051105
Document No:	M-688128-01-1
Guideline(s) followed in study:	Regulation (EC) No. 1107/2009 OECD (2017), Test No. 247: Bumblebees, Acute Oral Toxicity Test, OECD Guidelines for the Testing of Chemicals
Deviations from current test guideline:	Yes OECD 247 guideline (2017) The stock solution was not analysed due to human error. This deviation did not have any detrimental impact on 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

Bumblebees (*Bombus terrestris* L) were exposed to spiroxamine technical in a 48-hour oral toxicity study. The purpose of the study was to determine the effects of spiroxamine technical on the behaviour and survival of the test organisms.

Bumblebees were exposed to spiroxamine technical at concentrations of 50.9, 33.2, 19.9, 11.4 and 5.8 µg a.s./bumblebee (nominally 100, 50, 25, 12.5 and 6.25 µg a.s./bumblebee, respectively), a water control and solvent control and dimethoate as a reference item.

The NOED and LOED values were ≥50.9 µg a.s./bumblebee, respectively and the LD_{50} value was >50.9 µg a.s./bumblebee.

I. Materials and Methods

A. Materials

Test Material Spiroxamine technical

Lot/Batch #: AE 1344293-01-07

Purity: 97.0% w/w

Description: Light-yellow liquid

Reanalysis/Expiry date: 04 June 2021

Density: Not reported

Treatments

Test rates: Nominal: 6.25, 12.5, 25, 50 and 100 µg a.s./bumblebee
Measured: 5.8, 11.4, 19.9, 33.2 and 50.9 µg a.s./bumblebee

Solvent/vehicle: Tween80

Analysis of test concentrations: Yes, 93 – 97% of the nominal

Test organisms	
Species:	Bumblebees, *Bombus terrestris* L. (Insecta, Hymenoptera)
Source:	Koppert Deutschland GmbH, D-47638 Straelen
Acclimatisation period:	45.5 hours
Feeding:	50% w/v sucrose solution *ad libitum*
Test design	
Test vessel:	Cylindrical, latticed plastic cages (Nicot queen cages) with a length of approx. 7.3 cm and a diameter of 2.2 cm at the large and 1.7 cm at the small opening.
Replication:	30 per treatment goup/control
No. animals/vessel:	Individually housed
Duration of test:	48 hours
Environmental test conditions	
Temperature:	25 ± 2°C
Photoperiod:	Darkness (except during treatment procedures and observation)

B. Study Design

Bumblebees were exposed to spiroxamine technical in an acute oral test over 48 hours. The design of the study was based on OECD 247 (2017) and SANCO/3029/99. The test organisms were adult female worker *Bombus terrestris* L.

Acute oral toxicity of spiroxamine technical to adult bumblebees was assessed by exposing 30 worker bumblebees to nominal concentrations of 6.25, 12.5, 25, 50 and 100 µg a.s./bumblebee.

The test units consisted of cylindrical, latticed plastic cages with a length of 7.3 cm and a diameter of 2.2 cm at the large and 1.7 cm at the small opening; the test organisms were kept in the test units throughout the exposure period. Temperature and relative humidity were kept at 25.0 to 25.4 ºC and 58.7 to 62.6%, respectively during exposure. The bees were kept in darkness except for during observation. All test conditions were measured with suitable instruments.

The test item was applied to the bees as a dilution series of spiroxamine tech. in acetone and was transferred to 50 % w/v sucrose solution containing 1 % v/v Tween80. The final treated feeding solutions contained 50 % w/v sucrose, maximum 5 % acetone w/w and 1 % v/v Tween80. For the solvent control, 50 % w/v sucrose solution containing 5 % w/w acetone and 1 % v/v Tween80 was used. For the water control, 50 % w/v sucrose solution was used. For the reference treatment, dimethoate was diluted in 50 % w/v sucrose solution.

Approximately 40 µL food solution per bumblebee was provided in syringes which were weighed before and after introduction into the cages in order to determine the exact consumption.

The bees were observed after 4 (± 0.5 hours), 24 and 48 (± 2 hours) hours for mortality and behavioural abnormalities. Sub-lethal affects were defined by the categories of moribund (unable to walk, weak response to stimulus) and affected (reduced coordination).

Statistical analysis was performed on the data using ToxRat Professional, Version 3.2.1, ToxRat Solutions GmbH.

Analytical method

Samples of feeding diet were analysed using the validated analytical method M-688128-01-1, report reference M-688128-01-1 (**see Doc MCA Section 4**).

II. Results and Discussion

Validity criteria according to the OECD 247 guideline (2017) were met.

- Mortality in the water control should be ≤10% at the end of the test. If included, also solvent control mortality should be ≤10% at the end of the test (actual: 0% in both the water and the solvent controls)
- Mortality in the toxic reference substance group should be ≥50% at the end of the test (actual: 100%)

Analytical verification of the feeding solutions at the highest and lowest test concentrations (nominally 100 and 6.25 µg a.s./bumblebee) confirmed the correct dosing of the bees with analytical recoveries of 97% and 93%, respectively.

At test termination, no mortality was observed for the treated bumblebees at any test concentration applied. Similarly, no mortality was observed in either the water or solvent control. There were no behavioural abnormalities observed at any test concentration or in either of the controls.

For the reference item, at test termination there was 100% mortality in the organisms tested.

Table CA 8.3.1.1.1/02-1 Summary of mortality and behavioural data observed

Treatment group (µg a.s./bumblebee)	4 hours		24 hours		48 hours	
	Mortality (% mean)	Abnormal behaviour (% mean)	Mortality (% mean)	Abnormal behaviour (% mean)	Mortality (% mean)	Abnormal behaviour (% mean)
Water control	0	0	0	0	0	0
Solvent control	0	0	0	0	0	0
Spiroxamine technical: 5.8	0	0	0	0	0	0
Spiroxamine technical: 11.4	0	0	0	0	0	0
Spiroxamine technical: 19.9	0	0	0	0	0	0
Spiroxamine technical: 33.2	0	0	0	0	0	0
Spiroxamine technical: 50.9	0	0	0	0	0	0
Reference item: 4.5	0	96.4	100	-	100	-

In the oral test the target dose levels of 6.25, 12.5, 25, 50 and 100 µg a.s./bumblebee would have been achieved if exactly 40 mg treated feeding solution were consumed by each exposed bumblebee. The mean food uptake was calculated considering all replicates per treatment group. However, actual food uptake in the treatment groups ranged between 5 and 51 mg per bumblebee. Bumblebees which did not consume at least 80 % of the mean food uptake per treatment group were excluded from the derivation of the end points, as well as from the calculation of the actual mean oral doses in the test and reference item treatment groups. This was done to avoid potentially overestimating the final endpoints. The actual mean oral doses following this adjustment for non-feeding bumblebees were 5.8, 11.4, 19.9, 33.2 and 50.9 µg a.s./bumblebee.

For the 5.8, 11.4, 19.9, 33.2 and 50.9 µg a.s./bumblebee test item treatment group 27, 22, 18, 17 and 14 bumblebees were considered for the evaluation. For the water control (50 % w/v sucrose solution) and solvent control (50 % w/v sucrose solution containing 5 % w/w acetone and 1 % v/v Tween80) treatment groups 29 and 25 bumblebees were considered for the evaluation. At the end of the oral toxicity test (48 hours after application) 5.8, 11.4, 19.9, 33.2 and 50.9 µg a.s./bumblebee led to no mortality. No mortality

occurred also in the water control (50 % w/v sucrose solution) and in the solvent control (50 % w/v sucrose solution containing 5 % w/w acetone and 1 % v/v Tween80) treatment groups. No test item induced behavioural effects were observed at any time during the oral test.

The reference item target dose level of 4 µg dimethoate/bumblebee would have been achieved if exactly 40 mg treated feeding solution was consumed per bumblebee. Considering bumblebees with a food uptake of at least 80 % of the mean food uptake the measured consumption corresponded to an actual oral dose of 4.5 µg dimethoate/bumblebee. For the reference item treatment group 28 bumblebees were considered for the evaluation. The mortality in the reference item treatment group was 100 % (24 hours after application).

As the mortality in the test item treatment groups did not reach or exceed 10% at test termination, the LD_{50}, LD_{20} and LD_{10} were not statistically calculated. Thus, the LD_{50}, LD_{20} and LD_{10} for Spiroxamine technical were all considered to be >50.9 µg a.s./bumblebee.

III. Conclusion

Bumblebees (*Bombus terrestris* L) were exposed to spiroxamine technical in a 48-hour oral toxicity study. The purpose of the study was to determine the effects of spiroxamine technical on the behaviour and survival of the test organisms.

As the mortality in the test item treatment groups did not reach or exceed 10% at test termination, the LD_{50}, LD_{20} and LD_{10} were not statistically calculated. Thus, the LD_{50}, LD_{20} and LD_{10} were all considered to be >50.9 µg a.s./bumblebee.

The oral NOED and LOED were calculated to be >50.9 µg a.s./bumblebee, respectively.

Assessment and conclusion by applicant:

Validity criteria according to the current OECD 247 guideline (2017) were met. This is the version of test guideline to which the study was conducted.

- Mortality in the water control should be ≤10% at the end of the test. If included, also solvent control mortality should be ≤10% at the end of the test (actual: 0% in both the water and the solvent controls)
- Mortality in the toxic reference substance group should be >50% at the end of the test (actual: 100%)

The guideline validity criteria were met therefore the study is considered to be acceptable.

The LD_{50} was considered to be >50.9 µg a.s./bumblebee.

CA 8.3.1.1.2 Acute contact toxicity

Data Point:	KCA 8.3.1.1.2/01
Report Author:	[redacted]
Report Year:	2015
Report Title:	Effects of spiroxamine tech. (acute contact) on bumblebees (Bombus terrestris L.) in the laboratory
Report No:	88621105
Document No:	M-510841-01-1
Guideline(s) followed in study:	No specific guidelines available; study design based on OECD 214 (1998) Van der Steen (2001) and ICPPR non-apis group (2014)
Deviations from current test guideline:	Yes OECD 246 (2017) It is noted that the study did not include analytical verification of the dosing solution used to treat the bees which is a requirement of the current OECD 246 test guideline, however this study was conducted prior to the issue of this formal OECD test guideline and is therefore considered to be valid and based on the accepted test methodology at the time.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Bumblebees (*Bombus terrestris* L.) were exposed to spiroxamine technical in a 48-hour contact toxicity study. Exposure was at 100 µg a.s./bumblebee, along with a water control and a solvent control. Dimethoate at 12 µg a.s./bee as a reference item was also tested.

Mortality in the treatment concentration of 100 µg a.s./bumblebee was 8% after 48 hours therefore the NOED and LD_{50} values have been considered to be ≥100 and >100 µg a.s/bumblebee, respectively.

I. Materials and Methods

A. Materials

Test Material	Spiroxamine technical
Lot/Batch #:	EDTH008883
Purity:	98.7%
Description:	Colourless liquid
Stability of test compound:	Not reported
Reanalysis/Expiry date:	13/06/2016
Density:	0.93 g/cm³ (20°C)
Treatments	
Test rates:	100 µg a.s./L
Solvent/vehicle:	Acetone
Analysis of test concentrations:	No

Test organisms

Species:	Bumblebee, *Bombus terrestris* L.
Source:	Bumblebee colonies, healthy and queen-right, obtained from Biobest Belgium N.V., Ilse Velden 18, 2260 Westerlo, Belgium
Acclimatisation period:	19 hours 40 minutes
Feeding:	50% w/v sucrose solution *ad libitum*; given directly after treatment using syringes.
Treatment for disease:	None reported

Test design

Test vessel:	Cylindrical, latticed plastic cages with a length of approximately 7 cm and a diameter of 2.2 and 1.7 cm at the large and small openings, respectively.
Replication:	50 per treatment group/control
No. animals/vessel:	Individually housed
Duration of test:	48 hours

Environmental test conditions

Temperature:	25 ± 2°C
Photoperiod:	Darkness (except during observation)

B. Study Design

Bumblebees were exposed to spiroxamine technical in an acute contact test over 48 hours. The design of the study was based OECD 214 (1998), Van der Steen (2001) and ICPPR non-*Apis* group (2014).

The test organisms were adult female *Bombus terrestris* L., with a mean weight of 276 mg (SD 46.9).

The bumblebees were kept in the test units and the contact application was conducted outside of the test unit. Temperature and relative humidity were kept at 22 to 26°C and 59 to 74%, respectively during acclimatisation and 22 to 25°C and 53 to 75% respectively during exposure. The bees were kept in darkness except for during observation.

The bees were anesthetised for application of a 5 µL droplet at a concentration of 100 µg a.s./bumblebee to the dorsal bumblebee thorax. The reference item used was 12 µg dimethoate/bumblebee, the water control was tap water with 0.5% Tween80 and the solvent control was 5µL acetone/bee. Each treatment group consisted of 50 bees.

The bees were observed after 4 (±0.5 hours), 24 and 48 (±2 hours) hours for mortality and behavioural abnormalities. Sub-lethal affects were defined by the categories of moribund (unable to walk, weak response to stimulus), affected (reduced coordination) and cramps (contracting abdomen or whole body).

II. Results and Discussion

The validity criteria set out in the study report were met:

- Control mortality <10% (actual: water control had 2% mortality and solvent control was 0%)
- LD_{50} of the reference item ≥50% (actual: 96%)

At test termination (48 hours) there was 8.0 % mortality at 100 µg a.s./bumblebee. 2.0% mortality occurred in the water control group (water + 0.5% Tween80) and there was no mortality in the solvent control group (acetone). No sub-lethal effects were observed in the test item treatment group.

Table CA 8.3.1.1.2/01-1 Mean mortality and behavioural abnormalities of the bumblebees in the contact toxicity test

Treatment group (µg a.s. /bumblebee)	After 4 hours		After 24 hours		After 48 hours	
	Mortality (%)	Behavioural abnormality (%)	Mortality (%)	Behavioural abnormality (%)	Mortality (%)	Behavioural abnormality (%)
Water control	0.0	0.0	0.0	0.0	2.0	0.0
Solvent control	0.0	0.0	0.0	0.0	0.0	0.0
100	0.0	0.0	4.0	0.0	8.0	0.0
Dimethoate	6.0	2.0	84.0	12.0	96.0	4.0

Due to the limited effects on mortality after 48 hours in the 100 µg a.s./bumblebee treatment, the NOED was considered to be ≥100 µg a.s./bumblebee and the contact LD_{50} value was >100 µg a.s./bumblebee.

III. Conclusion

After 48-hours of exposure to spiroxamine technical, the contact NOED value for *Bombus terrestris* L. was considered to be ≥100 µg a.s./bumblebee. The contact LD_{50} value was >100 µg a.s./bumblebee.

Assessment and conclusion by applicant:

This study is a new study and has not been previously reviewed.

The validity criteria defined in the current OECD 246 (2017) guideline were met:

- Control mortality <10% (actual: water control had 2% mortality and solvent control was 0%)
- LD_{50} of the reference item ≥50% (actual: 96%)

It is noted that the study did not include analytical verification of the dosing solution used to treat the bees which is a requirement of the current OECD 246 test guideline, however this study was conducted prior to the issue of this formal OECD test guideline and is therefore considered to be valid and based on the accepted test methodology at the time. The study is therefore considered to be acceptable.

The contact LD_{50} value was determined to be >100 µg a.s./bumblebee.

CA 8.3.1.2 Chronic toxicity to bees

No chronic adult honeybee oral toxicity test data using spiroxamine technical are available. However, data are available using the representative formulation Spiroxamine EC 500. A full summary has been provided in Document M-CP Section 10.

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:	2018
Report Title:	Spiroxamine tech. - Honey bee (Apis mellifera L.) 22 day larval toxicity test (repeated exposure)
Report No:	S17-02467
Document No:	M-623462-01-1
Guideline(s) followed in study:	OECD Guidance Document 239 on Honey bee (Apis mellifera) Larval Toxicity Test, Repeated Exposure (2016)
Deviations from current test guideline:	Yes Methods: SANCO/3029/99 rev. 4 Accuracy n=1 or 3, no precision data for n=1 Ecotoxicology: OECD (2016) For the toxic reference item group(s) mortality but no other observations were assessed. No emergence boxes were used as from Day 15 to enable the assignment of each emerged bee to the respective replicate.
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 objective of this study was to determine the effects of spiroxamine technical on the emergence of adult honey bees, *Apis mellifera* L., from repeated feeding exposure in a 22-day laboratory test and to determine the cumulative mortalities during the larval phase and the pupation phase as well as the adult emergence rate.

Synchronised honeybee larvae (first instar, L1) were housed individually in well plates, where they were fed a standardised amount of artificial diet. From day three until day six spiroxamine technical was administered daily to the larvae in the diet at concentrations of 2.64, 7.93, 23.8, 71.3 and 214 mg spiroxamine/kg diet (equivalent to cumulative doses of 0.4, 1.2, 3.7, 11 and 33 µg spiroxamine/larva per developmental period) plus a control and a solvent control.

Since there was no statistically significant difference between any test item group and the control group, a LOEC of >214 mg spiroxamine/kg diet, equivalent to a LOED of >33 µg spiroxamine/larva per developmental period is considered. The EC_{10}/ED_{10}, EC_{20}/ED_{20} and EC_{50}/ED_{50} could not be calculated, but can be regarded as >214 mg spiroxamine/kg diet, equivalent to >33 µg spiroxamine/larva per developmental period.

I. Materials and Methods

A. Materials

Test Material Spiroxamine technical

Lot/Batch #:	EDTH008883
Purity analysed:	98.7% w/w
Description:	Liquid / colourless
Stability of test compound:	Sufficient based on the expiration date

Reanalysis/Expiry date:	18 May 2018
Density:	0.93 g/cm³
Treatments	
Test rates:	2.64, 7.93, 23.8, 71.3 and 214 mg spiroxamine/kg diet
Solvent/vehicle:	Acetone
Analysis of test concentrations:	Measured concentrations of spiroxamine in the larval diet were 90 – 110% across all test substance groups
Test organisms	
Species:	*Apis mellifera carnica*
Source:	Not reported – colonies located at testing facility
Feeding:	50% weight of fresh royal jelly and 50% weight of an aqueous solution containing varying amounts of yeast extract, glucose and fructose
Test design	
Test vessel:	Larvae were transferred into crystal polystyrene grafting cells having a diameter of 9 mm and a depth of 8 mm.
Test medium:	Bees exposed via the diet
Housing:	For each treatment group, 48 test organisms from three different hives were tested over 22 days. Each hive equates to one replicate, 16 larvae from each replicate were used.
Number of organisms per vessel:	As above
Duration of test:	22 days
Environmental test conditions	
Temperature:	Mean values 33.4 to 34.2°C
Relative humidity:	Mean values 66.0 to 79.4%
Photoperiod:	During the entire test period the test organisms were kept under constant darkness except during grafting, feeding and assessments.

B. Study Design

The objective of this study was to determine the effects of spiroxamine technical on the emergence of adult honey bees, *Apis mellifera* L., from repeated feeding exposure in a 22-day laboratory test and to determine the cumulative mortalities during the larval phase and the pupation phase as well as the adult emergence rate.

The study was conducted as a dose response test with a duration of 22 days from grafting on day one to the final assessment on day 22. The study comprised of one control group, one solvent control group and five different test item concentrations of, 2.64, 7.93, 23.8, 71.3 and 214 mg spiroxamine/kg diet (equivalent to cumulative doses of 0.4, 1.2, 3.7, 11 and 33 µg spiroxamine/larva per developmental period) and one dimethoate reference item group with 48.0 mg dimethoate/kg diet, equivalent to a cumulative dose of 7.39 µg dimethoate/larva per developmental period.

The test item and reference item were measured using a balance. For the preparation of the test item stock solution and dilutions were prepared. Acetone was used as solvent. A new pipette tip was used for each dilution step. The test item stock solution was prepared freshly at each application day. For the preparation of the dimethoate reference item stock solution and dilution, autoclaved water was used as solvent. A new pipette tip was used for each dilution step. All solutions were homogenised by shaking.

For each treatment group, 48 test organisms from three different hives were tested over 22 days. Each hive equates to one replicate, 16 larvae from each replicate were used.

Incubation was at mean temperatures of 32.9 to 34.5°C and relative humidity ranged from 40.4 to 100.0%. The larvae were kept in in complete darkness (except during grafting, feeding and assessments).

Larvae were transferred into crystal polystyrene grafting cells having a diameter of 9 mm and a depth of 8 mm. Cells were initially sterilised by submerging for 30 min in ethanol 70% (v/v), and then dried. Each cell was placed into a well of a sterile 48-well cellular culture plate (Greiner Bio One). The open plates were placed into a hermetically sealed desiccator, containing a dish filled with a saturated potassium sulphate (K_2SO_4) solution in order to keep a water saturated atmosphere from day 1 until day 8. On day 8 the plates were transferred into a second desiccator containing a dish filled with a saturated sodium chloride (NaCl) solution. The desiccators were placed in an incubator with forced air circulation. On day 15, each plate was covered by its lid and transferred from the desiccator into an incubator with automated humidity control.

The larval diet was prepared with deionised, 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

Assessment of mortality during the larval phase was conducted before feeding from day 4 until day 8. Larvae were recorded as dead, if no respiration (movement of spiracles) was observed. Assessment of mortality during the pupation phase was conducted on day 15 and day 22. On day 15 dead larvae, pupae and larvae that have not transformed into pupae were recorded as dead. On day 22 pupae that have not emerged were recorded as dead. Assessment of adult emergence was conducted on day 22. Bees were counted as successfully emerged if they showed signs of adult eclosion. This included the presence of differentiated wings and hair in the absence of the pupal skin. At each assessment time dead larvae and pupae were removed for sanitary reasons. On day 8 the presence of uneaten food was qualitatively recorded. Other observations and any other adverse effects were qualitatively recorded to aid in the interpretation of mortality in comparison to the solvent control group.

Analytical samples were taken from all control and test substance groups, directly from the prepared diets.

Analytical method

Samples of diet were analysed using the validated analytical method M-623462-01-1, report reference M-623462-01-1 (**see Doc MCA Section 4**).

II. Results and Discussion

The study was conducted to the OECD (2016): Series on Testing and Assessment Number 239: Guidance Document on Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure

The study was considered valid since validity criteria for control mortality and reference item mortality on day 8 as well as control emergence on day 22 were met.

- Control mortality: The cumulative larval mortality from day 3 until day 8 was ≤15% across all replicates (actual: 2.1% in control and 4.2% in the solvent control).
- Control emergence: On day 22 the adult emergence rate was ≥70% across all control replicates (actual: 81.3% in control and 77.1% in the solvent control)
- Reference item mortality: In the dimethoate reference item group the cumulative larval mortality was ≥50% across all replicates on day 8 (actual: 80.9%).

Spiroxamine was analysed in the test item treated larval diet of each test item group (T1-T5) and the control groups by liquid chromatography and mass spectrometric detection (HPLC-MS/MS). In the larval diet of the control groups no spiroxamine was detectable; the concentrations of the test item were below the limit of quantification of 0.01 mg spiroxamine/kg diet. The measured concentrations of spiroxamine in the larval diet were between recoveries of 90 % and 110 % across all test item groups. The mean measured concentrations of the test item in the larval diet were within ±20% of nominal for each test item group. Therefore, the concentrations of the test item in the larval diet were confirmed and the endpoints are based on nominal concentrations.

On day 8, larval mortality was 2.1% and 4.2% in the control and solvent control group and 81.3% in the reference item group.

On day 22, the adult emergence rates in the control and solvent control group were 81.3 and 77.1%, respectively. Consequently, validity criteria for the control and reference item groups were met and the test was considered valid.

During the assessments of mortality and emergence no other test item related observations such as deviating sizes, appearances and malformations of the test organisms were made.

On day 8, uneaten food was observed in the two highest test item groups of 71.3 and 214 mg spiroxamine/kg diet and in the reference item group.

Summarised results of the evaluation of mortality and emergence over the test period are presented in the following tables.

Table CA 8.3.1.3/01-1 The effects of spiroxamine technical on the larval mortality of the honey bee, *Apis mellifera carnica*, from repeated exposure

Concentration (mg spiroxamine/kg diet[a])	Cumulative dose (µg spiroxamine/larva per developmental period[a] [b])	Larval mortality on day 8	
		[%]	Corrected [%]
Control	-	2.1	-
Solvent control	-	4.2	-
2.64	0.4	0.0	-4.4
7.93	1.2	0.0	-4.4
23.8	3.7	2.1	-2.2
71.3	11	4.2	0.0
21.4	33	10.4	6.5

[a] Based on the analysed purity

[b] Based on the cumulative feeding volume from day 3 until day 6 of 140 µL diet/larva and a density of the diet of 1.1 g/cm^3

Table CA 8.3.1.3/01-2 The effects of spiroxamine technical on the pupal stage and on the adult emergence of the honey bee, *Apis mellifera carnica*, from repeated exposure and the corresponding endpoints

Concentration (mg a.s./kg diet[a])	Cumulative dose (µg a.s./larva per developmental period[a b])	Mortality on day 15		Pupal mortality from days 8 – 22		Adult emergence on day 22[a]
		[%]	Corr.[%]	[%]	Corr.[%]	
Control		12.5	-	17.0	-	81.3
Solvent control		16.7	-	19.6	-	77.1
2.64	0.4	16.7	0.0	18.8	-1.0	81.3
7.93	1.2	14.6	-2.5	18.8	-1.0	81.3
23.8	3.7	18.8	2.5	17.0	-3.2	81.3
71.3	11	18.8	2.5	19.6	0.0	77.1
214	33	22.9	7.4	16.3	-4.1	75.0

[a] Based on the analysed purity

[b] Based on the cumulative feeding volume from day 3 until day 6 of 140 µL diet/larva and a density of the diet of 1.1 g/cm³

Table CA 8.3.1.3/01-3 Assessments of larval and pupal mortality and adult emergence from day four until day 22 including presence of uneaten food on day eight

Treatment groups	Cumulative number of dead larvae					Alive larvae with uneaten food	Cumulative number of dead larvae/pupae	Cumulative number of dead larvae / pupae and non-emerged bees	Number of emerged bees
	D4	D5	D6	D7	D8	D8	D15	D22	D22
Control(s):									
C	0	1	1	1	1	1	6	9	39
SC	0	2	2	2	2	0	7	11	37
Test item: spiroxamine tech. [mg spiroxamine/kg diet]									
2.64	0	0	0	0	0	0	8	9	39
7.93	0	0	0	0	0	0	7	9	39
23.8	0	0	1	1	1	0	9	9	39
71.3	0	0	0	0	0	0	9	11	37
21.4	1	3	4	5	5	1	11	12	36
Reference item: dimethoate [mg dimethoate/kg diet]									
48.0	30	34	38	39	8				

Table CA 8.3.1.3/01-4 A summary of relevant endpoints

Endpoints for day 22				
LOEC	NOEC	EC_{10}	EC_{20}	EC_{50}
[mg spiroxamine/kg diet][b]				
>214	≥214	>214[d]	>214[d]	>214[d]
[µg spiroxamine/larva per developmental period][b c]				
>33	≥33	>33[d]	>33[d]	>33[d]

[a] statistical evaluation for non-emergence

[b] Based on the analysed purity

[c] Based on the cumulative feeding volume from day 3 until day 6 of 140 µL diet/larva and a density of the diet of 1.1 g/cm³

[d] The EC_{10}/ED_{10}, EC_{20}/ED_{20} and EC_{50}/ED_{50} could not be calculated due to the lack of inhibition in emergence >10%, but can be regarded as above the highest concentration/dose tested

III. Conclusion

In a repeated exposure larval toxicity test with spiroxamine technical and a duration of 22 days the NOEC for adult emergence on day 22 was determined as ≥214 mg spiroxamine/kg diet, equivalent to a NOED of ≥33 µg spiroxamine/larva per developmental period.

Since there was no statistically significant difference between any test item group and the solvent control group, a LOEC of >214 mg spiroxamine/kg diet, equivalent to a LOED of >33 µg spiroxamine/larva per developmental period is considered.

The EC_{10}/ED_{10}, EC_{20}/ED_{20} and EC_{50}/ED_{50} could not be calculated, but can be regarded as >214 mg spiroxamine/kg diet, equivalent to >33 µg spiroxamine/larva per developmental period.

Assessment and conclusion by applicant:

This is a new study that has not been previously evaluated.

The study was conducted to the OECD (2016): Series on Testing and Assessment Number 239: Guidance Document on Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure

The study was considered valid since validity criteria for control mortality and reference item mortality on day eight as well as control emergence on day 22 were met.

- Control mortality: The cumulative larval mortality from day 3 until day 8 was ≤15% across all replicates (actual: 2.1% in control and 4.2% in the solvent control).
- Control emergence: On day 22 the adult emergence rate was ≥70% across all control replicates (actual: 81.3% in control and 77.1% in the solvent control).
- Reference item mortality: In the dimethoate reference item group the cumulative larval mortality was ≥50% across all replicates on day 8 (actual: 80.9%).

The study is therefore considered acceptable.

The NOEC for adult emergence on day 22 was determined as ≥214 mg spiroxamine/kg diet, equivalent to a NOED of ≥33 µg spiroxamine/larva per developmental period.

CA 8.3.1.4 Sub-lethal effects

Tunnel test data and field study data are available using Spiroxamine EC 500. Please refer to Document M-CP Section 10 for full details.

Relevant literature on bees

No relevant scientifically peer-reviewed open literature could be found on spiroxamine or its major metabolites, from an ecotoxicological perspective, on bees. Details of the literature search undertaken can be found in the M-CA Section 9 of the current submission.

CA 8.3.2 Effects on non-target arthropods other than bees

CA 8.3.2.1 Effects on *Aphidius rhopalosiphi*

No data are available using spiroxamine technical but data are available using the representative formulations. Please refer to Document M-CP Section 10 for full details.

CA 8.3.2.2 Effects on *Typhlodromus pyri*

No data are available using spiroxamine technical but data are available using the representative formulations. Please refer to Document M-CP Section 10 for full details.

Relevant literature on non-target arthropods

No relevant scientifically peer-reviewed open literature could be found on spiroxamine or its major metabolites, from an ecotoxicological perspective, on non-target arthropods. Details of the literature search undertaken can be found in the M-CA Section 9 of the current submission.

CA 8.4 Effects on non-target soil meso and macrofauna

CA 8.4.1 Earthworm, sub-lethal effects

No earthworm reproduction data using spiroxamine technical are available. However, data are available using the representative formulations and full summaries have been provided in Document M-CP Section 10.

The available data for earthworms are presented in the table below.

Table CA 8.4.1-1 Summary of earthworm toxicity studies with spiroxamine metabolites

Organism	Test item	Test type	Endpoints		Reference
Earthworm (*Eisenia fetida*)	KWG 4168-desethyl (M01)	56 d Chronic toxicity; 5% peat	NOEC 100 mg/kg soil dw	EU	M-281615-01-1
		Statistical Re-analysis	EC_{10} 93.8 mg/kg soil dw EC_{20} 120 mg/kg soil dw	NEW	M-760435-01-1
Earthworm (*Eisenia andrei*)	KWG 4168-despropyl (M02)	56 d Chronic toxicity; 10% peat	NOEC 100 mg/kg soil dw; $NOEC_{corr}$ 50 mg/kg soil dw[1]; EC_{10} >100 mg/kg soil dw; $EC_{10\,corr}$ >50 mg/kg soil dw[1]	NEW	M-680755-01-2
Earthworm (*Eisenia fetida*)	KWG 4168-N-oxide (M03)	56 d Chronic toxicity; 5% peat	NOEC 100 mg/kg soil dw	EU	M-281617-01-1
		Statistical Re-analysis	EC_{10} 245 mg/kg soil dw EC_{20} 287 mg/kg soil dw	NEW	M-760434-01-1
Earthworm (*Eisenia fetida*)	KWG 4168-acid (M06)	56 d Chronic toxicity; 10% peat	NOEC 100 mg/kg soil dw; EC_{10} >100 mg/kg soil dw;	NEW	M-727123-01-1

EU: previously evaluated as part of the original EU review and listed in EFSA conclusion and DAR
NEW: new study or data generated since the previous EU review or previously not submitted
[1] The NOEC from the study, which was conducted using soil with a 10% peat content, has been divided by 2 to account for lipophilic effects from compounds with a Log Pow >2

KWG 4168-desethyl (M01)

Data Point:	KCA 8.4.1/01
Report Author:	[redacted]
Report Year:	2007
Report Title:	KWG 4168-Desethyl (technical): Effects on survival, growth and reproduction on the earthworm Eisenia fetida tested in artificial soil with 5 % peat
Report No:	LRT-RG-R-26/06
Document No:	M-281615-01-1
Guideline(s) followed in study:	ISO 11268-2: 1998 (E) and OECD 222: April 13, 2004
Deviations from current test guideline:	Yes OECD 222 (2016) 5% sphagnum peat was added to the artificial substrate, however, the guidance suggested 10% sphagnum peat to be added, the survival of the worms would suggest this had no impact to the study
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The purpose of this study was to assess the effect of KWG 4168-desethyl (technical) on survival, growth, and reproduction on the earthworm *Eisenia fetida* during an exposure into an artificial soil with 5 different test concentrations.

Eight replicates with 10 worms each were prepared for the control, whereas each test item rate consisted of four replicates. Earthworms were weighed and assessed for mortality and abnormal behaviour 28 days after test start. At the end of the test after 8 weeks, the number of surviving juveniles per test vessel was determined.

The NOEC and LOEC for both growth and reproduction were 100 and 316 mg test item/kg soil dry weight, respectively.

I. Materials and Methods

A. Materials

Test Material KWG 4168-desethyl

Lot/Batch #: 921103E-LB02

Content of a.s.: 98% w/w

analysed: Substance is a mixture of diastereomers and split into two peaks (56% Isomer A : 42% Isomer B)

Description: Clear brown oily liquid

Stability of test compound: Sufficient for test period based on the expiry date

Reanalysis/Expiry date: 2009-12-07

Density: Not reported

Treatments

Test rates: 10, 32, 100, 316 and 1000 mg test item/kg dry weight soil

Solvent/vehicle:	Quartz sand
Analysis of test concentrations:	n/a
Test organisms	
Species:	*Eisenia fetida*
Source:	In-house culture
Test design	
Test vessel:	Non-re-usable plastic boxes (16.5 x 12 x 6 cm, area approximately 200 cm^2) containing approx. 500 g soil (dry weight) to a depth approx. 5 cm
Test soil:	5% sphagnum peat (shredded), 20% kaolinite clay, 73.85% industrial quartz sand, 0.15% calcium carbonate, 1% dried ground cow manure (food)
Replication:	Four per treatment group
Number of organisms per vessel:	Ten animals per test vessel
Duration of test:	The study consisted of two parts. Adult earthworms were exposed to the test item for a period of 4 weeks (first part); after this period, the adults were removed from the test vessels and the cocoons and juvenile earthworms remained in the test vessels for an additional 4 weeks (second part). The total duration of each run of the study was 8 weeks
Environmental test conditions	
Temperature:	18 – 22°C
pH:	6.85 – 7.04
Water content:	The soil was moistened with deionised water to reach a water content of 58% of the maximum water holding capacity
Photoperiod:	16-hour light to 8-hour darkness photoperiod and a light intensity at light period between approximately 400 - 800 Lux

B. Study Design

The purpose of this study was to assess the effect of KWG 4168-desethyl (technical) on survival, growth, and reproduction on the earthworm *Eisenia fetida* during an exposure into an artificial soil with five different test concentrations.

Nominal test concentrations were 10, 32, 100, 316 and 1000 mg test item/kg dry weight soil.

Ten worms were added to each of the four replicate test vessels. Test vessels were non-re-usable plastic boxes (length x width x height *ca.* 16.5 cm x 12 cm x 6 cm, area approximately 200 cm^2) were used as test vessels. Each test vessel contained an amount of approximately 500 g artificial soil (dry weight) to obtain a depth of approximately 5 cm soil in the test vessels.

Incubation was at 18 to 22°C with a photoperiod of 16 hours light to 8 hours dark at approximately 400 to 800 lux. The measured mean light intensity was 599 lux at day 0, 603 lux at day 28 and 624 lux at day 56 of the study.

After 4 weeks of exposure, the content of each test vessel was emptied and the living adult earthworms were counted and checked for any abnormal behaviour or other adverse effects (*e.g.* lack of movement, rigidity *etc.*). Those earthworms, which did not move after gentle mechanical stimulus were considered to be dead. Also missing earthworms (compared to the number of initially placed test organisms) were considered to have died.

At the beginning (prior to exposure) and at the end of the 4 weeks of exposure, the adult test organisms of each vessel were weighed (at the start each individually, at the end together of each test vessel). Before weighing, the earthworms were quickly washed with water; surplus water was absorbed on filter paper.

At the end of the test after 8 weeks, the number of surviving juveniles per test vessel was determined. The test vessels were placed in a water bath at 50-60 °C for approximately 15-20 minutes. By this treatment, alive juvenile earthworms rose to the soil surface. The emerging earthworms were removed and counted. Afterwards the content of each test vessel was checked additionally by carefully stirring up the artificial soil with the help of tweezers.

II. Results and Discussion

The data were assessed against the criteria of the OECD and ISO test guidelines to which the study was conducted. All validity criteria were met:

- Adult mortality over the initial 4 weeks of the test to be ≤10% (actual: 0%)
- Mean change in growth of the adult earthworms in the control during the exposure period of four weeks should not exceed -20% (actual: +62.0%)
- Each replicate (containing 10 adults) to have produced ≥30 juveniles by the end of the test (actual: 180.6)
- The coefficient of variation of reproduction to be ≤30% (actual: 10.7%)

The study is therefore, considered acceptable.

No mortality of adult earthworms were observed after 28 days test duration in the control group and at the test concentrations of 10, 32, 100 and 316 mg test item/kg dry weight artificial soil of KWG 4168-Desethyl (technical). A mortality rate of 95% compared to the control was observed at the highest test concentration of 1000 mg test item/kg dry weight artificial soil.

Adult earthworms were observed on the soil surface of the replicates of the highest test concentration of 1000 mg test item/kg dry weight artificial soil on day 1, 7, 14, 21 and 28. No adult earthworms were observed during this test period on the soil surface of the control group and of the test concentrations of 10, 32, 100 and 316 mg test item/kg dry weight artificial soil.

Table CA 8.4.1/01-1 Number of surviving adult earthworms and percent mortality at day 28

Test concentration (mg test item/kg dry weight soil)	Number of earthworms exposed day 0	Number of earthworms survived day 28	Sum of dead earthworms	Mortality per test concentration (%)
Control	80	80	0	0
10	40	40	0	0
32	40	40	0	0
100	40	40	0	0
316	40	40	0	0
1000	40	2*	38	95

* Worms were inactive

The mean body weight of the adult earthworms in the control group had increased during the 4 weeks of exposure. The mean body weight was 0.55 g per worm (+ 62.0% of the mean initial weight).

The mean changes in body weight of the test concentrations of 10, 32 and 100 mg test item/kg dry weight artificial soil were not statistically significantly different relative to the control (+ 63.1%, +

70.4% and + 55.8%). The mean changes in body weight of the test concentrations of 316 and 1000 mg test item/kg dry weight artificial soil were statistically significantly different relative to the control (+ 38.0% and – 57.9%). (Results of a Dunnett´s multiple t-test, two-sided, α = 0.05).

Table CA 8.4.1/01-2 Mean body wet weight of adult earthworms at the test start and after 28 days (values in this table are rounded values)

Test concentration (mg test item/kg dry weight soil)	Number of surviving worms		Mean weight per worm (g)		Mean change of body weight (%) ± SD
	Day 0	Day 28	Day 0	Day 28	
Control	40	40	0.34	0.56	62.0 ± 8.8
10	40	40	0.34	0.55	63.1 ± 11.7
32	40	40	0.34	0.57	70.4 ± 5.6
100	40	40	0.34	0.52	55.8 ± 8.6
316	40	40	0.33	0.52	38.0* ± 3.8
1000	40	2	0.34	0.14	-57.9* ± 2.7

* Statistically significantly reduced according to Dunnett's multiple t-test, two-sided, $p < 0.05$

In the control group, on average 180.6 juvenile earthworms per test vessel were found (corresponding to a mean reproduction rate of 18.1 juveniles per surviving adult).

In the treatment groups exposed to the test item KWG 4168-Desethyl (technical) up to and including the highest test concentration of 1000 mg test item/kg dry weight artificial soil, the mean reproduction rate was in the range of 0.0% to 100.3% of the control value.

No statistically significantly different values for the number of juveniles per test vessel relative to the control were observed at the test concentrations of 10, 32 and 100 mg test item/kg dry weight artificial soil. Statistically significantly different values for the number of juveniles per test vessel relative to the control were observed at the test concentrations of 316 and 1000 mg test item/kg dry weight artificial soil (Results of a Dunnett´s multiple t-test, one-sided smaller, α = 0.05).

Table CA 8.4.1/01-3 Reproduction of the earthworms

Test concentration (mg test item/kg dry weight soil)	Reproduction rate (per surviving adult)	Juvenile earthworms per test box		
	Mean ± SD	Mean ± SD	CV (%)	% of control
Control	18.1 ± 1.9	180.6 ± 19.3	10.7	-
10	18.1 ± 1.9	181.3 ± 25.4	14.0	100.3
32	15.5 ± 2.5	155.3 ± 24.7	15.9	86.0
100	16.0 ± 1.7	160.0 ± 17.2	10.7	88.6
316	3.4 ± 0.6	34.0 ± 5.9	17.3	18.8*
1000	0.0 ± 0.0	0.0 ± 0.0	-	0.0*

* Statistically significantly reduced compared to the control (Dunnett's Multiple t-test, one-sided smaller, $p < 0.05$)

To verify the sensitivity of the test system, the reference item (Carbendazim 360 g/L) was tested at concentrations of 1.25, 2.5 and 5.0 mg a.s./kg soil dry weight in a separate study. In the most recent GLP study, the number of juveniles was statistically significantly reduced compaed to the control at rates of 1.25, 2.5 and 5.0 mg a.s./kg soil dry weight. The effects on the reduction of reproduction showed that the test system was sensitive and reflected the expected toxicity as given in the OECD 222 test guideline (significant effects observed between 1 and 5 mg a.s./kg soil).

III. Conclusion

The purpose of this study was to assess the effect of KWG 4168-desethyl (technical) on survival, growth, and reproduction on the earthworm *Eisenia fetida* during an exposure into an artificial soil with 5 different test concentrations.

Exposure to the test item at the test concentrations of 10, 32, 100 and 316 mg test item/kg dry weight artificial soil did not affect mortality of *Eisenia fetida*. A mortality rate of 95% compared to the control was observed at the highest test concentration of 1000 mg test item/kg dry weight artificial soil.

The NOEC and LOEC for both growth and reproduction were 100 and 316 mg test item/kg soil dry weight, respectively.

Assessment and conclusion by applicant:

The study was conducted to the OECD 222: April 13, 2004: "OECD Guideline for the Testing of Chemicals – Earthworm Reproduction Test (*Eisenia fetida* / *Eisenia andrei*)".

The study was also assessed to current guidance: OECD 222: 29 July, 2016: "OECD Guideline for the Testing of Chemicals – Earthworm Reproduction Test (*Eisenia fetida* / *Eisenia andrei*)", as this is the most recent version: All validity criteria were met:

- Each replicate (containing 10 adults) to have produced >30 juveniles by the end of the test (actual: 180.6)
- The coefficient of variation of reproduction to be ≤30% (actual: 10.7%)
- Adult mortality over the initial 4 weeks of the test to be ≤10% (actual: 0%)

The reference item also demonstrated sufficient sensitivity of the test organisms. The study is therefore considered to be acceptable.

The NOEC, based on growth and reproduction was determined to be 100 mg test item/kg soil dry weight.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.4.1/04
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10 and EC20 values for Eisenia fetida with KWG 4168-desethyl TG in a reproduction study
Report No:	0471836-EC011
Document No:	M-760435-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary

The report M-281615-01-1 on the effects of KWG 4168-desethyl TG in the earthworm (*Eisenia fetida*) reproduction study did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013. The resulting EC_{10}, and EC_{20} values for reproduction were 93.826 (95% CL: 62.026 – 128.981) and 120.139 (95% CL: 87.489 – 154.476) mg/kg dws, respectively.

I. Methods

The statistical evaluation was performed with statistical software ToxRat Standard v3.3.0. Due to high mortality levels in the top concentration, data on reproduction for the 1000 mg/kg dws was subsequently

removed from all statistical analyses. EC_{10} and EC_{20} values for reduction were determined using a Probit function with maximum likelihood regression. The confidence limits were determined by bootstrapping (1000 resamplings); bias-corrected.

II. Results and Discussion

Regarding the calculation of EC_{10} and EC_{20} values for reproduction of the earthworms, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.00, showing no significant deviation between fit and data, and a statistically significant concentration/response was found ($p(F) < 0.001$) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table.

Table CA 8.4.1/04-1 Results of the Probit analysis (max. likelihood regression) with reproduction: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (by bootstrapping (1000 resamplings); bias-corrected)

Parameter	Reproduction	
	EC_{10} (95 % confidence interval) [mg/kg dws]	EC_{20} (95 % confidence interval) [mg/kg dws]
Reproduction	93.826 (62.026 – 128.981)	120.139 (87.489 – 154.476)

The resulting EC_{10} and EC_{20} values of 93.826 (95% CL: 62.026 – 128.981) and 120.139 (95% CL: 87.489 – 154.476) mg/kg dws, respectively, meet the goodness of fit criteria and therefore the estimated EC_{10} value is considered reliable for use in the risk assessment.

III. Conclusion

The resulting EC_{10} and EC_{20} values for reproduction were 93.826 (95% CL: 62.026 – 128.981) and 120.139 (95% CL: 87.489 – 154.476) mg/kg dws, respectively.

Assessment and conclusion by applicant:

The statistical re-evaluation of the reproduction data has determined an EC_{10} of 93.8 mg/kg dws.

As the EC_{10} is lower than the established NOEC value, the EC_{10} of 93.8 mg/kg dws shall be used in the risk assessment as the most critical endpoint from this study.

The values determined in the re-evaluation work are considered to be fully valid.

KWG 4168-despropyl (M02)

Data Point:	KCA 8.4.1/05
Report Author:	[redacted]
Report Year:	2019
Report Title:	KWG4168-despropyl: Effects on reproduction and growth of earthworms Eisenia andrei in artificial soil
Report No:	143071022
Document No:	M-680755-01-2
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 (2009) OECD Guideline no. 222 ISO-Guideline 11268-2
Deviations from current test guideline:	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 purpose of this study was to assess the effect of KWG 4168-despropyl on the reproduction and growth on the earthworm *Eisenia andrei* in artificial soil.

In an 8 week study, earthworms were exposed to KWG 4168-despropyl at nominal concentrations of 6.25, 12.5, 25, 50 and 100 mg test item/kg soil dry weight. There were 40 earthworms per treatment group, weighing between 310 and 598 mg.

Exposure to KWG 4168-despropyl did not show significant lethal effects to the earthworm in artificial soil up to the highest test concentration of 100 mg test item/kg soil dry weight.

There were no statistically significant differences in growth data up to and including the highest test concentration (Dunnett's t-test, $\alpha = 0.05$, two-sided). There were no statistically significant differences in reproduction data up to and including the highest test concentration (Welsh t-test after Bonferroni-Holm, $\alpha = 0.05$, one-sided smaller).

There were no abnormal behaviours observed in any of the treatment groups.

The NOEC and EOEC values for mortality, weight change and reproduction of the earthworm *Eisenia andrei* were determined to be ≥100 and >100 mg test item/kg soil, respectively. The LC_{50}, EC_{10}, EC_{20} and EC_{50} values were all considered to be >100 mg test item/kg soil dry weight.

I. Materials and Methods

A. Materials

Test Material KWG 4168-despropyl

Lot/Batch #: AE 1344303-PU-01

Purity: 99.1% w/w

Description: Colourless liquid

Reanalysis/Expiry date: 13 May 2022

Treatments

Test rates: 6.25, 12.5, 25, 50 and 100 mg KWG 4168-despropyl/kg soil

Test organisms

Species:	Earthworm (*Eisenia andrei*) weighing 310 – 598 mg
Source:	Not reported
Acclimatisation period:	One day in artificial soil, under test conditions
Feeding:	Finely ground animal manure

Test design

Test vessel:	Plastic boxes (18.3 x 13.6 x 6 cm) with perforated plastic lids
Test medium:	Artificial soil according to OECD 222, 500 g dry weight, 648.4 g wet weight
Replication:	Four per treatment group
No. animals/vessel:	Ten animals per test vessel
Duration of test:	Eight weeks

Environmental test conditions

Temperature:	18 – 22°C
Water content:	Test start: 30.3 – 30.9% (53.2 – 54.2% of the WHC_{max}) Test end: 30.6 – 33.3% (53.8 – 58.3% of the WHC_{max})
pH:	Test start: 5.7 Test end: 5.9 – 6.0
Photoperiod:	16 hours light, 8 hours dark (light intensity: 400 – 800 lux)

B. Study Design

This study was conducted in order to assess the effect of KWG 4168-despropyl on the reproduction and growth on the earthworm *Eisenia andrei* during an exposure into an artificial soil at five different test concentrations.

Ten earthworms were added to each of the four replicate test vessels. Test vessels were plastic boxes (18.3 x 13.6 x 6 cm) with perforated plastic lids.

The test soil consisted of 69.6% fine quartz-sand, 20% kaolinite clay, 10% sphagnum peat and 0.4% calcium carbonate. Prepared soil consisted of approximately 500 g of dry weight and 648.4 g wet weight.

The earthworms were exposed to nominal concentrations of 6.25, 12.5, 25, 50 and 100 mg test item/kg soil.

Incubation was at 20 ± 2°C with a photoperiod of 16 hours light and 8 hours dark at approximately 400 to 800 lux.

After 4 weeks of exposure, the content of each test vessel was emptied and the adult worms were counted, removed and weighed before the substrate was returned to the respective test units minus the worms. Mortality and behavioural abnormalities (*e.g.* lack of movement and rigidity) were observed at this stage (28 days after application).

At test initiation and after 4 weeks of exposure, the adult test organisms of each vessel were weighed. Weights were determined by washing the worms and placing them on filter paper to absorb surplus water.

At test termination, the number of surviving juveniles per test vessel was determined by placing the test vessels in a water bath of 50 to 60°C and counting all emerging worms. Reproduction was determined by the number of alive juveniles at test termination.

The earthworms were fed finely ground cattle manure which was added to the soil throughout the duration of the test.

Mortality data were statistically evaluated using the Fisher's Exact Binomial Test (multiple comparison with Bonferroni Correction, α = 0.05, one-sided greater).

Growth and reproduction data were tested for normal distribution and homogeneity of variance (α = 0.05) using the Shapiro-Wilk's test and the Levene's test, respectively. As the growth change data were homogenous and normally distributed but did not follow a monotonicity trend, the Dunnett's t-test was used to compare the treatment and control values (multiple comparison, α = 0.05, one-sided smaller). As the reproduction data were homogenous and normally distributed, the Welch t-test After Bonferroni-Holm was used to compare the treatment and control values (multiple comparison, α = 0.05, one-sided smaller).

II. Results and Discussion

Validity criteria according to the OECD 222 guideline (2016) were met.

- Each replicate (containing 10 adults) to have produced ≥30 juveniles by the end of the test (actual: 187 - 221)
- The coefficient of variation to be ≤30% (actual: 5.5%)
- Adult mortality over the initial 4 weeks of the test to be ≤10% (actual: 0%)

A slight mortality of 2.5% was observed at the test concentration of 50 mg test item/kg soil, which was not statistically significantly different compared to the control (Fisher's Exact Test, one-sided greater, α = 0.05). There were no statistically significant mortalities observed at any test concentration as compared to the control.

Table CA 8.4.1/05-1 Mortality and survival data observed after 28 days exposure

Treatment group (mg test item/kg dry weight soil)	Mean number of live adults		Mean mortality (%) ± SD[1]
	Start	4 weeks	
Control	10	10	0.0 ± 0.0
6.25	10	10	0.0 ± 0.0
12.5	10	10	0.0 ± 0.0
25	10	10	0.0 ± 0.0
50	10	10	2.5 ± 5.0
100	10	10	0.0 ± 0.0

- = not relevant

[1] = Mean ± standard deviation of 4 replicates (8 in the control)

The body weight changes in the treated groups were not statistically significantly different as compared to the control up to and including the highest test concentration (Dunnett's t-test, α = 0.05, two-sided).

Table CA 8.4.1/05-2 Body weight data observed after 28 days exposure

Treatment group (mg test item/kg dry weight soil)	Body weight range (mg)		Body weight change	
	Start	4 weeks	Mean ± SD[1] (mg)	Mean ± SD[1] (%)
Control	418.3-501.2	519.7-577.7	83 ± 21	18.3 ± 5.2
6.25	430.5-485.3	516.2-584.8	90 ± 44	19.9 ± 10.8
12.5	437.5-484.6	533.0-555.1	84 ± 14	18.3 ± 3.9
25	438.7-482.2	560.3-616.0	123 ± 8	26.6 ± 1.3

Treatment group (mg test item/kg dry weight soil)	Body weight range (mg)		Body weight change	
	Start	4 weeks	Mean ± SD[1] (mg)	Mean ± SD[1] (%)
50	443.4-480.4	495.6577.0	88 ± 26	19.0 ± 5.5
100	444.3-478.8	550.5-593.3	113 ± 32	24.6 ± 7.7

The results represent rounded values calculated on the exact raw data

[1] = Mean ± standard deviation of 4 replicates (8 in the control)

The reproduction data in the treated groups were not statistically significantly different as compared to the control up to and including the highest test concentration (Welsh t-test After Bonferroni-Holm, α = 0.05, one-sided smaller).

Table CA 8.4.1/05/-3 Reproduction data observed after 28 days exposure

Treatment group (mg test item/kg dry weight soil)	Number of juvenile earthworms	
	Mean ± SD[1]	% of control
Control	201 ± 11	-
6.25	193 ± 19	96.1
12.5	205 ± 11	102.2
25	196 ± 25	97.6
50	183 ± 39	91.3
100	176 ± 39	87.7

The results represent rounded values calculated on the exact raw data
[1] = Mean ± standard deviation of 4 replicates (8 in the control)
- = Not relevant

To verify the sensitivity of the test system, the reference item carbendazim was tested in a separate study. There were statistically significant effects on reproduction at a concentration of 0.695 mg a.s./kg soil and above. The EC_{50} for reproduction was calculated as 0.92 mg a.s./kg soil. The effects on the reduction of reproduction showed that the test system was sensitive and reflected the expected toxicity as given in the OECD 222 test guideline (significant effects observed between 1 and 5 mg a.s./kg soil).

III. Conclusion

In an 8 week earthworm reproduction and growth study with KWG 4168-despropyl, the NOEC and LOEC values for mortality, growth and reproduction were determined to be ≥100 and >100 mg test item/kg soil dry weight, respectively. The EC_{50} was estimated to be >100 mg test item/kg soil dry weight. Due to the lack of a clear concentration-response relationship, no reliable EC_X-calculation was possible. The EC_{10}, EC_{20} and EC_{50} values were all considered to be >100 mg test item/kg soil dry weight.

Assessment and conclusion by applicant:

This is a new study that has not been previously submitted for evaluation.

Validity criteria according to the OECD 222 guideline (2016) were met.

- Each replicate (containing 10 adults) to have produced ≥30 juveniles by the end of the test (actual: 187 - 221)
- The coefficient of variation to be ≤30% (actual: 5.5%)
- Adult mortality over the initial 4 weeks of the test to be ≤10% (actual: 0%)

The reference item also demonstrated sufficient sensitivity of the test organisms. The study is therefore considered acceptable.

The NOEC, based on growth and reproduction was determined to be 100 mg test item/kg soil dry weight.

KWG 4168-N-oxide (M03)

Data Point:	KCA 8.4.1/02
Report Author:	[redacted]
Report Year:	2007
Report Title:	KWG 4168-N-Oxid (technical): Effects on survival, growth and reproduction on the earthworm Eisenia fetida tested in artificial soil with 5 % peat
Report No:	LRT-RG-R-27/06
Document No:	M-281617-01-1
Guideline(s) followed in study:	ISO 11268-2: 1998 (E) and OECD 222: April 13, 2004
Deviations from current test guideline:	Yes OECD 222 (2016) 5% sphagnum peat was added to the artificial substrate, however, the guidance suggested 10% sphagnum peat to be added, the survival of the worms would suggest this had no impact to the study.
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The purpose of this study was to assess the effect of KWG 4168-N-Oxid (technical) on survival, growth, and reproduction on the earthworm *Eisenia fetida* during an exposure into an artificial soil with 5 different test concentrations.

Eight replicates with 10 worms each were prepared for the control, whereas each test item rate consisted of four replicates. Earthworms were weighed and assessed for mortality and abnormal behaviour 28 days after test start. At the end of the test after 8 weeks, the number of surviving juveniles per test vessel was determined.

The NOEC and LOEC for growth were 100 and 316 mg test item/kg soil dry weight, the NOEC and LOEC for reproduction were 316 and 1000 mg test item/kg soil dry weight.

I. Materials and Methods

A. Materials

Test Material KWG 4168-N-oxide (technical)

Lot/Batch #: KTS 10324-1-2

Content of a.s. analysed: 86.6 % w/w

Description: Colourless viscous oil

Stability of test compound: Sufficient based on expiry date

Reanalysis/Expiry date: 2007-03-07

Density: Not reported

Treatments

Test rates: 10, 32, 100, 316 and 1000 mg test item/kg dry weight soil

Solvent/vehicle:	Quartz sand
Analysis of test concentrations:	NA
Test organisms	
Species:	*Eisenia fetida*
Source:	In-house culture
Test design	
Test vessel:	Non-re-usable plastic boxes (16.5 x 12 x 6 cm, area approximately 200 cm^2) were used as test vessels. Each test vessel contained an amount of approximately 500 g artificial soil (dry weight) to obtain a depth of approximately 5 cm soil in the test vessels
Test soil:	5% sphagnum peat (shredded), 20% kaolinite clay, 73.82% industrial quartz sand, 0.18% calcium carbonate, 1% dried ground cow manure (food)
Replication:	Four replicates
Number of organisms per vessel:	Ten worms per vessel
Duration of test:	The study consisted of 2 parts. Adult earthworms were exposed to the test item for a period of 4 weeks (first part); after this period, the adults were removed from the test vessels and the cocoons and juvenile earthworms remained in the test vessels for additional 4 weeks (second part). The total duration of each run of the study was 8 weeks.
Environmental test conditions	
Temperature:	Range: 20 ± 2°C
pH:	6.91 – 7.07
Water content:	Then, the soil was moistened with deionised water to reach a water content of 58 % of the maximum water holding capacity
Photoperiod:	16-hour light to 8-hour darkness photoperiod and a light intensity at light period between approximately 400 - 800 Lux

B. Study Design

The purpose of this study was to assess the effect of KWG 4168-N-Oxid (technical) on survival, growth, and reproduction on the earthworm *Eisenia fetida* during an exposure into an artificial soil with 5 different test concentrations.

Nominal test concentrations were 10, 32, 100, 316 and 1000 mg test item/kg dry weight soil.

Ten worms were added to each of the four replicate test vessels. Test vessels were non-re-usable plastic boxes (length x width x height *ca.* 16.5 cm x 12 cm x 6 cm, area approximately 200 cm^2). Each test vessel contained an amount of approximately 500 g artificial soil (dry weight) to obtain a depth of approximately 5 cm soil in the test vessels.

After 4 weeks of exposure, the content of each test vessel was emptied and the living adult earthworms were counted and checked for any abnormal behaviour or other adverse effects (*e.g.* lack of movement, rigidity *etc.*). Those earthworms, which did not move after gentle mechanical stimulus were considered

to be dead. Also missing earthworms (compared to the number of initially placed test organisms) were considered to have died, since dead earthworms completely decompose in the soil in a short time and hence cannot always be found.

At the beginning (prior to exposure) and at the end of the 4 weeks of exposure, the adult test organisms of each vessel were weighed (at the start each individually, at the end together for each test vessel). Before weighing, the earthworms were quickly washed with water; surplus water was absorbed on filter paper.

Changes in body weight values of the surviving test organisms of the treatment groups during the test period were compared to the values of the control group.

At the end of the test after 8 weeks, the number of surviving juveniles per test vessel was determined. The test vessels were placed in a water bath at 50-60 °C for approximately 15-20 minutes. By this treatment, alive juvenile earthworms rose to the soil surface. The emerging earthworms were removed and counted. Afterwards the content of each test vessel was checked additionally by carefully stirring up the artificial soil with the help of tweezers.

The reproduction of the surviving test organisms per test vessel at the end of the study was compared to the control values.

At each feeding date, the amount of food consumed by the adult earthworms was visually estimated for each test vessel.

During the test period, the temperature was in the range of 18 to 22°C.

The measured mean light intensity was 592 lux at day 0, 664 lux at day 28 and 576 lux at day 56 of the study.

II. Results and Discussion

The data were assessed against the criteria of the OECD and ISO test guidelines to which the study was conducted. All validity criteria were met.

- Adult mortality over the initial 4 weeks of the test to be ≤10% (actual: 0%)
- Mean change in growth of the adult earthworms in the control during the exposure period of four weeks should not exceed 20% (actual: +53.7%)
- Each replicate (containing 10 adults) to have produced ≥30 juveniles by the end of the test (actual: 174.9)
- The coefficient of variation of reproduction to be ≤30% (actual: 20.9%)

No mortality of adult earthworms were observed after 28 days test duration in the control group and at any test concentration, including the highest concentration of 1000 mg test item/kg dry weight artificial soil.

Table CA 8.4.1/02-1 Number of surviving adult earthworms and % mortality at day 28

Test concentration (mg test item/kg dry weight soil)	Number of earthworms exposed day 0	Number of earthworms survived day 28	Sum of dead earthworms	% mortality per replicate	% mortality per test concentration
Control	80	80	0	0	0
10	40	40	0	0	0
32	40	40	0	0	0
100	40	40	0	0	0
316	40	40	0	0	0
1000	40	40	0	0	0

The mean body weight of the adult earthworms in the control group had increased during the 4 weeks of exposure. The mean body weight was 0.52 g per worm (+ 53.7% of the mean initial weight).

The mean changes in body weight of the test concentrations of 10, 32 and 100 mg test item/kg soil dry weight were not statistically significantly different relative to the control (+ 51.8%, + 44.1% and + 45.9%). The mean changes in body weight of the test concentrations of 316 and 1000 mg test item/kg soil dry weight were statistically significantly different relative to the control (+ 37.2% and + 23.7%). (Results of a Dunnett´s multiple t-test, two-sided, α = 0.05).

Table CA 8.4.1/02-2 Mean body wet weight of adult earthworms at the test start and after 28 days (values in this table are rounded values)

Test concentration (mg test item/kg dry weight soil)	Number of surviving worms		Mean weight per worm (g)		Mean change of body weight (%)
	Day 0	Day 28	Day 0	Day 28	
Control	80	80	0.34	0.52	53.7 ± 7.4
10	40	40	0.34	0.52	51.8 ± 8.8
32	40	40	0.35	0.50	44.1 ± 3.6
100	40	40	0.34	0.49	45.9 ± 8.6
320	40	40	0.34	0.46	37.2 ± 4.6*
1000	40	40	0.34	0.42	23.7 ± 7.0*

s. Mean value statistically significantly different compared to the control ($p < 0.05$)

In the control group, on average 174.9 juvenile earthworms per test vessel were found (corresponding to a mean reproduction rate of 17.5 juveniles per surviving adult).

In the treatment groups exposed to the test item KWG 4168-N-Oxid (technical) up to and including the highest test concentration of 1000 mg test item/kg soil dry weight, the mean reproduction rate was in the range of 0.4% to 109.2% of the control value.

No statistically significantly different values for the number of juveniles per test vessel relative to the control were observed at the test concentrations of 10, 32, 100 and 316 mg test item/kg soil dry weight. A statistically significantly different value for the number of juveniles per test vessel relative to the control was observed at the test concentration of 1000 mg test item/kg soil dry weight. (Results of a Dunnett´s multiple t-test, one-sided smaller, α = 0.05).

Table CA 8.4.1/02-3 Reproduction of the earthworms

Test concentration (mg test item/kg dry weight soil)	Reproduction rate (per surviving adult)	Juvenile earthworms per test box		
	Mean ± SD	Mean ± SD	CV (%)	% of control
Control	17.5 ± 3.7	174.9 ± 36.6	20.9	100.0
10	16.0 ± 3.3	160.3 ± 33.2	20.7	91.6
32	19.1 ± 4.7	191.0 ± 46.7	24.5	109.2
100	18.3 ± 3.7	183.3 ± 36.7	20.0	104.8
316	12.5 ± 5.9	125.3 ± 59.5	47.5	71.6
1000	0.1 ± 0.1	0.8 ± 1.0	127.7	0.4*

*Mean value statistically significantly different compared to the control ($p < 0.05$)

To verify the sensitivity of the test system, the reference item carbendazim was tested in a separate study. There were statistically significant effects on reproduction at concentrations of ≥1.25 mg a.s./kg soil. The effects on the reduction of reproduction showed that the test system was sensitive and reflected the expected toxicity as given in the OECD 222 test guideline (significant effects observed between 1 and 5 mg a.s./kg soil).

III. Conclusion

No statistically significantly different values for the growth relative to the control were observed at the test concentrations of 10, 32 and 100 mg test item/kg soil dry weight. Statistically significantly different

values for the growth relative to the control were observed at the test concentrations of 316 and 1000 mg test item/kg soil dry weight.

No statistically significantly different values for the number of juveniles per test vessel relative to the control were observed at the test concentrations of 10, 32, 100 and 316 mg test item/kg soil dry weight. A statistically significantly different value for the number of juveniles per test vessel relative to the control was observed at the test concentration of 1000 mg test item/kg soil dry weight.

Considering all measured parameters and endpoints of the study, the overall NOEC is determined to be 100 mg test item/kg soil dry weight. Thus, the overall LOEC is determined to be 316 mg test item/kg soil dry weight.

Assessment and conclusion by applicant:

The study was assessed to current guidance: OECD 222: 29 July, 2016: "OECD Guideline for the Testing of Chemicals – Earthworm Reproduction Test (*Eisenia fetida / Eisenia andrei*)", as this is the most recent version:

- Each replicate (containing 10 adults) to have produced ≥30 juveniles by the end of the test (actual: 174.9)
- The coefficient of variation of reproduction to be ≤30% (actual: 20.9%)
- Adult mortality over the initial 4 weeks of the test to be ≤10% (actual: 0%)

The reference item also demonstrated sufficient sensitivity of the test organisms. The study is therefore considered acceptable.

The NOEC, based on growth, was determined to be 100 mg test item/kg soil dry weight.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.4.1/06
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10 and EC20 values for Eisenia fetida with KWG 4168-N-oxid TG in a reproduction study
Report No:	0471836-ECO12
Document No:	M-760434-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary

The report M-281617-01-1 on the effects of KWG 4168-N-Oxid TG in the earthworm *(Eisenia fetida)* reproduction study did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013. The resulting EC_{10} and EC_{20} values were determined to be 244.53 (95% CL: 236.89 – 250.87) and 286.64 (95% CL: 282.94 - 289.71) mg/kg dws, respectively and are considered reliable as the criteria for goodness of fit were met.

I. Methods

The statistical evaluation was performed with statistical software ToxRat Standard v3.3.0. To calculate ECx values, Probit analysis using linear maximum likelihood regression was performed along with 95% EC_X confidence limits based on Fieller's Theorem.

II. Results

Regarding the calculation of EC_{10} and EC_{20} values for reproduction of the earthworms, the criteria for goodness of fit were met as the P(Chi^2) value was 1.00, showing no significant deviation between fit and data, and a statistically significant concentration/response was found (p(F) < 0.001) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table below.

Table CA 8.4.1/06-1 Results of the Probit analysis (max. likelihood regression) with reproduction at 56 d: Selected effective concentrations (EC_x) of the test item and their 95% confidence limits (according to Fieller`s theorem)

Parameter	Reproduction at test end (56 days)	
	EC_{10} (95 % confidence interval) [mg/kg dws]	**EC_{20} (95 % confidence interval) [mg/kg dws]**
Effect on reproduction	244.53 (236.89 – 250.87)	286.64 (282.94 – 289.71)

The resulting EC_{10} and EC_{20} values were determined to be 244.53 (95% CL: 236.89 – 250.87) and 286.64 (95% CL: 282.94 - 289.71) mg/kg dws, respectively, and are considered reliable as the criteria for goodness of fit were met.

III. Conclusion

The resulting EC_{10} and EC_{20} values were determined to be 244.53 (95% CL: 236.89 – 250.87) and 286.64 (95% CL: 282.94 - 289.71) mg/kg dws, respectively.

Assessment and conclusion by applicant:

The statistical re-evaluation of the reproduction data has determined an EC_{10} of 245 mg/kg dws.

As the EC_{10} is lower than the established NOEC value, the EC_{10} of 245 mg/kg dws would be used in the risk assessment. However, the NOEC based on growth of 100 mg/kg dws remains the most critical endpoint from this study and has therefore been used in the risk assessment.

The values determined in the re-evaluation work are considered to be fully valid.

KWG 4168-carboxylic acid (M06)

Data Point:	KCA 8.4.1/07
Report Author:	[redacted]
Report Year:	2020
Report Title:	KWG 4168-carboxylic acid: Effects on reproduction and growth of earthworms Eisenia andrei in artificial soil
Report No:	152521022
Document No:	M-727123-01-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 (2009) OECD 222: Guideline for the testing of chemicals - Earthworm Reproduction Test (Eisenia fetida/Eisenia andrei; adopted July 29, 2016) ISO-Guideline 11268-2: Soil quality - Effects of pollutants on earthworms - Part 2: Determination of effects on reproduction of Eisenia fetida/Eisenia andrei, 2012
Deviations from current test guideline:	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 purpose of this study was to assess the effect of KWG 4168-carboxylic acid on the reproduction and growth of the earthworm *Eisenia andrei* in artificial soil.

In an 8-week study, earthworms were exposed to KWG 4168-carboxylic acid at nominal concentrations of 1.63, 2.94, 5.29, 9.53, 17.1, 30.9, 55.6 and 100 mg/kg soil. There were 40 earthworms per treatment group, weighing between 302 and 600 mg.

Exposure to KWG 4168-carboxylic acid showed slight mortality of 2.5% and 7.5% at the test concentrations of 30.9 and 55.6 mg/kg, respectively, which was not statistically significantly different as compared to the pooled control (Welsh t-test after Bonferroni Holm, α = 0.05, one-sided greater).

There were no statistically significant differences in growth data up to and including the highest test concentration of 100 mg/kg (Dunnett's t-test, α = 0.05, two-sided). There were no statistically significant differences in reproduction data up to and including the highest test concentration of 100 mg/kg (Dunnett's t-test, α = 0.05, one-sided smaller).

There were no abnormal behaviours observed in any of the treatment groups.

The NOEC and LOEC values for mortality, weight change and reproduction of the earthworm *Eisenia andrei* were determined to be ≥100 and >100 mg/kg, respectively. The LC_{50} and EC_{50} values were both determined to be >100 mg/kg.

I. Materials and Methods

A. Materials

Test Material KWG 4168-carboxylic acid

Lot/Batch #: AE 1344313-01-03

Purity: 90.6%

Description: Turbid brown liquid

Reanalysis/Expiry date: 13 March 2021

Treatments	
Test rates:	1.63, 2.94, 5.29, 9.53, 17.1, 30.9, 55.6 and 100 mg/kg soil
Test organisms	
Species:	Earthworm (*Eisenia andrei*) weighing 302 – 600 mg, approximately 8 months old
Source:	In-house culture
Acclimatisation period:	4 days in artificial soil, under test conditions
Feeding:	Finely ground cattle manure
Test design	
Test vessel:	Plastic boxes (18.3 x 13.6 x 6 cm) with perforated plastic lids
Test medium:	Artificial soil according to OECD 222, 500 g dry weight, 654 g wet weight
Replication:	4 per treatment group, 8 per control
No. animals/vessel:	10 per replicate
Duration of test:	8 weeks
Environmental test conditions	
Temperature:	18 – 22°C
Water content:	Test start: 29.5% – 31.2% (50.0 – 53.0% of the WHC_{max}) Test end: 32.8% – 35.9% (55.5 – 60.9% of the WHC_{max})
pH:	Test start: 6.3 – 6.4 Test end: 6.3 – 6.4
Photoperiod:	16 hours light, 8 hours dark (400 – 800 lux)

B. Study Design

The study was conducted in order to assess the effect of KWG 4168-carboxylic acid on the reproduction and growth of the earthworm *Eisenia andrei* during an exposure into artificial soil at eight different test concentrations.

Ten earthworms were added to each of the four replicate test vessels in the treatment groups and to each of the eight replicate test vessels in the controls. Test vessels were plastic boxes (18.3 x 13.6 x 6 cm) with perforated plastic lids.

The test soil consisted of 69.6% fine quartz sand, 20% kaolinite clay, 10% sphagnum peat and 0.4% calcium carbonate. Prepared soil consisted of approximately 500 g of dry weight and 654 g wet weight.

The earthworms were exposed to nominal concentrations of 1.63, 2.94, 5.29, 9.53, 17.1, 30.9, 55.6 and 100 mg/kg soil.

Incubation was within 18 and 22ºC with a photoperiod of 16 hours light and 8 hours dark at approximately 400 to 800 lux.

After 4 weeks of exposure, the content of each test vessel was emptied and the adult worms were counted, removed and weighed before the substrate was returned to the respective test units minus the worms. Mortality and behavioural abnormalities (e.g. lack of movement and rigidity) were observed at this stage (28 days after application).

At test initiation and after 4 weeks of exposure, the adult test organisms of each vessel were weighed.

At test termination, the number of surviving juveniles per test vessel was determined by placing the test vessels in a water bath of 50 to 60ºC and counting all emerging worms. Reproduction was determined by the number of alive juveniles at test termination.

The earthworms were fed finely ground cattle manure which was added to the soil throughout the duration of the test.

Mortality data were tested for normal distribution and homogeneity of variance ($\alpha = 0.01$) using the Shapiro-Wilk's test and the Levene's test, respectively. As the data were normally distributed and heterogeneous, Welsh t-test after Bonferroni-Holm was used to compare the treatment and pooled control values (multiple comparison, one-sided greater).

Growth and reproduction data were tested for normal distribution and homogeneity of variance ($\alpha = 0.01$) using the Shapiro-Wilk's test and the Levene's test, respectively. Since both the growth and reproduction data were normally distributed and homogeneous but did not follow a monotonicity trend, the Dunnett's t-test was used to compare the treatment and pooled control (growth; multiple comparison, $\alpha = 0.05$, two-sided) and solvent control (reproduction; multiple comparison, $\alpha = 0.05$, one-sided smaller) values.

II. Results and Discussion

The data were assessed against the criteria of the OECD and ISO test guidelines to which the study was conducted. All validity criteria were met:

- Each replicate (containing 10 adults) to have produced ≥30 juveniles by the end of the test (actual: 162 - 183)
- The coefficient of variation to be ≤30% (actual: 4.0%)
- Adult mortality over the initial 4 weeks of the test to be ≤10% (actual: 0%)

A mortality of 0% was observed in the control and solvent control. No mortality was observed in the test item treated groups except at the test concentration of 30.9 mg test item/kg soil where 2.5% mortality was observed and at the test concentration of 55.6 mg test item/kg soil where 7.5% mortality was observed. The mortality was not statistically significantly different compared to the pooled control (Welsh t-test after Bonferroni-Holm, one-sided greater, $\alpha = 0.05$). The Welsh t-test after Bonferroni-Holm showed no significant statistical difference at the two test concentrations where mortality was observed compared to the pooled control. At the other test concentrations without mortality, the test could not be performed due to mathematical reasons. Nevertheless, as there was no mortality observed, no test item related effect was observed.

Table CA 8.4.1/07-1 Summary of adult mortality following 4 weeks of exposure

Treatment group (mg/kg)	Mean number of live adults		Mean mortality (%) ± SD[1]	Significance
	Start	4 weeks		
Control	10	10	0.0 ± 0.0	-
Solvent control	10	10	0.0 ± 0.0 Pooled control 0.0 ± 0.0	n.s. [1)]
1.63	10	10	0.0 ± 0.0	n.s.[+]
2.94	10	10	0.0 ± 0.0	n.s.[+]
5.29	10	10	0.0 ± 0.0	n.s.[+]
9.53	10	10	0.0 ± 0.0	n.s.[+]
17.1	10	10	0.0 ± 0.0	n.s.[+]
30.9	10	9.75	2.5 ± 5.0	n.s.
55.6	10	9.25	7.5 ± 9.6	n.s.
100	10	10	0.0 ± 0.0	n.s.[+]

- not relevant

[1] mean ± standard deviation of 4 replicates (8 in the controls)
n.s.[1)] not significantly different compared to the control, Fisher's exact test, α = 0.05, two-sided
n.s.[+] test could not be performed due to mathematical reasons, but as no mortality was observed they are determined to be not statistically significantly different compared to the pooled control
n.s. not significantly different compared to the pooled control, Welsh t-test after Bonferroni Holm, α = 0.05, one-sided greater

The body weight changes in the test item treated groups were not statistically significantly different compared to the pooled control up to and including the highest test concentration of 100 mg/kg (Dunnett's t-test, α = 0.05, two-sided).

Table CA 8.4.1/07-2 Body weight data observed following 4 weeks exposure

Treatment group (mg/kg)	Body weight range (mg)		Body weight change		Significance
	Start	4 weeks	Mean ± SD[1] (mg)	Mean ± SD[1] (%)	
Control	359.7 – 424.8	468.0 – 509.8	89 ± 26	23.0 ± 7.3	-
Solvent control	360.3 – 430.2	461.4 – 493.2	97 ± 18 Pooled control 93 ± 22	24.8 ± 5.4 Pooled control 23.9 ± 6.3	n.s.[1)]
1.63	360.5 – 418.5	458.1 – 490.8	89 ± 15	22.9 ± 4.9	n.s.
2.94	362.3 – 416.9	494.8 – 543.1	119 ± 30	30.6 ± 8.9	n.s.
5.29	365.0 – 412.2	444.3 – 513.3	96 ± 21	24.5 ± 5.3	n.s.
9.53	367.6 – 409.0	494.6 – 524.3	102 ± 24	26.4 ± 6.7	n.s.
17.1	370.4 – 408.1	478.2 – 496.3	95 ± 17	24.6 ± 5.3	n.s.
30.9	374.7 – 408.0	491.9 – 513.5	114 ± 18	29.4 ± 5.3	n.s.
55.6	375.1 – 404.1	455.2 – 519.1	104 ± 33	26.8 ± 9.1	n.s.
100	375.5 – 402.9	477.2 – 515.4	100 ± 19	25.7 ± 5.1	n.s.

- not relevant
[1] mean ± standard deviation of 4 replicates (8 in the controls)
n.s.[1)] not significantly different compared to the control, Student t-test, α = 0.05, two-sided
n.s. not significantly different compared to the pooled control, Dunnett's t-test, α = 0.05, two-sided

The reproduction rates were not statistically significantly different compared to the solvent control up to and including the highest test concentration (Dunnett's t-test, α = 0.05, one-sided smaller).

Table CA 8.4.1/07-3 Reproduction data observed following 4 weeks exposure

Treatment group (mg/kg)	Number of juvenile earthworms		Significance
	Mean ± SD[1]	% of solvent control	
Control	173 ± 7	-	-
Solvent control	158 ± 17		*
1.63	152 ± 20	96.6	n.s.
2.94	129 ± 22	82.0	n.s.
5.29	148 ± 21	94.1	n.s.
9.53	162 ± 20	102.5	n.s.
17.1	163 ± 22	103.3	n.s.
30.9	161 ± 14	102.1	n.s.
55.6	145 ± 10	92.0	n.s.
100	160 ± 20	101.2	n.s.

- not relevant
[1] mean ± standard deviation of 4 replicates (8 in the controls)
* significantly different compared to the control, Student t-test, α = 0.05, two-sided
n.s. not significantly different compared to the solvent control, Dunnett's t-test, α = 0.05, one-sided smaller

To verify the sensitivity of the test system, the reference item carbendazim was tested in a separate study. There were statistically significant effects on reproduction at a concentration of 0.695 mg a.s./kg soil and above. The EC_{50} for reproduction was calculated as 0.92 mg a.s./kg soil. The effects on the

reduction of reproduction showed that the test system was sensitive and reflected the expected toxicity as given in the OECD 222 test guideline (significant effects observed between 1 and 5 mg a.s./kg soil).

III. Conclusion

In an earthworm reproduction and growth study with KWG 4168-carboxylic acid, the NOEC for mortality of the earthworm *Eisenia andrei* was determined to be ≥100 mg/kg soil. The LOEC for mortality was estimated to be >100 mg/kg soil. The LC_{50} was estimated to be >100 mg/kg soil.

The NOEC for growth was determined to be ≥100 mg/kg soil. The LOEC for growth was estimated to be >100 mg/kg soil.

The NOEC for reproduction was determined to be ≥100 mg/kg soil. The LOEC for reproduction was estimated to be >100 mg/kg soil. Due to the lack of a concentration-response relationship, no reliable EC_x-calculation was possible. Therefore no EC_{10} can be reported, and the EC_{20} and EC_{50} values were estimated to be >100 mg/kg soil as there were no effects >20% or >50% effects in reproduction observed at any test item concentration.

Assessment and conclusion by applicant:

This is a new study that has not been previously submitted for evaluation.

Validity criteria according to the OECD 222 guideline (2016) were met.

- Each replicate (containing 10 adults) to have produced ≥30 juveniles by the end of the test (actual: 162 - 183)
- The coefficient of variation to be ≤30% (actual: 4.0%)
- Adult mortality over the initial 4 weeks of the test to be ≤10% (actual: 0%)

The reference item also demonstrated sufficient sensitivity of the test organisms. The study is therefore considered acceptable.

The NOEC, based on reproduction and growth, was determined to be ≥100 mg/kg soil.

Acute earthworm studies

Acute studies using earthworms are no longer a data requirement but for completeness the available acute data with spiroxamine technical have been presented below as supporting information only.

Data Point:	KCA 8.4.1/03
Report Author:	[redacted]
Report Year:	1993
Report Title:	Toxicity of KWG 4168 (techn.) to earthworms
Report No:	HBF/RG 181
Document No:	M-008806-01-1
Guideline(s) followed in study:	OECD Guideline No. 207: OECD Guideline for Testing of Chemicals, 'Earthworm, Acute Toxicity Tests' (4 April 1984)
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (1997), RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

The purpose of this study was to assess the acute toxicity of KWG 4168 (tech.) on survival of the earthworm *Eisenia fetida* during an exposure into an artificial soil with five different test concentrations.

Adult earthworms more than two months old were used for the test. The test substance was applied to the substrate using acetone as a vehicle, and later sprayed onto the artificial soil. Four replicates with 10 worms each were prepared for the control and for the test groups. Earthworms were weighed at test start and at Day 14. Mortality observations were conducted at Days 7 and 14. Moisture content and maximum water holding capacity of the test substrate was determined at the start and end of the test.

The 14-day LC_{50} determined for earthworms exposed to KWG 4168 (tech.) was >1000 mg a.s./kg dry weight substrate.

The lowest observed effect concentration (LOEC) was determined to be 562 mg a.s./kg dry weight substrate and the no observed effect concentration (NOEC) was determined to be 316 mg a.s./kg dry weight substrate.

The lowest lethal concentration (LLC) was determined to be >1000 mg a.s./kg dry weight substrate. The observed effect threshold (MATC) was determined to be 421 mg a.s./kg dry weight substrate.

I. Materials and Methods

A. Materials

Test Material	KWG 4168 (tech.)
Lot/Batch #:	898 114 002
Content of a.s.:	97.8%
Description:	Colourless liquid
Stability of test compound:	Not reported
Reanalysis/Expiry date:	Not reported
Density:	Not reported
Treatments	
Test rates:	100, 178, 316, 562 and 1000 mg a.s./kg dry weight substrate
Solvent/vehicle:	Acetone
Analysis of test concentrations:	NA
Test organisms	
Species:	*Eisenia fetida*
Source:	Strain of Prof. Graff, Forschungsanstalt für Landwirtschaft, 38104 Braunschweig, Germany). This strain has been held in the laboratory of the testing facility for several years at 22 ± 1°C, 70 – 90% relative humidity, 12:12 hours light-dark cycle
Test design	
Test vessel:	1.5 L preserving jars, covered with glass lids, and labelled
Test soil:	10% dried, finely ground sphagnum peat, 20% kaolinite clay, 69% fine quartz sand, 1% calcium carbonate
Replication:	Four replicates per concentration
Number of organisms per vessel:	Ten worms per vessel

Duration of test:	14 days
Environmental test conditions	
Temperature:	Range: 20 ± 1°C
pH:	Test start: 6.16 – 6.20 Test end: 6.15 – 6.18
Water content:	Test stat: approx. 31% Test end: 30.0 – 30.3%
Photoperiod:	16-hour light to 8-hour darkness photoperiod and a light intensity of approximately 400 – 800 lux

B. Study Design

The purpose of this study was to assess the effect of KWG 4168 (tech.) on survival of the earthworm *Eisenia fetida* during an exposure into an artificial soil with five different test concentrations.

Nominal test concentrations were 100, 178, 316, 562 and 1000 mg a.s./kg dry weight soil.

Ten worms were added to each of the four replicate test vessels. Test containers were 1.5 Litre preserving jars, covered with glass lids. Five hundred grams dry weight substrate (725 g wet weight) was added to each test container.

Incubation was at 20 ± 1°C with a constant photoperiod at approximately 400 – 800 lux.

Seven days after the start of the study, the number of surviving earthworms was counted by emptying the substrate out onto an inert surface and removing the earthworms by hand. The animals were then returned to the test container with the substrate. After 14 days, the weight as well as the number of surviving earthworms were determined. Earthworms which show no reaction upon being prodded with a blunt probe are considered dead.

The moisture content of the peat and the test substrate as well as the maximum water capacity of the test substrate were determined with the aid of a hydrometer at 105°C, the pH with an electronic measuring instrument.

II. Results and Discussion

The test was conducted in accordance with the OECD Guideline No. 207: OECD Guideline for Testing of Chemicals, 'Earthworm, Acute Toxicity Tests' (4 April 1984). The following guidelone validity criterion was met:

- The mortality in the controls should not exceed 10% (actual: 0%)

The study is therefore considered acceptable.

No mortality of adult earthworms were observed after 14 days test duration in the control group and at any test concentration, including the highest concentration of 1000 mg a.s./kg dry weight artificial soil.

The LC_{50} of the reference substance was within the usual range. The test conditions are therefore equivalent to the standard.

Table CA 8.4.1/03-1 Individual figures obtained in the study with KWG 4168 (tech.)

Concentration (mg a.s./kg dry weight substrate)	Number of surviving worms			Mean weight of worms (g)	
	Day 0	Day 7	Day 14	Day 0	Day 14
Control	40	40	40	0.43	0.41
100	40	40	40	0.43	0.41
178	40	40	40	0.44	0.42
316	40	40	40	0.43	0.38

Concentration (mg a.s./kg dry weight substrate)	Number of surviving worms			Mean weight of worms (g)	
	Day 0	Day 7	Day 14	Day 0	Day 14
562	40	40	40	0.44	0.36
1000	40	40	40	0.44	0.30

III. Conclusion

The 14-day LC_{50} determined for earthworms exposed to KWG 4168 (tech.) was >1000 mg a.s./kg dry weight substrate.

Related to weight alteration and symptoms the no-observed effect concentration (NOEC) was 316 mg a.s./kg dry weight substrate, the lowest observed effect concentration (LOEC) was 562 mg a.s./kg and the observed effects threshold 421 mg a.s./kg (MATC, geometric mean of NOEC and LOEC). The lowest tested concentration with mortality (LLC) was >1000 mg a.s./kg dry weight substrate.

The values given are nominal concentrations.

Assessment and conclusion by applicant:

The test was conducted in accordance with the OECD Guideline No. 207: OECD Guideline for Testing of Chemicals, 'Earthworm, Acute Toxicity Tests' (4 April 1984) which is still the current version. The following validity criterion applies:

- The mortality in the controls should not exceed 10% (actual: 0%)

The study is therefore considered acceptable on the basis that the validity criterion has been met. It is noted that acute earthworm studies are no longer a data requirement therefore this study has been submitted for completeness but considered to be supporting information only.

CA 8.4.2 Effects on non-target soil meso and macrofauna (other than earthworms)

The available data for meso- and macro-fauna other than arthworms are presented in the table below.

Table CA 8.4.2-01 Summary of soil macro-organism (other than earthworm) toxicity studies with spiroxamine and metabolites

Organism	Test item	Test type	Endpoints		Reference
Folsomia candida	Spiroxamine	28 d Chronic toxicity; 5% peat	NOEC 32 mg a.s./kg soil dw;	EU	M-289274-01-1
		Statistical Re-analysis	EC_{10}/EC_{20} not determinable	NEW	M-760433-01-1
Folsomia candida	Spiroxamine	28 d Chronic toxicity; 5% peat	NOEC 75 mg a.s./kg soil dw;	NEW	M-405276-01-1
		Statistical Re-analysis	EC_{10} 175 mg a.s./kg soil dw EC_{20} 258 mg a.s./kg soil dw	NEW	M-761559-01-1
Folsomia candida	KWG 4168-desethyl (M01)	28 d Chronic toxicity; 5% peat	NOEC 316 mg/kg soil dw;	EU	M-289321-01-1
		Statistical Re-analysis	EC_{10}/EC_{20} not determinable	NEW	M-760431-01-1
Folsomia candida	KWG 4168-despropyl (M02)	28 d Chronic toxicity; 5% peat	NOEC 316 mg/kg soil dw;	EU	M-288905-01-1

Organism	Test item	Test type	Endpoints		Reference
		Statistical Re-analysis	EC_{10} 308 mg/kg soil dw EC_{20} 402 mg/kg soil dw	NEW	M-760410-01-1
Folsomia candida	KWG 4168-N-oxide (M03)	28 d Chronic toxicity; 5% peat	NOEC 100 mg/kg soil dw; EC_{10} >100 mg/kg soil dw;	NEW	M-687854-01-1
Folsomia candida	KWG 4168-acid (M06)	28 d Chronic toxicity; 5% peat	NOEC 1000 mg/kg soil dw; EC_{10} >1000 mg/kg soil dw;	NEW	M-727126-01-1
Hypoaspis aculeifer	KWG 4168-desethyl (M01)	14 d Chronic toxicity; 5% peat	NOEC 50 mg/kg soil dw; EC_{10} 94.1 mg/kg soil dw;	NEW	M-680684-01-1
Hypoaspis aculeifer	KWG 4168-despropyl (M02)	14 d Chronic toxicity; 5% peat	NOEC 100 mg/kg soil dw; EC_{10} >100 mg/kg soil dw;	NEW	M-680694-01-1
Hypoaspis aculeifer	KWG 4168-N-oxide (M03)	14 d Chronic toxicity; 5% peat	NOEC 100 mg/kg soil dw; EC_{10} >100 mg/kg soil dw;	NEW	M-680687-01-1
Hypoaspis aculeifer	KWG 4168-acid (M06)	14 d Chronic toxicity; 5% peat	NOEC 1000 mg/kg soil dw; EC_{10} >1000 mg/kg soil dw;	NEW	M-727128-02-1

EU: previously evaluated as part of the original EU review and listed in EFSA conclusion and DAR
NEW: new study or data generated since the previous EU review or previously not submitted

CA 8.4.2.1 Species level testing

Data Point:	KCA 8.4.2.1/01
Report Author:	[redacted]
Report Year:	2007
Report Title:	KWG 4168 tech.: Influence on the reproduction of the collembola species Folsomia candida tested in artificial soil with 5 percent peat
Report No:	FRM-COLL-52/07
Document No:	M-289274-01-1
Guideline(s) followed in study:	ISO 11267 (1999)
Deviations from current test guideline:	Yes OECD 232 (2016) The reference item used was not boric acid, as recommended by the OECD 232 test guideline, however the reference item used was acceptable under the ISO guideline to which the study was conducted and was considered to sufficiently demonstrate the sensitivity of the test organism.
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Collembola (*Folsomia candida*) aged 10 to 12 days were exposed to KWG 4168 technical incorporated into soil in a 4-week study to assess effects on mortality and reproduction.

Test organisms were exposed to 10, 32, 100, 316 and 1000 mg a.s./kg soil dry weight and to a water control. Betosip (a.s. phenmedipham) was used as a toxic standard in accordance with ISO 11267 (1999) guidelines.

A statistically significant reduction in number of juveniles compared to the control was observed in the treatment groups with 100, 316 and 1000 mg a.s./kg soil dry weight, resulting in reductions of 21.4, 21.9 and 95.6%, respectively.

The NOEC and LOEC for reproduction were 32 and 100 mg a.s./kg soil dry weight, respectively.

I. Materials and Methods

A. Materials

Test Material

Test Material	KWG 4168 tech.
Lot/Batch #:	EDTH004650
Purity:	95.1%
Description:	Light brown clear, oily liquid
Stability of test compound:	Not reported
Reanalysis/Expiry date:	14 August 2007
Density:	Not reported

Treatments

Test rates:	10, 32, 100, 316 and 1000 mg a.s./kg soil dry weight
Solvent/vehicle:	Because the test item was poorly soluble in water, a test item quartz sand mixture for each concentration was prepared

Analysis of test concentrations:	No
Test organisms	
Species:	*Folsomia candida,* Collembola, Isotomidae
Source:	Bred at Bayer CropScience since April 2002. The strain was originally obtained from Ibacon, Institute for Analytic and Consulting, GmbH, D – 64380 Rossdorf.
Acclimatisation period:	None reported
Feeding:	Approximately 2 mg granulated dry yeast at the start of the study and after 14 days
Treatment for disease:	None reported
Test design	
Test vessel:	Glass vessels (volume: 140 mL, diameter: 5 cm) covered with glass lids
Test medium:	Artificial soil according to OECD 207 (1984). With respect to the properties of the test item (log P_{ow} 2) 5% peat instead of 10% peat were used considering the influence on bioavailability (EPPO 2002).
Replication:	5 (+1 without Collembola for measurement of soil moisture during the test and pH and soil moisture at the end of the study)
No. animals/vessel:	10
Duration of test:	28 days
Environmental test conditions	
Temperature:	20 ± 2°C
Photoperiod:	16 h light : 8 h dark at 450 – 645 lux

B. Study Design

Collembola (*Folsomia candida*) were exposed to KWG 4168 technical over 4 weeks to assess the effects on mortality and reproduction.

The Collembola were 10 to 12 days old at the start of the study. For each replicate, 10 of the juvenile Collembola were placed in the glass test vessels, which had been prepared with the test item quartz sand and artificial soil. The soil was aligned with OECD 207 (1984) standard, but with 5% peat instead of 10% due to considerations on the influence on bioavailability with respect to the test item. The required amount of the test item was mixed thoroughly with 5 g quartz sand. If less than 50 mg test item was required, the test item had to be mixed with quartz sand and a stock mixture was prepared and diluted with quartz sand to reach the demanded test concentrations. Water was added to the soil mix until 50% water holding capacity was achieved.

The artificial soil was kept at 18 to 22°C, with the temperature continuously recorded by a thermo-hygrograph integrated in the climatic chamber. The test vessels were kept at 450 to 645 lux under a photoperiod of 16 h light : 8 h dark, monitored by an integrated luxmeter of the climatic chamber.

Application rates in this study were 10, 32, 100, 316 and 1000 mg a.s./kg soil dry weight. Five replicates were exposed to control (water) treatment, and five replicated to 10, 32, 100, 316 and 1000 mg a.s./kg soil dry weight. During the study, they were fed with granulated dry yeast.

A reference test with the toxic standard, Betosip, was performed at least once a year to ensure that the laboratory test conditions were adequate and to verify that the response of the test organisms does not change significantly over time.

Water content was checked 14 days after application by reweighing the additional vessels. If the water loss exceeded 2% of the initial water content, the missing amount of water was added to all vessels of the treatment group. At the same time, the food was checked and the Collembola were fed again if necessary. Mortality and reproduction were reported after 28 days and were determined by the number of dead adult Collembola and the number of living juveniles detected using digital images.

II. Results and Discussion

Validity criteria, according to the test guideline that the study was conducted to, were assessed. In the control, the following criteria were met:

- Mean adult mortality <20% at the end of the test (actual: 12%);
- The mean number of juveniles per vessel ≥100 at the end of the test (actual: 742);
- The coefficient of variation calculated for the number of juveniles ≤30% (actual: 21%).

Mortality did not vary from the control greater than 6% at test item concentrations between 10 and 316 mg a.s./kg soil dry weight. At the highest test item concentration, 1000 mg a.s./kg soil dry weight, 86% mortality was observed in adult Collembola.

Table CA 8.4.2.1/01-1 Survival of adult Collembola after 4 weeks treatment (n=10/replicate)

	mg a.s./kg soil dry weight					
	Control	**10**	**32**	**100**	**316**	**1000**
Mean[1]	8.8	8.8	9.6	9.2	9.2	1.4
SD[1]	1.1	1.6	0.5	0.8	1.3	0.5
% mortality[2]	12.0	12.0	4.0	8.0	8.0	86.0

1 mean and standard deviation (SD) of five replicates
2 formula: ((initial placed organisms per vessel – mean of surviving adults per vessel) / 10) * 100

Reproduction in the Collembola was statistically different to the control at 100, 316 and 1000 mg test item/kg artificial soil dry weight treatment groups. At the highest test item concentration, 1000 mg test item/kg artificial soil dry weight, reproduction was at 4.4% of the control.

Table CA 8.4.2.1/01-2 Reproduction of the Collembola after 4 weeks treatment (juveniles/replicate)

	mg test item/kg artificial soil dry weight					
	Control	**10**	**32**	**100**	**316**	**1000**
Mean[1]	741.6	864.8	726.2	583.0	579.0	32.4
SD[1]	156.9	79.4	124.5	112.2	101.1	32.9
CV[2]	21.2	-	-	-	-	-
% of control[3]	-	116.6	97.9	78.6*	78.1*	4.4*

1 mean and standard deviation (SD) of five replicates
2 Coefficient of Variation
3 formula: mean number of juveniles per treatment group * 100 / mean number of juveniles per control group
- = not applicable
* = significantly different compared to the control (Dunnett's Test, one-sided-smaller, α = 0.05)

To demonstrate the sensitivity of the test system Betosip (phenmedipham 15.4 %) as a toxic standard was regularly tested (once a year) at concentrations of 89, 133, 200 and 300 mg test item/kg artificial soil dry weight. In the most recent test the mortality rate of adult Collembola was 8 %, 14 %, 22 % and 32 % at 89, 133, 200 and 300 mg Betosip/kg artificial soil dry weight. In all treatment groups the number of juveniles was statistically significant reduced in comparison to the control. The $NOEC_{reproduction}$ was < 89 mg Betosip (13.7 mg a.s)/kg artificial soil dry weight and the $LOEC_{reproduction}$ was 89 mg Betosip (13.7 mg a.s)/kg artificial soil dry weight. The results were considered to demonstrate the sensitivity of the test system.

III. Conclusion

In the control group, 12% of the adult Collembola died which is within the tolerated range of ≤20% mortality recommended by the guideline. The highest mortality rate of 86% was found in the test item concentration of 1000 mg a.s./kg soil dry weight. Concerning the number of juveniles, statistical analysis revealed significant differences between the control and the treatment groups at 100, 316 and 1000 mg a.s./kg soil dry weight.

The NOEC for reproduction: 32 mg a.s./kg soil dry weight

The LOEC for reproduction: 100 mg a.s./kg soil dry weight.

Assessment and conclusion by applicant:

Validity criteria according to the current OECD 232 guideline (2016) were met. In the control, the following criteria were met:

- Mean adult mortality <20% at the end of the test (actual: 12%);
- The mean number of juveniles per vessel >100 at the end of the test (actual: 742);
- The coefficient of variation calculated for the number of juveniles <30% (actual: 21%).

The study was not specifically conducted to the OECD 232 test guideline, however the test design and the parameters assessed are consistent with the OECD test guideline. Furthermore the OECD validity criteria have been met.

The reference item used was not boric acid, as recommended by the OECD 232 test guideline, however the reference item used was acceptable under the ISO guideline to which the study was conducted and was considered to sufficiently demonstrate the sensitivity of the test organism.

The study is therefore considered acceptable.

The NOEC for reproduction: 32 mg a.s./kg soil dry weight

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.4.2.1/04
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10 and EC20 values for Folsomia candida with spiroxamine TG in a reproduction study
Report No:	0471836-ECO14
Document No:	M-760433-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary

The report M-289274-01-1 on the effects of spiroxamine TG in a springtail (*Folsomia candida*) reproduction study did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013. Due to the wide span of the confidence limits, unfit EC_{10} and EC_{20} obtained values and due to the lack of a significant dose response with some

models, when compared to the control, it was not possible to calculate reliable EC_{10} and EC_{20} values for reproduction.

I. Methods

The statistical evaluation was performed with statistical software ToxRat Standard v3.3.0. For the calculation of EC_{10} and EC_{20} values, several statistical models were used. However, due to the wide span of the confidence limits, unfit EC_{10} and EC_{20} obtained values and due to the lack of a significant dose response with some models, when compared to the control, it was not possible to calculate reliable EC_{10} and EC_{20} values for reproduction.

II. Results

Due to the wide span of the confidence limits, unfit EC_{10} and EC_{20} obtained values and due to the lack of a significant dose response with some models, when compared to the control, it was not possible to calculate reliable EC_{10} and EC_{20} values for reproduction.

III. Conclusion

Due to the wide span of the confidence limits, unfit EC_{10} and EC_{20} obtained values and due to the lack of a significant dose response with some models, when compared to the control, it was not possible to calculate reliable EC_{10} and EC_{20} values for reproduction.

Assessment and conclusion by applicant:

The statistical re-evaluation of the reproduction data could not calculate reliable EC_{10} and EC_{20} values.

The NOEC of 32 mg a.s./kg dws shall be used in the risk assessment as the most critical endpoint from this study.

The values determined in the re-evaluation work are considered to be fully valid.

Data Point:	KCA 8.4.2.1/05
Report Author:	[redacted]
Report Year:	2011
Report Title:	Spiroxamine a.s.: Influence on the reproduction of the collembolan species Folsomia candida tested in artificial soil
Report No:	FRM-COLL-119/11
Document No:	M-405276-01-1
Guideline(s) followed in study:	OECD 232 adopted, September 07, 2009: OECD Guidelines for Testing Chemicals - Collembolan Reproduction Test in Soil
Deviations from current test guideline:	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

Collembola (*Folsomia candida*) aged 10 to 12 days were exposed to spiroxamine technical incorporated into soil in a 4-week study to assess effects on mortality and reproduction.

Test organisms were exposed to 18.75, 37.5, 75, 150, 300 and 600 mg a.s./kg soil dry weight and to a water control.

The highest mortality rate of 35% was found in the test item concentration of 600 mg a.s./kg soil dry weight. The LC_{50} for mortality was considered to be >600 mg a.s./kg soil dry weight.

A statistically significant reduction in number of juveniles compared to the control was observed in the treatment groups with 150, 300 and 600 mg a.s./kg soil dry weight, resulting in reductions of 13, 22 and 61%, respectively.

The NOEC and LOEC for reproduction were 75 and 150 mg a.s./kg soil dry weight, respectively. The EC_{50} for reproduction was determined to be 501 mg a.s./kg soil dry weight.

I. Materials and Methods

A. Materials

Test Material	Spiroxamine
Lot/Batch #:	EDTH008883
Purity:	98.2% w/w
Description:	Light yellow liquid
Stability of test compound:	Not reported
Reanalysis/Expiry date:	16 December 2012
Density:	Not reported
Treatments	
Test rates:	18.75, 37.5, 75, 150, 300 and 600 mg a.s./kg soil dry weight
Solvent/vehicle:	None. A test item quartz sand mixture for each concentration was prepared.
Analysis of test concentrations:	No
Test organisms	
Species:	*Folsomia candida*, Collembola, Isotomidae
Source:	Bred at Bayer CropScience since April 2002. The strain was originally obtained from Ibacon, Institute for Analytic and Consulting, GmbH, D – 64380 Rossdorf.
Acclimatisation period:	None reported
Feeding:	Approximately 2 mg granulated dry yeast at the start of the study and after 14 days
Treatment for disease:	None reported
Test design	
Test vessel:	Glass vessels (volume: 140 mL, diameter: 5 cm) covered with glass lids each containing 30 g test soil.
Test medium:	Artificial soil according to OECD 232 (74.8% fine quartz sand, 5% sphagnum peat, 20% kaolin clay and 0.2% $CaCO_3$)
Replication:	8 for control and 4 for each test item concentration (one additional replicate also prepared without Collembola for measurement of soil moisture during the test and pH and soil moisture at the end of the study)

No. animals/vessel:	10
Duration of test:	28 days
Environmental test conditions	
Temperature:	20 ± 2°C
Photoperiod:	16 h light : 8 h dark at 545 – 663 lux

B. Study Design

Collembola (*Folsomia candida*) were exposed to spiroxamine technical over 4 weeks to assess the effects on mortality and reproduction.

The Collembola were 10 to 12 days old at the start of the study. For each replicate, 10 of the juvenile Collembola were placed in the glass test vessels, which had been prepared with the test item quartz sand and artificial soil. The soil was prepared in accordance with the OECD 232 test guideline. The required amount of the test item was mixed thoroughly with quartz sand and then mixed thoroughly with the artificial soil.

The artificial soil was kept at 18 to 22°C, with the temperature continuously recorded in the climatic chamber. The test vessels were kept at 545 to 663 lux under a photoperiod of 16 h light : 8 h dark.

Application rates in this study were 18.75, 37.5, 75, 150, 300 and 600 mg a.s./kg soil dry weight. Eight replicates were exposed to control (water) treatment, and four replicated to the test item treatments. During the study, they were fed with granulated dry yeast.

At test start each test vessel was weighed for the determination of water loss. After 14 days the loss of water was determined by reweighing the test vessels. The vessels were rewetted with the approximately 2-fold amount of the missing water. The test vessels were set up randomised in a climatic test room. After 7, 14 and 21 days the test vessels were re-randomised.

Mortality and reproduction were reported after 28 days and were determined by the number of dead adult Collembola and the number of living juveniles detected using digital images.

II. Results and Discussion

Validity criteria, according to the test guideline that the study was conducted to, were assessed. In the control, the following criteria were met:

- Mean adult mortality ≤20% at the end of the test (actual: 5%);
- The mean number of juveniles per vessel ≥100 at the end of the test (actual: 1570);
- The coefficient of variation calculated for the number of juveniles ≤30% (actual: 12%).

Mortality did not vary from the control greater than 7.5% at test item concentrations between 18.75 and 300 mg a.s./kg soil dry weight. At the highest test item concentration, 600 mg a.s./kg soil dry weight, 35% mortality was observed in adult Collembola. A LC_{50} could not be calculated and was therefore considered to be > 600 mg test item/kg dry weight artificial soil.

Table CA 8.4.2.1/05-1 Survival of adult Collembola after 4 weeks treatment (n=10/replicate)

	mg a.s./kg soil dry weight						
	Control	**18.75**	**37.5**	**75**	**150**	**300**	**600**
Mean[1]	9.5	9.3	9.7	9.5	9.8	9.8	6.5
SD[1]	0.5	1.0	0.6	0.6	0.5	0.5	2.4
% mortality[2]	5.0	7.5	3.3	5.0	2.5	2.5	35.0

[1] mean and standard deviation (SD) of 8 replicates in the control and 4 replicates per test item concentration
[2] formula: ((initial placed organisms per vessel – mean of surviving adults per vessel) / 10) * 100

Reproduction in the Collembola was statistically different to the control at 150, 300 and 600 mg a.s./kg artificial soil dry weight treatment groups. At the highest test item concentration, 600 mg a.s./kg artificial soil dry weight, reproduction was at 39.5% of the control.

The No-Observed-Effect-Concentration ($NOEC_{reproduction}$) was 75 mg a.s./kg artificial soil dry weight. The Lowest-Observed-Effect-Concentration ($LOEC_{reproduction}$) was 150 mg a.s./kg artificial soil dry weight. The EC_{50} for reproduction, determined by probit analysis, was 501 mg a.s./kg artificial soil dry weight (95 % confidence limit 422 – 637 mg a.s./kg artificial soil dry weight).

Table CA 8.4.2.1/05-2 Reproduction of the Collembola after 4 weeks treatment (juveniles/replicate)

	mg test item/kg artificial soil dry weight						
	Control	**18.75**	**37.5**	**75**	**150**	**300**	**600**
Mean[1]	1569.6	1587.8	1520.7	1578.0	1370.3	1220.0	619.8
SD[1]	188.3	207.6	136.9	92.2	96.1	120.0	74.2
CV[2]	12.0						
% of control[3]	-	101.2	96.9	100.5	87.4*	77.7*	39.5*

[1] mean and standard deviation (SD) of 8 replicates in the control and 4 replicates per test item concentration
[2] Coefficient of Variation
[3] formula: mean number of juveniles per treatment group * 100 / mean number of juveniles per control group
- = not applicable
* = significantly different compared to the control (Dunnett's Test, one-sided-smaller, $\alpha = 0.05$)

To demonstrate the sensitivity of the test system, boric acid as a toxic standard was tested at concentrations of 44, 67, 100, 150 and 225 mg test item/kg artificial soil dry weight. In the most recent test boric acid showed an EC_{50} of 91 mg test item/kg artificial soil dry weight (95 % confidence limits from 80 mg to 104 mg Boric acid/kg artificial soil dry weight) for reproduction. The result is in the recommended range of the guideline (about 100 mg Boric acid/kg artificial soil dry weight). The $NOEC_{reproduction}$ was calculated to be 44 mg Boric acid/kg artificial soil dry weight and accordingly the $LOEC_{reproduction}$ is 67 mg Boric acid/kg artificial soil dry weight according Williams-Test multiple t-test procedure, $\alpha = 0.05$, one-sided smaller. This demonstrated that the test organisms were sufficiently sensitive.

III. Conclusion

In the control group, 5% of the adult Collembola died which is within the tolerated range of ≤20% mortality recommended by the guideline. The highest mortality rate of 35% was found in the test item concentration of 600 mg a.s./kg soil dry weight. Concerning the number of juveniles, a statistically significant reduction in number of juveniles compared to the control was observed in the treatment groups with 150, 300 and 600 mg a.s./kg soil dry weight, resulting in reductions of 13, 22 and 61%, respectively.

The LC_{50} for mortality was considered to be > 600 mg a.s./kg soil dry weight.

The NOEC and LOEC for reproduction were 75 and 150 mg a.s./kg soil dry weight, respectively. The EC_{50} for reproduction was determined to be 501 mg a.s./kg soil dry weight.

Assessment and conclusion by applicant:

Validity criteria according to the current OECD 232 guideline (2016) were met. In the control, the following criteria were met:

- Mean adult mortality ≤20% at the end of the test (actual: 5%);
- The mean number of juveniles per vessel ≥100 at the end of the test (actual: 1570);
- The coefficient of variation calculated for the number of juveniles ≤30% (actual: 12%).

The reference item also demonstrated sufficient sensitivity of the test organisms. The study is therefore considered to be acceptable.

The NOEC for reproduction was determined to be 75 mg a.s./kg soil dry weight.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.4.2.1/06
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10 and EC20 values for Folsomia candida with spiroxamine TG in a reproduction study
Report No:	0471836-ECO22
Document No:	M-761559-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary

The report M-405276-01-1 on the effects of spiroxamine TG in a springtail (*Folsomia candida*) reproduction study did not provide estimates of EC_{10} or EC_{20}. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013. The resulting EC_{10} and EC_{20} values of 175.34 (95% CL: 109.54 – 225.95) and 258.31 (95% CL: 191.18 – 308.29) mg a.s./kg dws, respectively, are considered reliable as the criteria for goodness of fit were met.

I. Methods

The statistical evaluation was performed with statistical software ToxRat Professional v3.3.0. To calculate EC_X values, logit analysis using linear maximum likelihood regression was performed along with 95% EC_X confidence limits based on Fieller's theorem.

II. Results

For the calculation of EC_{10} and EC_{20} values, the criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.00, showing no significant deviation between fit and data, and a statistically significant concentration/response was found ($p(F) = 0.001$) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table below.

Table CA 8.4.2.1/06-1 Results of the Logit analysis (max. likelihood regression) with reproduction at 28 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (according to Fieller`s theorem)

Parameter	Reproduction at test end (28 days)	
	EC_{10} (95 % confidence interval) [mg a.s./kg dws]	**EC_{20} (95 % confidence interval) [mg a.s./kg dws]**
Effect on reproduction	175.34 (109.54 – 225.95)	258.31 (191.18 – 308.29)

The resulting EC_{10} and EC_{20} values of 175.34 (95% CL: 109.54 – 225.95) and 258.31 (95% CL: 191.18 – 308.29) mg a.s./kg dws, respectively, for the springtail (*Folsomia candida*) in a spiroxamine TG reproduction test (28 days period), even if outside the predicted visual estimated range are considered

reliable as the criteria for goodness of fit were met and no better fit was found with any other available statistical model.

III. Conclusion

The resulting EC_{10} and EC_{20} values of 175.34 (95% CL: 109.54 – 225.95) and 288.31 (95% CL: 191.18 – 308.29) mg a.s./kg dws, respectively, are considered reliable as the criteria for goodness of fit were met.

Assessment and conclusion by applicant:

The statistical re-evaluation of the reproduction data has determined an EC_{10} of 175 mg/kg dws.

As the NOEC is lower than the EC_{10}, the NOEC of 75 mg a.s./kg dws shall be used in the risk assessment as the most critical endpoint from this study.

The values determined in the re-evaluation work are considered to be fully valid.

Metabolites

KWG 4168-desethyl (M01)

Data Point:	KCA 8.4.2.1/02
Report Author:	[redacted]
Report Year:	2007
Report Title:	KWG 4168-desethyl (Metabolite of KWG 4168): Influence on the reproduction of the collembola species Folsomia candida tested in artificial soil with 5 percent peat
Report No:	FRM-COLL-53/07
Document No:	M-289321-01-1
Guideline(s) followed in study:	ISO 11267 (1999)
Deviations from current test guideline:	Yes OECD 232 (2016) The reference item used was not boric acid, as recommended by the OECD 232 test guideline, however the reference item used was acceptable under the ISO guideline to which the study was conducted and was considered to sufficiently demonstrate the sensitivity of the test organism
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Collembola (*Folsomia candida*) aged 10 to 12 days were exposed to KWG 4168-desethyl incorporated into soil in a 4-week study to assess effects on reproduction.

Test organisms were exposed to 10, 32, 100, 316 and 1000 mg test item/kg soil dry weight and to a water control. Betosip (a.s. phenmedipham) was used as a toxic standard in accordance with ISO 11267 (1999) guidelines.

A statistically significant reduction in number of juveniles compared to the control was observed in the treatment group at 1000 mg/kg soil dry weight, resulting in a reduction of 76.1%, respectively.

The NOEC and LOEC for reproduction were 316 and 1000 mg/kg soil dry weight, respectively.

I. Materials and Methods

A. Materials

Test Material	KWG 4168-desethyl (metabolite of KWG 4168)
Lot/Batch #:	921103ELB02
Purity:	98%
Description:	Clear brown, oily liquid
Stability of test compound:	Not reported
Reanalysis/Expiry date:	7 December 2009
Density:	Not reported
Treatments	
Test rates:	10, 32, 100, 316 and 1000 mg test item/kg soil dry weight
Solvent/vehicle:	Because the test item was poorly soluble in water, a test item quartz sand mixture for each concentration was prepared
Analysis of test concentrations:	No
Test organisms	
Species:	*Folsomia candida,* Collembola, Isotomidae
Source:	Bred at Bayer CropScience. The strain was originally obtained from Ibacon, Institute for Analytic and Consulting, GmbH, D – 64380 Rossdorf.
Acclimatisation period:	None reported
Feeding:	Approximately 2 mg granulated dry yeast at the start of the study and after 14 days
Treatment for disease:	None reported
Test design	
Test vessel:	Glass vessels (volume: 140 mL, diameter: 5 cm) covered with glass lids
Test medium:	Artificial soil according to OECD 207 (1984). With respect to the properties of the test item (log P_{ow} ≥2) 5% peat instead of 10% peat were used considering the influence on bioavailability (EPPO 2002).
Replication:	5 (+1 without Collembola for measurement of soil moisture during the test and pH and soil moisture at the end of the study)
No. animals/vessel:	10
Duration of test:	4 weeks
Environmental test conditions	
Temperature:	20 ± 2°C

Photoperiod: 16 h light: 8 h dark at 524-606 lux

B. Study Design

This study was conducted in order to assess the effects on reproduction of KWG 4168-desethyl on Collembola (*Folsomia candida*) over 4 weeks.

The Collembola were 10 to 12 days old at the start of the study. For each replicate, 10 of the juvenile Collembola were placed in the glass test vessels, which had been prepared with the test item quartz sand and artificial soil. The soil was aligned with OECD 207 (1984) standard, but with 5% peat instead of 10% due to considerations on the influence on bioavailability with respect to the test item. The required amount of the test item was mixed thoroughly with 20 g quartz sand. If less than 25 mg test item had to be mixed with quartz sand a stock mixture was prepared and diluted with quartz sand to reach the demanded test concentrations. Water was added to the soil until 50% water holding capacity was achieved.

The artificial soil was kept at 18 to 22°C, with the temperature continuously recorded by a thermo-hygrograph integrated in the climatic chamber. The test vessels were exposed to 524-606 lux under a photoperiod of 16 h light: 8 h dark, monitored by an integrated luxmeter in the climatic chamber.

Application rates in this study were 10, 32, 100, 316 and 1000 mg test item/kg soil dry weight. Five replicates were exposed to control (water) treatment, and five replicates to 10, 32, 100, 316 and 1000 mg test item/kg soil dry weight treatments. During the study the Collembola were fed with granulated dry yeast.

A reference test with the toxic standard, Betosip, was performed at least once a year to ensure that the laboratory test conditions were adequate and to verify that the response of the test organism does not change significantly over time.

Water content was checked 14 days after application by reweighing the additional vessels. If the water loss exceeded 2% of the initial water content, the missing amount of water was added to all vessels of the treatment group. At the same time, the food was checked and the Collembola were fed again if necessary. Mortality and reproduction were reported after 28 days and were determined by the number of dead adult Collembola and the number of living juveniles detected using digital images.

II. Results and Discussion

Validity criteria, according to the test guideline that the study was conducted to, were assessed. In the control, the following criteria were met:

- Mean adult mortality ≤20% at the end of the test (actual: 10%);
- The mean number of juveniles per vessel >100 at the end of the test (actual: 826);
- The coefficient of variation calculated for the number of juveniles <30% (actual: 18%).

Mortality did not vary from the control greater than 6% at test item concentrations between 10 and 316 mg test item/kg soil dry weight. At the highest test item concentration, 1000 mg test item/kg soil dry weight, 54% mortality was observed in adult Collembola.

Table CA 8.4.2.1/02-1 Survival of Adult Collembola after 4 weeks treatment (n=10/replicate)

	mg test item/kg soil dry weight					
	Control	**10**	**32**	**100**	**316**	**1000**
Mean[1]	9.0	9.4	9.4	9.4	9.6	4.6
SD[1]	1.4	0.9	0.9	0.9	0.5	1.3
% mortality[2]	10.0	6.0	6.0	6.0	4.0	54.0

[1] mean and standard deviation (SD) of five replicates
[2] formula: ((initial placed organisms per vessel – mean of surviving adults per vessel) / 10) * 100

Reproduction in the Collembola was statistically different to the control at 1000 mg test item/kg soil dry weight treatment group at which reproduction was at 23.9% of the control.

Table CA 8.4.2.1/02-2 Reproduction of the Collembola after 4 weeks treatment (juveniles/replicate)

	mg test item/kg soil dry weight					
	Control	**10**	**32**	**100**	**316**	**1000**
Mean[1]	825.6	676.8	675.6	700.0	643.8	197.6
SD[1]	149.1	127.1	127.2	166.8	135.6	97.8
CV[2]	18.1	-	-	-	-	
% of control[3]	-	82.0	81.8	84.8	78.0	23.9*

1 mean and standard deviation (SD) of five replicates
[2] Coefficient of Variation
[3] formula: mean number of juveniles per treatment group * 100 / mean number of juveniles per control group
- = not applicable
* = significantly different compared to the control (Dunnett's Test, one-sided-smaller, $\alpha = 0.05$)

To demonstrate the sensitivity of the test system Betosip (phenmedipham 15.4 %) as a toxic standard was regularly tested (once a year) at concentrations of 89, 133, 200 and 300 mg test item/kg artificial soil dry weight. In the most recent test the mortality rate of adult Collembola was 8 %, 14 %, 22 % and 32 % at 89, 133, 200 and 300 mg Betosip/kg artificial soil dry weight. In all treatment groups the number of juveniles was statistically significant reduced in comparison to the control. The $NOEC_{reproduction}$ was < 89 mg Betosip (13.7 mg a.s)/kg artificial soil dry weight and the $LOEC_{reproduction}$ was 89 mg Betosip (13.7 mg a.s)/kg artificial soil dry weight. The results were considered to demonstrate the sensitivity of the test system.

III. Conclusion

In the control group, 10% of the adult Collembola died which is within the tolerated range of ≤20% mortality recommended by the test guideline. The highest mortality rate of 54% was found in the test item concentration of 1000 mg test item/kg soil dry weight. Concerning the number of juveniles, statistical analysis revealed significant differences between the control and the highest treatment group at 1000 mg test item/kg soil dry weight.

The NOEC for reproduction: 316 mg test item/kg soil dry weight.

The LOEC for reproduction: 1000 mg test item/kg soil dry weight.

Assessment and conclusion by applicant:

Validity criteria according to the current OECD 232 guideline (2016) were met. In the control, the following criteria were met:

- Mean adult mortality ≤20% at the end of the test (actual: 10%);
- The mean number of juveniles per vessel ≥100 at the end of the test (actual: 826);
- The coefficient of variation calculated for the number of juveniles <30% (actual: 18%).

The study was not specifically conducted to the OECD 232 test guideline, however the test design and the parameters assessed are consistent with the OECD test guideline. Furthermore the OECD validity criteria have been met.

The reference item used was not boric acid, as recommended by the OECD 232 test guideline, however the reference item used was acceptable under the ISO guideline to which the study was conducted and was considered to sufficiently demonstrate the sensitivity of the test organism.

The study is therefore considered acceptable.

The NOEC for reproduction was determined to be 316 mg test item/kg soil dry weight.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.4.2.1/07
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10 and EC20 values for Folsomia candida with KWG 4168-desethyl in a reproduction study
Report No:	0471836-ECO15
Document No:	M-760431-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary

The report M-289321-01-1 on the effects of KWG 4168-desethyl (metabolite of KWG 4168) in a springtail (*Folsomia candida*) reproduction study did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values have been calculated in accordance with the Annex to Com. Reg 283/2013. Due to the lack of a significant dose response, it was not possible to determine reliable EC_{10} or EC_{20} values for reproduction.

I. Methods

The statistical evaluation was performed with statistical software ToxRat Standard v3.3.0. Due to the lack of a significant dose response on reproduction when compared to the control, it was not possible to calculate reliable EC_{10} or EC_{20} values.

II. Results

Due to the lack of a significant dose response on the reproduction, when compared to the control, it was not possible to calculate reliable EC_{10} or EC_{20} values.

III. Conclusion

Due to the lack of a significant dose response, it was not possible to determine reliable EC_{10} or EC_{20} values for reproduction.

Assessment and conclusion by applicant:

The statistical re-evaluation of the reproduction data could not calculate reliable EC_{10} and EC_{20} values.

The NOEC of 316 mg/kg dws from the original study report shall be used in the risk assessment as the most critical endpoint from this study.

The values determined in the re-evaluation work are considered to be fully valid.

Data Point:	KCA 8.4.2.1/08
Report Author:	[redacted]
Report Year:	2019
Report Title:	KWG4168-desethyl: Effects on reproduction of the predatory mite hypoaspis aculeifer in artificial soil
Report No:	143061089
Document No:	M-680684-01-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 (2009)
Deviations from current test guideline:	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 purpose of the study was to determine the effects of KWG 4168-desethyl on mortality and reproduction of the predatory mite *Hypoaspis aculeifer*.

KWG 4168-desethyl was added to soil at concentrations of 6.25, 12.5, 25, 50 and 100 mg test item/kg soil dry weight.

KWG 4168-desethyl caused no statistically significant effects on mortality of *Hypoaspis aculeifer* up to and including the concentration of 100 mg test item/kg soil. Therefore, the No Observed Effect Concentration (NOEC) for mortality was determined to be ≥100 mg test item/kg soil. The Lowest Observed Effect Concentration (LOEC) for mortality was estimated to be >100 mg test item/kg soil. The LC_{50} was estimated to be >100 mg test item/kg soil.

The NOEC for reproduction was determined to be 50 mg test item/kg soil and the LOEC for reproduction was determined to be 100 mg test item/kg soil. The EC_{10} was determined to be 94.1 mg test item/kg soil, the EC_{20} was determined to be 102.1 mg test item/kg soil, and the EC_{50} was determined to be 117.3 mg test item/kg soil.

I. Materials and Methods

A. Materials

Test Material KWG 4168-desethyl

Lot/Batch #: AE 1344302-PU-01

Purity: 95.8% w/w

Description: Amber liquid

Stability of test compound: Sufficient based on expiration date

Reanalysis/Expiry date: 24 January 2020

Density: Not reported

Treatments

Test rates: 6.25, 12.5, 25, 50 and 100 mg test item/kg soil

Solvent/vehicle: Acetone

Analysis of test concentrations:	No
Test design	
Test species:	*Hypoaspis aculeifer* (age: approximately 14 days at test start)
Test vessel:	100 mL glass containers (volume: 100 mL; diameter: 5 cm) with tight screw cap
Test substrate:	5% Sphagnum-peat, 20% kaolin clay, 74.8% fine quartz-sand, 0.2% Calcium carbonate
Replication:	Four per treatment group and eight for the control
No. of animals/vessel:	10
Duration of test:	14 days
Environmental test conditions	
Temperature:	20 ± 2°C
pH:	Test start: 5.8 to 6.0 Test end: 5.9 to 6.0
Photoperiod:	16 h light : 8 h dark (400 – 800 lux)
Water content:	Test start: 22.4% to 23.0% (49.8% to 51.1% of WHC_{max}) Test end: 22.1% to 22.8% (49.2% to 50.6% of WHC_{max})

B. Study Design

The purpose of the study was to determine the effects of KWG 4168-desethyl on mortality and reproduction of the predatory mite *Hypoaspis aculeifer*.

Nominal test concentrations were 6.25, 12.5, 25, 50 and 100 mg test item/kg soil.

Ten adult female mites were added to each of the four replicate test vessels (eight for the control). Test vessels were glass containers (volume: 100 mL, diameter: 5 cm), tight screw top closure to avoid water evaporation, filled with approximately 20 g ± 1.0 g artificial soil dry weight. The height of the soil layer in the containers was 1.5 to 2 cm.

One spatula of cheese mites (*Tyrophagus putrescentiae*) was provided as food at experimental start and on days 2, 4, 7, 9 and 11.

Incubation was at 18 to 22°C with a photoperiod of 16 hours light to 8 hours dark at approximately 400 to 800 lux.

After 14 days exposure the soil was filled into Millipore pots with attached plastic containers for collecting the escaping mites. These extraction units were placed in a heat extractor. The soil including the mites was exposed to a temperature of approximately 25°C and 30°C for approximately 2 days. Escaping mites were collected in a fixing liquid, cooled at a temperature of approximately 16°C. The fixing liquid contained glycol and a detergent.

Adult animals were counted once visually, juvenile animals were counted twice under binocular microscopes. One of the replicates was counted three times because the first two counts deviated more than 10% from their mean value.

II. Results and Discussion

Validity criteria according to the OECD 226 test guideline were met.

- Mean mortality in the controls to not exceed 20% (actual: 4%)

- The mean number of juvenile mites per replicate to be at least 50 (actual: 188 to 222)
- The coefficient of variation for reproduction to be ≤30% (actual: 7.0%)

Mortality of *Hypoaspis aculeifer* in the test item treated groups ranged from 0 to 8%. The values were not statistically significantly different compared to the control (Fisher's Exact Test, α = 0.05, one-sided greater). Therefore, the NOEC for mortality was determined to be ≥100 mg test item/kg soil dry weight. The LOEC for mortality was estimated to be >100 mg test item/kg soil dry weight.

No differences in morphology of the mites between the test item treated groups and the control were observed.

Table CA 8.4.2.1/08-1 Mortality of adult *Hypoaspis aculeifer* after 14 days

Treatment group (mg test item/kg soil dry weight)	Number of surviving females per group	Mean mortality [%]	Standard deviation [%]
Control	77	4	± 5
6.25	38	5	± 6
12.5	39	3	± 5
25	37	8	± 5
50	39	3	± 5
100	40	0	± 0

The results represent rounded values calculated from the exact raw data

There were no statistically significant effects on reproduction of *Hypoaspis aculeifer* up to and including the concentration of 50 mg test item/kg soil dry weight (Williams t-test, α = 0.05, one-sided smaller). At the concentration of 100 mg test item/kg soil dry weight a statistically significant reduction of reproduction was observed.

Therefore, the NOEC for reproduction was determined to be 50 mg test item/kg soil dry weight and the LOEC for reproduction was determined to be 100 mg test item/kg soil dry weight. The EC_{10} for *Hypoaspis aculeifer* in artificial soil was determined to be 94.1 mg test item/kg soil dry weight, the EC_{20} was determined to be 102.1 mg test item/kg soil dry weight, and the EC_{50} was determined to be 117.3 mg test item/kg soil dry weight. (95% confidence limits could not be determined due to mathematical reasons).

Table CA 8.4.2.1/08-2 Reproduction of *Hypoaspis aculeifer* after 14 days

Treatment group (mg test item/kg soil dry weight)	Mean number of juveniles per group	Standard deviation	% of control
Control	201	± 14	-
6.25	214	± 19	106
12.5	211	± 19	105
25	200	± 24	100
50	202	± 8	100
100	167	± 7	83*

*significantly different from the control
The results represent rounded values calculated from the exact raw data

To verify the sensitivity of the test system, the reference item (dimethoate) was tested at concentrations of 1.54, 2.23, 3.23, 4.69 and 6.80 mg a.s./kg soil dry weight in a separate study. In the most recent GLP study, an EC_{50} of 3.31 mg a.s./kg soil dry weight was determined for juvenile production. The effects on the reduction of reproduction showed that the test system was sensitive and reflected the expected toxicity as given in the OECD 226 test guideline (EC_{50} for reproduction should be between 3.0 and 7.0 mg a.s./kg soil dry weight).

III. Conclusion

Exposure to KWG 4168-desethyl caused no statistically significant effects on mortality of *Hypoaspis aculeifer* up to and including the concentration of 100 mg test item/kg soil dry weight. Therefore, the No Observed Effect Concentration (NOEC) for mortality was determined to be ≥100 mg test item/kg soil dry weight. The Lowest Observed Effect Concentration (LOEC) for mortality was estimated to be >100 mg test item/kg soil dry weight. The LC_{50} was estimated to be >100 mg test item/kg soil dry weight.

The NOEC for reproduction was determined to be 50 mg test item/kg soil dry weight and the LOEC for reproduction was determined to be 100 mg test item/kg soil dry weight. The EC_{10} was determined to be 94.1 mg test item/kg soil dry weight, the EC_{20} was determined to be 102.1 mg test item/kg soil dry weight, and the EC_{50} was determined to be 117.3 mg test item/kg soil dry weight.

Assessment and conclusion by applicant:

This is a new study that has not been previously evaluated.

Validity criteria according to the most recent OECD 226 test guideline (2016) were met.

- Mean mortality in the controls to not exceed 20% (actual: 4%)
- The mean number of juvenile mites per replicate to be at least 50 (actual: 188 to 222)
- The coefficient of variation for reproduction to be ≤30% (actual: 7.0%)

The reference item also demonstrated sufficient sensitivity of the test organisms. The study is therefore considered acceptable.

The NOEC for reproduction was determined to be 50 mg test item/kg soil dry weight.

KWG 4168-despropyl (M02)

Data Point:	KCA 8.4.2.1.03
Report Author:	[redacted]
Report Year:	2007
Report Title:	KWG 4168-despropyl (Metabolite of KWG 4168): Influence on the reproduction of the collembola species Folsomia candida tested in artificial soil with 5 percent peat
Report No:	FRM-COLL-54/07
Document No:	M-288905-01-1
Guideline(s) followed in study:	ISO 11267 (1999)
Deviations from current test guideline:	Yes OECD 232 (2016) The reference item used was not boric acid, as recommended by the OECD 232 test guideline, however the reference item used was acceptable under the ISO guideline to which the study was conducted and was considered to sufficiently demonstrate the sensitivity of the test organism
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Collembola (*Folsomia candida*) aged 10 to 12 days were exposed to KWG 4168-despropyl (metabolite of KWG 4168) in a 4-week study in artificial soil with 5% peat to assess effects on reproduction.

Test organisms were exposed to 10, 32, 100, 316 and 1000 mg test item/kg soil dry weight and to a control. Betosip (a.s. phenmedipham) was used as a toxic standard in accordance with ISO 11267 (1999) guidelines.

A statistically significant reduction in number of juveniles compared to the control was observed in the treatment group at 1000 mg test item/kg soil dry weight, resulting in reductions of 74.6%.

The NOEC and LOEC for reproduction were 316 and 1000 mg test item/kg soil dry weight, respectively.

I. Materials and Methods

A. Materials

Test Material	KWG 4168-despropyl
Lot/Batch #:	921103ELB03
Purity:	97%
Description:	Amber oily liquid
Stability of test compound:	Not reported
Reanalysis/Expiry date:	13 January 2008
Density:	Not reported
Treatments	
Test rates:	Nominal: 10, 32, 100, 316, 1000 mg test item/kg soil
Solvent/vehicle:	The test item was poorly soluble in water. A test item quartz sand mixture was prepared for each concentration.
Analysis of test concentrations:	No
Test organisms	
Species:	*Folsomia candida,* Collembola, Isotomidae
Source:	In-house culture originally from Ibacon, Institute for Analytic and Consulting, GmbH, D-64380, Rossdorf.
Feeding:	Approximately 2 mg granulated dry yeast at the start of the study and after 14 days.
Treatment for disease:	None reported.
Test design	
Test vessel:	Glass vessels (volume: 140 mL, diameter: 5 cm) covered with glass lids
Test medium:	Artificial soil according to OECD 207 (1984). With respect to the properties of the test item (log P_{ow} ≥2) 5% peat instead of 10% peat were used considering the influence on bioavailability (EPPO 2002). This is in line with OECD 232 (2016)
Replication:	5 replicates per treatment group. (+1 without Collembola for measurement of soil moisture during the test and pH and soil moisture at the end of the study
No. animals/vessel:	10

Duration of test:	4 weeks
Environmental test conditions	
Temperature:	20 ± 2°C
pH:	Recorded in each replicate of artificial soil. (5.84 - 6.11)
Photoperiod:	16 h light :8 h dark at 400-800 lux

B. Study Design

This study was conducted in order to assess the influence on reproduction of KWG 4168-despropyl on Collembola in an inhibition of reproduction test over 4 weeks.

The Collembola (*Folsomia candida*) were 10 to 12 days old at the start of the study. For each replicate 10 of the juvenile Collembola were placed in the test vessels, which had been prepared with the test item quartz sand and artificial soil. The soil was aligned with OECD 207 (1984) standard, but with 5% peat instead of 10% due to considerations on the influence on bioavailability with respect to the test item. The required amount of the test item was mixed thoroughly with 5 g quartz sand. If less than 50 mg test item had to be mixed with quartz sand a stock mixture was prepared and diluted with quartz sand to reach the demanded test concentrations. Water was added until 50% water holding capacity of the soil was achieved.

The artificial soil was kept at 18 to 22°C, with the temperature continuously recorded by a thermo hygrograph integrated in the climatic chamber. The test vessels were exposed to 559 to 622 lux under a photoperiod of 16 h light: 8 h dark, monitored by an integrated luxmeter of the climatic chamber.

Five replicates were exposed to control (water treated), 10, 32, 100, 316 and 1000 mg test item/kg soil dry weight. During the study, the test organisms were fed with granulated dry yeast.

A reference test with the toxic standard, Betosip, was performed at least once a year to ensure that the laboratory test conditions were adequate and to verify that the response of the test organisms does not change significantly over time.

After 14 days, water content was checked and replenished if water loss exceeded 2% of initial content. Food was also checked at this time and the Collembola were fed again if necessary. Mortality and reproduction were reported after 28 days and were determined by the number of dead adult Collembola and the number of living juveniles detected using digital images.

II. Results and Discussion

Validity criteria, according to the test guideline that the study was conducted to, were assessed. In the control, the following criteria were met:

- Mean adult mortality ≤20% at the end of the test (actual: 18%);
- The mean number of juveniles per vessel >100 at the end of the test (actual: 417);
- The coefficient of variation calculated for the number of juveniles <30% (actual: 10%).

At the highest application rate, 1000 mg test item/kg soil dry weight, 86% mortality was observed. The percent mortality in the control was 18%.

Table CA 8.4.2.1/03-1 Survival of adult Collembola after 4 weeks treatment (n=10/replicate)

	mg test item/kg soil dry weight					
	Control	**10**	**32**	**100**	**316**	**1000**
Mean[1]	8.2	6.6	8.4	6.4	7.6	1.4
SD[1]	1.3	2.8	1.3	1.1	0.9	0.5
% mortality[2]	18.0	34.0	16.0	36.0	24.0	86.0

1 mean and standard deviation (SD) of five replicates

	mg test item/kg soil dry weight					
	Control	10	32	100	316	1000

2 formula: ((initial placed organisms per vessel – mean of surviving adults per vessel) / 10) * 100

A statistically significant difference in reproduction compared to the control was observed at the highest application rate, 1000 mg test item/kg soil dry weight. Reproduction in this test item group was 25.4% of reproduction observed in the control.

Table CA 8.4.2.1/03-2 Reproduction of the Collembola after 4 weeks treatment (juveniles/replicate)

	mg test item/kg artificial soil dry weight					
	Control	10	32	100	316	1000
Mean[1]	416.8	491.2	628.0	491.4	372.0	105.8
SD[1]	41.7	88.4	76.8	113.3	47.1	16.4
CV[2]	10.0					
% of Control[3]	-	117.9	150.7	117.9	89.3	25.4*

1 mean and standard deviation (SD) of five replicates
2 Coefficient of Variation
3 formula: mean number of juveniles per treatment group * 100 / mean number of juveniles per control group
- = not applicable
* = significantly different compared to the control (Dunnett's Test, one-sided-smaller $\alpha = 0.05$)

To demonstrate the sensitivity of the test system Betosip (phenmedipham 15.4 %) as a toxic standard was regularly tested (once a year) at concentrations of 89, 133, 200 and 300 mg test item/kg artificial soil dry weight. In the most recent test the mortality rate of adult Collembola was 8 %, 14 %, 22 % and 32 % at 89, 133, 200 and 300 mg Betosip/kg artificial soil dry weight. In all treatment groups the number of juveniles was statistically significant reduced in comparison to the control. The $NOEC_{reproduction}$ was < 89 mg Betosip (13.7 mg a.s)/kg artificial soil dry weight and the $LOEC_{reproduction}$ was 89 mg Betosip (13.7 mg a.s)/kg artificial soil dry weight. The results were considered to demonstrate the sensitivity of the test system.

III. Conclusion

The highest mortality rate of 86% was found in the test item concentration of 1000 mg test item/kg soil dry weight. Concerning the number of juveniles, statistical analysis revealed significant differences between the control and the treatment group at 1000 mg test item/kg soil dry weight.

The NOEC for reproduction: 316 mg test item/kg soil dry weight.

The LOEC for reproduction: 1000 mg test item/kg soil dry weight.

Assessment and conclusion by applicant:

Validity criteria according to the current OECD 232 guideline (2016) were met. In the control, the following criteria were met:

- Mean adult mortality <20% at the end of the test (actual: 18%);
- The mean number of juveniles per vessel >100 at the end of the test (actual: 417);
- The coefficient of variation calculated for the number of juveniles <30% (actual: 10%).

The study was not specifically conducted to the OECD 232 test guideline, however the test design and the parameters assessed are consistent with the OECD test guideline. Furthermore the OECD validity criteria have been met.

The reference item used was not boric acid, as recommended by the OECD 232 test guideline, however the reference item used was acceptable under the ISO guideline to which the study was conducted and was considered to sufficiently demonstrate the sensitivity of the test organism.

The study is therefore considered acceptable.

The NOEC for reproduction was determined to be 316 mg test item/kg soil dry weight.

The data have been subjected to statistical re-evaluation and the results have been presented in the following study summary.

Data Point:	KCA 8.4.2.1/09
Report Author:	[redacted]
Report Year:	2020
Report Title:	Calculation of EC10 and EC20 values for Folsomia candida with KWG 4168-despropyl in a reproduction study
Report No:	0471836-ECO13
Document No:	M-760410-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary

The report M-288905-01-4 on the effects of KWG 4168-despropyl (metabolite of KWG 4168) in a springtail (*Folsomia candida*) reproduction study did not provide estimates of EC_{10} or EC_{20} values. Therefore, these values have been calculated in accordance with the Annex to Com. Reg. 283/2013. The resulting EC_{10} and EC_{20} values of 308.18 (95% CL: 306.07 – 310.27) and 402.24 (95% CL: 400.09-404.38) mg/kg dws, respectively, are considered reliable as the criteria for goodness of fit were met.

I. Methods

The statistical evaluation was performed with statistical software ToxRat Standard v3.3.0. To calculate EC_X values, probit analysis using linear maximum likelihood regression was performed along with 95% EC_X confidence limits based on Fieller's Theorem.

II. Results

The criteria for goodness of fit were met as the $P(Chi^2)$ value was 1.00, showing no significant deviation between fit and data, and a statistically significant concentration/response was found ($p(F) < 0.001$) for this parameter.

The resulting EC_{10} and EC_{20} values and the respective confidence intervals are represented in the following table.

Table CA 8.4.2.1/09-1 Results of the Probit analysis (max. likelihood regression) with reproduction at 28 d: Selected effective concentrations (EC_x) of the test item and their 95%-confidence limits (according to Fieller`s theorem)

Parameter	Reproduction at test end (28 days)	
	EC_{10} (95 % confidence interval) [mg/kg dws]	**EC_{20} (95 % confidence interval) [mg/kg dws]**
Effect on reproduction	308.18 (306.07 – 310.27)	402.24 (400.09 – 404.38)

The resulting EC_{10} and EC_{20} values of 308.18 (95% CL: 306.07 – 310.27) and 402.24 (95% CL: 400.09-404.38) mg/kg dws, respectively, for springtail (*Folsomia candida*) in a KWG 4168-despropyl

reproduction test (28 days period) are therefore considered reliable as the criteria for goodness of fit were met.

III. Conclusion

The resulting EC_{10} and EC_{20} values of 308.18 (95% CL: 306.07 – 310.27) and 402.24 (95% CL: 400.09-404.38) mg/kg dws, respectively, are considered reliable as the criteria for goodness of fit were met.

Assessment and conclusion by applicant:

The statistical re-evaluation of the reproduction data has determined an EC_{10} of 308 mg/kg dws.

As the EC_{10} is lower than the NOEC value, the EC_{10} of 308 mg/kg dws shall be used in the risk assessment as the most critical endpoint from this study.

The values determined in the re-evaluation work are considered to be fully valid.

Data Point:	KCA 8.4.2.1/10
Report Author:	[redacted]
Report Year:	2019
Report Title:	KWG4168-despropyl: Effects on reproduction of the predatory mite hypoaspis aculeifer in artificial soil
Report No:	143071089
Document No:	M-680694-01-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 (2009)
Deviations from current test guideline:	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 purpose of the study was to determine the effects of KWG 4168-despropyl on mortality and reproduction of the predatory mite *Hypoaspis aculeifer*.

KWG 4168-despropyl was added to soil at concentrations of 6.25, 12.5, 25, 50 and 100 mg test item/kg soil dry weight.

KWG 4168-despropyl caused no statistically significant effects on mortality or reproduction of *Hypoaspis aculeifer* up to and including the concentration of 100 mg test item/kg soil. Therefore, the No Observed Effect Concentration (NOEC) for mortality was determined to be ≥100 mg test item/kg soil dry weight and the Lowest Observed Effect Concentration (LOEC) was estimated to be >100 mg test item/kg soil dry weight.

The NOEC for reproduction was determined to be ≥100 mg test item/kg soil dry weight and the LOEC for reproduction was estimated to be >100 mg test item/kg soil dry weight. Due to the absence of a concentration-response relationship, the EC_{10}, EC_{20} and EC_{50} values have been estimated to be >100 mg test item/kg soil dry weight.

I. Materials and Methods

A. Materials

Test Material KWG 4168-despropyl

Lot/Batch #: AE 1344303-PU-01

Purity:	99.1% w/w
Description:	Colourless liquid
Stability of test compound:	Sufficient based on expiration date
Reanalysis/Expiry date:	13 May 2022
Density:	Not reported
Treatments	
Test rates:	6.25, 12.5, 25, 50 and 100 mg test item/kg soil dry weight
Solvent/vehicle:	Acetone
Analysis of test concentrations:	No
Test design	
Test species:	*Hypoaspis aculeifer*
Test vessel:	100 mL glass containers (volume: 100 mL; diameter: 5 cm), with tight screw cap
Test substrate:	5% Sphagnum-peat, 20% Kaolin clay, 74.8% fine quartz-sand, 0.2% Calcium carbonate
Replication:	Four per treatment group and eight for the control
No. of animals/vessel:	10
Duration of test:	14 days
Environmental test conditions	
Temperature:	20 ± 2°C
pH:	Test start: 5.8 to 6.0 Test end: 5.9 to 6.0
Photoperiod:	16 h light : 8 h dark (400 – 800 lux)
Water content:	Test start: 22.7% to 23.2% (50.5% to 51.5% of the WHC_{max}) Test end: 22.7% to 23.5% (50.4% to 52.2% of the WHC_{max})

B. Study Design

The purpose of the study was to determine the effects of KWG 4168-despropyl on mortality and reproduction of the predatory mite *Hypoaspis aculeifer*.

Nominal test concentrations were 6.25, 12.5, 25, 50 and 100 mg test item/kg soil dry weight.

Ten adult female mites were added to each of the four replicate test vessels (eight for the control). Test vessels were glass containers (volume: 100 mL, diameter: 5 cm), tight screw top closure to avoid water evaporation, filled with approximately 20 g ± 1.0 g artificial soil dry weight. The height of the soil layer in the containers was 1.5 to 2 cm.

One spatula of cheese mites (*Tyrophagus putrescentiae*) was provided as food at experimental start and on days 2, 4, 7, 9 and 11.

Incubation was at 18 to 22°C with a photoperiod of 16 hours light to 8 hours dark at approximately 400 to 800 lux.

After 14 days exposure the soil was filled into Millipore pots with attached plastic containers for collecting the escaping mites. These extraction units were placed in a heat extractor. The soil including the mites was exposed to a temperature of approximately 25°C and 30°C for approximately 2 days. Escaping mites were collected in a fixing liquid, cooled at a temperature of approximately 16°C. The fixing liquid contained glycol and a detergent.

Adult animals were counted once visually, juvenile animals were counted twice under binocular microscopes. None of the replicate counts deviated more than 10% from their mean value.

II. Results and Discussion

Validity criteria according to the OECD 226 guideline were met.

- Mean mortality in the controls to not exceed 20% (actual: 2.5%)
- The mean number of juvenile mites per replicate to be at least 50 (actual: 169 to 187)
- The coefficient of variation for reproduction to be ≤30% (actual: 3.3%)

Mortality of *Hypoaspis aculeifer* in the test item treated groups ranged from 0% to 2.5%. The values were not statistically significantly different compared to the control where 2.5% of the soil mites were dead (Fisher's Exact Test, α = 0.05, one-sided greater). Therefore, the NOEC for mortality was determined to be ≥100 mg test item/kg soil dry weight. The LOEC for mortality was estimated to be >100 mg test item/kg soil dry weight.

No differences in morphology of the mites between the test item treated groups and the control were observed.

Table CA 8.4.2.1/10-1 Mortality of adult *Hypoaspis aculeifer* after 14 days

Treatment group (mg test item/kg soil dry weight)	Number of surviving females per group	Mean mortality [%]	Standard deviation [%]
Control	78	2.5	± 5
6.25	40	0.0	± 0
12.5	39	2.5	± 5
25	40	0.0	± 0
50	40	0.0	± 0
100	39	2.5	± 5

The results represent rounded values calculated from the exact raw data

There were no statistically significant effects on reproduction of *Hypoaspis aculeifer* up to and including the concentration of 100 mg test item/kg soil dry weight (Dunnett's t-test, α = 0.05, one-sided smaller). Therefore, the NOEC for reproduction was determined to be ≥100 mg test item/kg soil dry weight and the LOEC for reproduction was estimated to be >100 mg test item/kg soil dry weight.

Due to the lack of a concentration-response relationship no reliable ECx-calculation is possible. Therefore, no EC_{10}/EC_{20}-value can be reported. The EC_{10}, EC_{20} and EC_{50} were estimated to be >100 mg test item/kg soil dry weight.

Table CA 8.4.2.1/10-2 Reproduction of *Hypoaspis aculeifer* after 14 days

Treatment group (mg test item/kg soil dry weight)	Mean number of juveniles per group	Standard deviation	% of control
Control	180	± 6	-
6.25	185	± 23	103
12.5	190	± 16	106
25	184	± 10	102

Treatment group (mg test item/kg soil dry weight)	Mean number of juveniles per group	Standard deviation	% of control
50	186	± 19	103
100	174	± 12	97

The results represent rounded values calculated from the exact raw data

To verify the sensitivity of the test system, the reference item (dimethoate) was tested at concentrations of 1.54, 2.23, 3.23, 4.69 and 6.80 mg a.s./kg soil dry weight in a separate study. In the most recent GLP study, an EC_{50} of 3.31 mg a.s./kg soil dry weight was determined for juvenile production. The effects on the reduction of reproduction showed that the test system was sensitive and reflected the expected toxicity as given in the OECD 226 test guideline (EC_{50} for reproduction should be between 3.0 and 7.0 mg a.s./kg soil dry weight).

III. Conclusion

KWG 4168-despropyl caused no statistically significant effects on mortality or reproduction of *Hypoaspis aculeifer* up to and including the concentration of 100 mg test item/kg soil dry weight. Therefore, the No Observed Effect Concentration (NOEC) for mortality was determined to be ≥100 mg test item/kg soil dry weight and the Lowest Observed Effect Concentration (LOEC) was estimated to be >100 mg test item/kg soil dry weight.

The NOEC for reproduction was determined to be ≥100 mg test item/kg soil dry weight and the LOEC for reproduction was estimated to be >100 mg test item/kg soil dry weight. Due to the absence of a concentration-response relationship, the EC_{10}, EC_{20} and EC_{50} values have been estimated to be >100 mg test item/kg soil dry weight.

Assessment and conclusion by applicant:

This is a new study that has not been previously evaluated.

Validity criteria according to the current OECD 226 guideline (2016) were met.

- Mean mortality in the controls to not exceed 20% (actual: 2.5%)
- The mean number of juvenile mites per replicate to be at least 50 (actual: 169 to 187)
- The coefficient of variation for reproduction to be ≤30% (actual: 3.3%)

The reference item also demonstrated sufficient sensitivity of the test organisms. The study was therefore considered acceptable.

The NOEC for reproduction was determined to be ≥100 mg test item/kg soil dry weight.

KWG 4168-N-oxide (M03)

Data Point:	KCA 8.4.2.1/11
Report Author:	[redacted]
Report Year:	2020
Report Title:	KWG4168-N-oxide: Effects on reproduction of the Collembola Folsomia candida in artificial soil 1st final report amendment
Report No:	143081016
Document No:	M-687854-01-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 (2009) US EPA OCSPP Not Applicable OECD-Guideline for testing chemicals No. 232 "Collembolan Reproduction Test in Soil" (adopted July 29, 2016) ISO 11267 Soil Quality – Inhibition of reproduction of Collembola (Folsomia candida) by soil contaminants, 2014
Deviations from current test guideline:	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

Collembola (*Folsomia candida*) aged 9 to 12 days were exposed to KWG 4168-N-oxide incorporated into soil in a 28-day study to assess effects on survival and reproduction.

Test organisms were exposed to 6.25, 12.5, 25, 50, and 100 mg test item/kg soil dry weight and to a water control.

Exposure to KWG 4168-N-oxide did not cause any statistically significant effects on the mortality and reproduction of *Folsomia candida* up to and including a concentration of 100 mg test item/kg soil dry weight.

The NOEC and LOEC for both mortality and reproduction are therefore ≥100 and >100 mg test item/kg soil dry weight, respectively. The LC_{50} for mortality and EC_{50} for reproduction were both determined to be >100 mg test item/kg soil. Due to a lack of a dose-response relationship, no reliable EC_{10} or EC_{20} could be calculated therefore these values are considered to be >100 mg test item/kg soil dry weight.

I. Materials and Methods

A. Materials

Test Material KWG 4168-N-oxide

Lot/Batch #: AE 1344305-00 1C74 0001 (origin batch no.: M26999)

Purity: 72.9% w/w

Description: Light yellow liquid

Stability of test compound: Not reported

Reanalysis/Expiry date: 28 November 2022

Density: Not reported

Treatments	
Test rates:	6.25, 12.5, 25, 50, and 100 mg test item/kg soil dw
Solvent/vehicle:	Deionised water
Analysis of test concentrations:	No significant deviations to the target concentration (<5%)
Test organisms	
Species:	Collembola *Folsomia candida*, 9 – 12 days old
Source:	In-house cultures
Acclimatisation period:	Reared under test conditions
Feeding:	Fed 2 mg dry yeast at test start and on day 14
Test design	
Test vessel:	100-mL glass vessels with a diameter of 5 cm, closed to avoid water evaporation. Vessels contained 30 ± 1.0 g test soil at a height of 2 – 2.5 cm
Test medium:	Artificial soil according to OECD 232
Replication:	Four replicates per concentration, eight in the control
No. animals/vessel:	10 collembola per vessel
Duration of test:	28 days
Environmental test conditions	
Temperature:	Within the range 18 – 22°C
pH:	Test start: 6.1 – 6.2 Test end: 6.2 – 6.3
Moisture content:	Test start: 26.3 – 27.6% (49.7 – 52.1% of WHC_{max}) Test end: 24.6 – 26.2% (46.4 – 49.4% of WHC_{max})
Photoperiod:	16 h light : 8 h dark in the range 400 – 800 lux

B. Study Design

This study was conducted in order to determine the effects of KWG 4168-N-oxide exposure on the mortality and reproduction of the collembola *Folsomia candida* in artificial soil over 28 days.

The collembola were aged between 9 and 12 days old, from a synchronised in-house cohort.

Test vessels were 100-mL glass vessels with a diameter of 5 cm, closed to avoid water evaporation. To each vessel was added 30 g test soil at a height of 2 to 2.5 cm

Test soil was prepared according to the OECD 232 guideline, with 5% sphagnum peat, 20% kaolin clay, 74.8% fine quartz sand, and 0.2% $CaCO_3$. Soil was pre-moistened to approximately half the final water content two days prior to application of the test substance, with the additional water added when applying the test item.

Test concentrations were prepared by serial dilution of a stock solution. Nominal test concentrations were 6.25, 12.5, 25, 50, and 100 mg test item/kg soil dw. No significant deviation to the target concentration >5% was found. Control soil was treated with deionised water only.

Assessment of adult mortality, behavioural effects, and reproduction was performed after 28 days.

II. Results and Discussion

Validity criteria according to OECD 232 were met in the controls:

- Adult mortality to be ≤20% at the end of the test (actual: 6%);
- Mean number of juveniles per vessel to be ≥100 at test end (actual: 437 to 625);
- The coefficient of variation for the number of juveniles to be <30% (actual: 11.2%).

Mortality of up to 13% was observed in the treated groups, however this was not statistically significantly different to the control. No abnormal behaviours were observed in any of the treatment groups.

Collembolan reproduction was not statistically significantly affected compared to the control at any test concentration.

Table CA 8.4.2.1/11-1 Mortality and reproduction of the Collembola after 28 days exposure

Treatment group (mg test item/kg soil dw)	Mean mortality (%) ±SD	Mean number of juveniles per replicate ±SD	Reproduction as % of control
Control	6 ± 5	544 ± 61	
6.25	3 ± 5	583 ± 20	107
12.5	0 ± 0	586 ± 38	108
25	8 ± 10	524 ± 123	96
50	13 ± 15	509 ± 64	93
100	8 ± 5	528 ± 76	97

To verify the sensitivity of the test system, the reference item (boric acid) was tested at concentrations of 30.5, 48.8, 78.1, 125 and 200 mg/kg soil dry weight in a separate study. In the most recent GLP study, an EC_{50} of 104.6 mg/kg soil dry weight was determined. The effects on the reduction of reproduction showed that the test system was sensitive and reflected the expected toxicity as given in the OECD 232 test guideline (50% reduction in reproduction at about 100 mg/kg soil dry weight).

III. Conclusion

Exposure to KWG 4168-N-oxide did not cause any statistically significant effects on the mortality and reproduction of *Folsomia candida* up to and including a concentration of 100 mg test item/kg soil dry weight.

The NOEC and LOEC for both mortality and reproduction are therefore ≥100 and >100 mg test item/kg soil dry weight, respectively. The LC_{50} for mortality and EC_{50} for reproduction were both determined to be >100 mg test item/kg soil dry weight. Due to a lack of a dose-response relationship, no reliable EC_{10} or EC_{20} could be calculated therefore these values are considered to be >100 mg test item/kg soil dry weight.

Assessment and conclusion by applicant:

This study has not been previously evaluated.

Validity criteria according to the current OECD 232 test guideline (2016) were met in the controls:

- Adult mortality to be ≤20% at the end of the test (actual: 6%);
- Mean number of juveniles per vessel to be ≥100 at test end (actual: 437 to 625);
- The coefficient of variation for the number of juveniles to be <30% (actual: 11.2%).

The reference item also demonstrated sufficient sensitivity of the test organisms. The study is therefore considered acceptable.

The NOEC for reproduction was determined to be 100 mg test item/kg soil dry weight.

Data Point:	KCA 8.4.2.1/12
Report Author:	[redacted]
Report Year:	2020
Report Title:	KWG4168-N-oxide: Effects on reproduction of the predatory Mite Hypoaspis aculeifer in artificial soil
Report No:	143081089
Document No:	M-680687-01-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 (2009)
Deviations from current test guideline:	Yes OECD 226 (2016) The EC50 determined in the reference test is slightly below the recommended range given in the test guideline, however, the results are considered to confirm that the test organisms at this test facility are sensitive to the effects of the reference substance and therefore the results achieved in this study are considered to be valid. The study is therefore considered acceptable.
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 purpose of the study was to determine the effects of KWG 4168-N-oxide on mortality and reproduction of the predatory mite *Hypoaspis aculeifer*.

KWG 4168-N-oxide was added to soil at concentrations of 6.25, 12.5, 25, 50 and 100 mg test item/kg soil dry weight.

KWG 4168-N-oxide caused no statistically significant effects on mortality or reproduction of *Hypoaspis aculeifer* up to and including the concentration of 100 mg test item/kg soil dry weight. Therefore, the No Observed Effect Concentration (NOEC) for mortality was determined to be ≥100 mg test item/kg soil dry weight and the Lowest Observed Effect Concentration (LOEC) was estimated to be >100 mg test item/kg soil dry weight.

The NOEC for reproduction was determined to be ≥100 mg test item/kg soil dry weight and the LOEC for reproduction was estimated to be >100 mg test item/kg soil dry weight. Due to the absence of a concentration-response relationship, the EC_{10}, EC_{20} and EC_{50} values have been estimated to be >100 mg test item/kg soil dry weight.

I. Materials and Methods

A. Materials

Test Material KWG 4168-N-oxide

Lot/Batch #: AE 1344305 00 1CV74 0001

Purity: 72.9 % w/w

Description: Light yellow liquid

Stability of test compound: Sufficient based on expiration date

Reanalysis/Expiry date: 28 November 2022

Density: Not reported

Treatments

Test rates:	6.25, 12.5, 25, 50 and 100 mg test item/kg soil dry weight
Solvent/vehicle:	Deionised water
Analysis of test concentrations:	No

Test design

Test species:	*Hypoaspis aculeifer*
Test vessel:	100 mL glass containers (volume: 100 mL; diameter: 5 cm), with tight screw cap
Test substrate:	5% Sphagnum-peat, 20% Kaolin clay, 74.8% fine quartz-sand, 0.2% Calcium carbonate
Replication:	Four per treatment group and eight for the control
No. of animals/vessel:	10
Duration of test:	14 days

Environmental test conditions

Temperature:	20 ± 2°C	
pH:	Test start:	5.9
	Test end:	5.8 to 6.0
Photoperiod:	16 h light : 8 h dark (400 – 800 lux)	
Water content:	Test start:	27.7% to 27.9% (52.3% to 52.7% of WHC_{max})
	Test end:	26.7% to 27.4% (50.4% to 51.7% of WHC_{max})

B. Study Design

The purpose of the study was to determine the effects of KWG 4168-N-oxide on mortality and reproduction of the predatory mite *Hypoaspis aculeifer*.

Nominal test concentrations were 6.25, 12.5, 25, 50 and 100 mg test item/kg soil dry weight.

Ten adult female mites were added to each of the four replicate test vessels (eight for the control). Test vessels were glass containers (volume: 100 mL; diameter: 5 cm), tight screw top closure to avoid water evaporation, filled with approximately 20 g ± 1.0 g artificial soil dry weight. The height of the soil layer in the containers was 1.5 to 2 cm.

One spatula of cheese mites (*Tyrophagus putrescentiae*) was provided as food at experimental start and on days 2, 4, 7, 9 and 11.

Incubation was at 18 to 22°C with a photoperiod of 16 hours light to 8 hours dark at approximately 400 to 800 lux.

After 14 days exposure the soil was filled into Millipore pots with attached plastic containers for collecting the escaping mites. These extraction units were placed in a heat extractor. The soil including the mites was exposed to a temperature of approximately 25°C and 30°C for approximately 2 days. Escaping mites were collected in a fixing liquid, cooled at a temperature of approximately 16°C. The fixing liquid contained glycol and a detergent.

Adult animals were counted once visually, juvenile animals were counted twice under binocular microscopes. None of the replicate counts deviated more than 10% from their mean value.

II. Results and Discussion

Validity criteria according to the OECD 226 guideline were met.

- Mean mortality in the controls to not exceed 20% (actual: 0%)
- The mean number of juvenile mites per replicate to be at least 50 (actual: 173 to 226)
- The coefficient of variation for reproduction to be ≤30% (actual: 7.8%)

Mortality of *Hypoaspis aculeifer* in the test item treated groups ranged from 0 to 7.5%. The values were not statistically significantly different compared to the control (Fisher's Exact Test, $\alpha = 0.05$, one-sided greater). Therefore, the NOEC for mortality was determined to be ≥100 mg test item/kg soil dry weight. The LOEC for mortality was estimated to be >100 mg test item/kg soil dry weight.

No differences in morphology of the mites between the test item treated groups and the control were observed.

Table CA 8.4.2.1/12-1 Mortality of adult *Hypoaspis aculeifer* after 14 days

Treatment group (mg test item/kg soil dry weight)	Number of surviving females per group	Mean mortality [%]	Standard deviation [%]
Control	80	0	± 0
6.25	40	0	± 0
12.5	40	0	± 0
25	39	2.5	± 5
50	38	5.0	± 10
100	37	7.5	± 10

The results represent rounded values calculated from the exact raw data

There were no statistically significant effects on reproduction of *Hypoaspis aculeifer* up to and including the concentration of 100 mg test item/kg soil dry weight (Dunnett's t-test, $\alpha = 0.05$, one-sided smaller). Therefore, the NOEC for reproduction was determined to be ≥100 mg test item/kg soil dry weight and the LOEC for reproduction was estimated to be >100 mg test item/kg soil dry weight.

Due to the lack of a concentration-response relationship no reliable ECx-calculation was possible. Therefore, no EC_{10}/EC_{20}-value can be reported. The EC_{10}, EC_{20} and EC_{50} values were estimated to be >100 mg test item/kg soil dry weight.

Table CA 8.4.2.1/12-2 Reproduction of *Hypoaspis aculeifer* after 14 days

Treatment group (mg test item/kg soil dry weight)	Mean number of juveniles per group	Standard deviation	% of control
Control	192	± 15	-
6.25	183	± 12	96
12.5	184	± 20	96
25	195	± 9	102
50	197	± 18	103
100	169	± 22	88

The results represent rounded values calculated from the exact raw data

To verify the sensitivity of the test system, the reference item (dimethoate) was tested at concentrations of 1.54, 2.23, 3.23, 4.69 and 6.80 mg a.s./kg soil dry weight in a separate study. In the most recent GLP study, an EC_{50} of 2.47 mg a.s./kg soil dry weight was determined for juvenile production. The effects on the reduction of reproduction showed that the test system was sensitive and reflected the expected toxicity as given in the OECD 226 test guideline (EC_{50} for reproduction should be between 3.0 and 7.0 mg a.s./kg soil dry weight). The EC_{50} determined in the reference test is slightly below the recommended range given in the test guideline, however, the results are considered to confirm that the test organisms

at this test facility are sensitive to the effects of the reference substance and therefore the results achieved in this study are considered to be valid.

III. Conclusion

KWG 4168-N-oxide caused no statistically significant effects on mortality or reproduction of *Hypoaspis aculeifer* up to and including the concentration of 100 mg test item/kg soil dry weight. Therefore the No Observed Effect Concentration (NOEC) for mortality was determined to be ≥100 mg test item/kg soil dry weight and the Lowest Observed Effect Concentration (LOEC) was estimated to be >100 mg test item/kg soil dry weight. The LC_{50} was estimated to be >100 mg test item/kg soil dry weight.

The NOEC for reproduction was determined to be ≥100 mg test item/kg soil dry weight and the LOEC for reproduction was estimated to be >100 mg test item/kg soil dry weight. Due to the absence of a concentration-response relationship, the EC_{10}, EC_{20} and EC_{50} values have been estimated to be >100 mg test item/kg soil dry weight.

Assessment and conclusion by applicant:

This is a new study that has not been previously evaluated.

Validity criteria according to the current OECD 226 guideline (2016) were met.

- Mean mortality in the controls to not exceed 20% (actual: 0%)
- The mean number of juvenile mites per replicate to be at least 50 (actual: 173 to 226)
- The coefficient of variation for reproduction to be ≤30% (actual: 7.8%)

The EC_{50} determined in the reference test is slightly below the recommended range given in the test guideline, however, the results are considered to confirm that the test organisms at this test facility are sensitive to the effects of the reference substance and therefore the results achieved in this study are considered to be valid. The study is therefore considered acceptable.

The NOEC for reproduction was determined to be 100 mg test item/kg soil dry weight.

KWG 4168-carboxylic acid (M06)

Data Point:	KCA 8.4.2.1/13
Report Author:	[redacted]
Report Year:	2020
Report Title:	KWG 4168-carboxylic acid: Effects on reproduction of the collembola Folsomia candida in artificial soil
Report No:	152521016
Document No:	M-727126-01-1
Guideline(s) followed in study:	OECD-Guideline for testing chemicals No. 232 "Collembolan Reproduction Test in Soil" (adopted July 29, 2016) ISO 11267 Soil Quality – Inhibition of reproduction of Collembola (Folsomia candida) by soil contaminants, 2014
Deviations from current test guideline:	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

Collembola (*Folsomia candida*) aged 9 to 12 days were exposed to KWG 4168-carboxylic acid incorporated into artificial soil in a 4 week study in order to assess the effects on mortality and

reproduction. Test concentrations used were 62.5, 125, 250, 500 and 1000 mg/kg soil, with boric acid as a toxic standard

There were no statistically significant effects observed on the mortality or reproduction of *Folsomia candida* up to and including the test concentration of 1000 mg/kg as compared to the pooled controls.

The NOEC and LOEC values for mortality and reproduction were determined to be ≥1000 mg/kg soil and >1000 mg/kg soil, respectively.

I. Materials and Methods

A. Materials

Test Material	KWG 4168-carboxylic acid
Lot/Batch #:	AE 1344313-01-03
Purity:	90.6%
Description:	Turbid brown liquid
Reanalysis/Expiry date:	13 March 2021
Treatments	
Test rates:	62.5, 125, 250, 500 and 1000 mg/kg
Test organisms	
Species:	*Folsomia candida*, Collembola, Isotomidae, age 9 – 12 days
Source:	In-house culture
Acclimatisation period:	Not reported
Feeding:	2 mg of granulated dry yeast at test initiation and after 14 days
Test design	
Test vessel:	Glass containers (volume: 100 mL; diameter: 5 cm)
Test medium:	Artificial soil
Replication:	8 replicates for the control, 4 replicates per test concentration and 1 additional container per treatment to test the pH and water content of the soil at test termination
No. animals/vessel:	10 per replicate
Duration of test:	4 weeks
Environmental test conditions	
Temperature:	18 – 22°C
Water content:	Test start: 20.7% – 21.1% (51.7 – 52.8% of the WHC_{max}) Test end: 19.3% – 20.6% (48.2 – 51.5% of the WHC_{max})
pH:	Test start: 6.4 Test end: 6.5
Photoperiod:	16 hours light, 8 hours dark (400 – 800 lux)

B. Study Design

This study was conducted in order to assess the effects of KWG 4168-carboxylic acid on the reproduction of Collembola (*Folsomia candida*) over 4 weeks.

The Collembola were 9 to 12 days old at test initiation. Ten juvenile Collembola were introduced to the test vessels and placed onto the surface of treated artificial soil. The test soil was composed of 74.8% fine quartz sand, 20% kaolinite clay, 5% sphagnum peat and 0.2% calcium carbonate.

The artificial soil was kept within 18 to 22ºC and the test vessels were held under a 16 hours light, 8 hours dark photoperiod at 400 to 800 lux in a controlled environment chamber. Water content was checked 14 days after application by reweighing the additional test vessels.

Test concentrations of 62.5, 125, 250, 500 and 1000 mg/kg were applied to the artificial soil. Eight replicates were exposed to the control treatment and four replicates were exposed to the treatment groups.

At test initiation and after 14 days, the Collembola were fed with approximately 2 mg of granulated dry yeast.

A reference test with the toxic standard, Boric acid, was performed at least once a year to ensure that the laboratory test conditions were adequate and to verify that the response of the test organisms does not change significantly over time.

Reproduction and mortality data were observed at test termination. Behavioural abnormalities were also recorded at test termination. Statistical analysis was performed on the reproduction data using Shapiro-Wilk's test and Levene's test ($\alpha = 0.01$) to test for normal distribution and homogeneity. Since the reproduction data were normally distributed and homogeneous but did not follow a monotonicity trend (contrast trend), the Dunnett's t-test was used to compare treatment and control values (multiple comparison, $\alpha = 0.05$, one-sided smaller). Statistical analysis was performed on the mortality data using Step-down Cochran-Armitage Test ($\alpha = 0.05$, one-sided greater).

The software used to perform the statistical analysis was ToxRat Professional, Version 3.3.0, ToxRat® Solutions GmbH.

II. Results and Discussion

Validity criteria according to the OECD 232 test guideline (2016) were met.

- Mean adult mortality should not exceed 20% at the end of the test (actual: 9% in untreated control, 5% in solvent control and 7% in pooled control)
- The mean number of juveniles per vessel should be at least 100 at the end of the test (actual: 614 – 1137 in untreated control, 637 – 968 in solvent control and 614 – 1137 in pooled control)
- The coefficient of variation calculated for the number of juveniles should be less than 30% at the end of the definitive test (actual: 21.3% in untreated control, 13.6% in solvent control and 17.3% in pooled control)

There were no statistically significant differences between the untreated control and the solvent control (Fisher's Exact test, $\alpha = 0.05$, two-sided). Therefore, the test item treatments were compared with the pooled data of both controls. Mortality of *Folsomia candida* was not statistically significantly different compared to the pooled control up to and including the highest test concentration of 1000 mg/kg (Step-down Cochran-Armitage Test, $\alpha = 005$, one-sided greater).

Table CA 8.4.2.1/13-1 Mortality data observed after 28 days exposure

Treatment group (mg/kg)	Mean mortality (%)	Standard deviation	Significance
Control	9	± 10%	-
Solvent control	5	± 5%	n.s.[1)]
Pooled control	7	± 8%	-

Treatment group (mg/kg)	Mean mortality (%)	Standard deviation	Significance
62.5	3	± 5%	n.s.[2)]
125	10	± 8%	n.s.[2)]
250	5	± 10%	n.s.[2)]
500	18	± 5%	n.s.[2)]
1000	8	± 10%	n.s.[2)]

n.s.[1)] not significantly different compared to the control, Fisher's Exact Test, two-sided, α = 0.05
n.s.[2)] not significantly different compared to the pooled control, Step-down Cochran-Armitage Test, one-sided greater, α = 0.05

There were no statistically significant differences between the untreated control and the solvent control (Student t-test, α = 0.05, two-sided). Therefore, the test item treatments were compared with the pooled data of both controls. There were no statistically significant effects on reproduction of *Folsomia candida* up to and including the highest test concentration of 1000 mg/kg (Dunnett's t-test, α = 0.05, one-sided smaller).

Table CA 8.4.2.1/13-2 Reproduction data observed after 28 days exposure

Treatment group (mg/kg)	Mean	Standard deviation	% of control	% of pooled control	Significance
Control	821	± 175	-	-	-
Solvent control	830	± 119	101	-	n.s.[1)]
Pooled control	826	± 143	-	-	
62.5	794	± 99	-	96	n.s.[2)]
125	905	± 218	-	110	n.s.[2)]
250	869	± 195	-	105	n.s.[2)]
500	809	± 254	-	98	n.s.[2)]
1000	698	± 267	-	85	n.s.[2)]

n.s.[1)] not significantly different compared to the control, Student's t-test, α = 0.05, two-sided
n.s.[2)] not significantly different compared to the pooled control, Dunnett's t-test, α = 0.05, one-sided smaller
- not applicable

To verify the sensitivity of the test system, the reference item (boric acid) was tested at concentrations of 30.5, 48.8, 78.1, 125 and 200 mg/kg soil dry weight in a separate study. In the most recent GLP study, an EC_{50} of 104.6 mg/kg soil dry weight was determined. The effects on the reduction of reproduction showed that the test system was sensitive and reflected the expected toxicity as given in the OECD 232 test guideline (50% reduction in reproduction at about 100 mg/kg soil dry weight).

III. Conclusion

KWG 4168-carboxylic acid caused no statistically significant effects on mortality and reproduction of *Folsomia candida* up to and including the highest test concentration of 1000 mg/kg.

Therefore, the NOEC for mortality was determined to be ≥1000 mg/kg and the LOEC was estimated to be >1000 mg/kg. The NOEC for reproduction was determined to be ≥1000 mg/kg and the LOEC was estimated to be >1000 mg/kg.

The LC_{50} was estimated to be >1000 mg/kg. Due to the lack of a concentration-response relationship, no reliable EC_x calculation was possible. Therefore, no EC_{10} can be reported, and the EC_{20} and EC_{50} values were estimated to be >1000 mg/kg as there were no effects >20% or >50% in reproduction observed at any test item concentration.

Assessment and conclusion by applicant:

This is a new study that has not been previously submitted or evaluated.

Validity criteria according to the OECD 232 guideline (2016) were met.

- Mean adult mortality should not exceed 20% at the end of the test (actual: 9% in untreated control, 5% in solvent control and 7% in pooled control)

- The mean number of juveniles per vessel should be at least 100 at the end of the test (actual: 614 – 1137 in untreated control, 637 – 968 in solvent control and 614 – 1137 in pooled control)
- The coefficient of variation calculated for the number of juveniles should be less than 30% at the end of the definitive test (actual: 21.3% in untreated control, 13.6% in solvent control and 17.3% in pooled control)

The reference item also demonstrated sufficient sensitivity of the test organisms. The study is therefore considered acceptable.

The NOEC for reproduction was determined to be 1000 mg/kg soil dry weight.

Data Point:	KCA 8.4.2.1/14
Report Author:	[redacted]
Report Year:	2020
Report Title:	Amendment no. 01: KWG 4168-carboxylic-acid: Effects on reproduction of the predatory mite Hypoaspis aculeifer in artificial soil
Report No:	152521089
Document No:	M-727128-02-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 (2009) OECD 226: Guidelines for the testing of chemicals - Predatory Mite (Hypoaspis (Geolaelaps) aculeifer) reproduction test in soil, adopted July 29, 2016
Deviations from current test guideline:	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

Adult *Hypoaspis aculeifer* were exposed to KWG 4168-carboxylic acid in a 14-day study to assess the effects on mortality and reproduction.

Hypoaspis aculeifer were exposed in artificial soil to a control and to test concentrations of 62.5, 125, 250, 500 and 1000 mg/kg soil, according to the guidelines set out in OECD 226 (2016).

The NOEC and LOEC values for mortality were determined to be ≥1000 and >1000 mg/kg soil, respectively.

The NOEC and LOEC values for reproduction were determined to be ≥1000 and >1000 mg/kg soil, respectively.

The LC_{50} and EC_{50} values for mortality and reproduction respectively were both estimated to be >1000 mg/kg soil.

I. Materials and Methods

A. Materials

Test Material	KWG 4168-carboxylic acid
Lot/Batch #:	AE 1344313-01-03
Purity:	90.6%
Description:	Turbid brown liquid
Reanalysis/Expiry date:	13 March 2021

Density:	Not reported
Treatments	
Test rates:	62.5, 125, 250, 500 and 1000 mg/kg soil
Solvent/vehicle:	Acetone
Test organisms	
Species:	*Hypoaspis aculeifer*, predatory mite, Laelapidae, adult
Source:	In-house culture
Feeding:	One spatula of cheese mites (*Tyrophagus putrescentiae*) at test initiation and on test days 2, 5, 7, 9 and 12
Test design	
Test vessel:	Glass containers (volume: 100 mL, diameter: 5 cm) with tight screw top lids
Test medium:	Each test vessel was filled with 20 ± 1.0 g dry weight artificial soil (height of soil layer: approximately 1.5 – 2 cm)
Replication:	8 replicates for the control, 4 replicates per treatment group and 1 additional container per treatment to test the pH and water content of the test substrate at test termination
No. animals/vessel:	10 per test vessel
Duration of test:	14 days
Environmental test conditions	
Temperature:	18 to 22°C
Water content:	Test start: 20.7% – 21.1% (51.7 – 52.8% of the WHC_{max}) Test end: 19.9% – 20.9% (49.7 – 52.3% of the WHC_{max})
pH:	Test start: 6.4 Test end: 6.3 – 6.4
Photoperiod:	16 hours light, 8 hours dark (400 – 800 lux)

B. Study Design

This study was conducted in order to assess the effects of KWG 4168-carboxylic acid on the reproduction of *Hypoaspis aculeifer* over 14 days.

Ten adult female *Hypoaspis aculeifer* per replicate (8 replicates for the control group and 4 replicates for reach treatment group) were exposed to the control and treatments. Concentrations of 62.5, 125, 250, 500 and 1000 mg/kg soil were mixed into artificial soil. The test soil was composed of 74.8% fine quartz-sand, 20% kaolinite clay, 5% sphagnum peat and 0.2% calcium carbonate. The soil was prepared according to the guideline OECD 226 (2016).

Test vessels comprised of glass containers with a volume of 100 mL and a diameter of 5 cm; the height of the soil layer was approximately 1.5 to 2 cm. Throughout the test, the temperature was maintained within 18 and 22°C under a 16 hours light, 8 hours dark photoperiod at 400 to 800 lux.

A reference test with the toxic standard, dimethoate, was performed at least once a year to ensure that the laboratory test conditions weree adequate and to verify that the response of the test organisms does not change significantly over time.

Water content was checked 7 days after application by reweighing the additional test vessels. If the water content did not deviate by more than 2% from the initial water content, the vessels were not compensated for water loss.

Reproduction data were observed at test termination. Statistical analysis was performed on the reproduction data using Shapiro-Wilk's test and Levene's test ($\alpha = 0.01$) to test for normal distribution and homogeneity. Since the reproduction data were normally distributed but did not follow a monotonicity trend (contrast trend), the Dunnet's t-test was used to compare treatment and solvent control values (multiple comparison, $\alpha = 0.05$, one-sided smaller).

Mortality data were observed at test termination. Missing adult mites were assumed dead and degraded. Statistical analysis was performed on the mortality data using Chi² Test (multiple comparison, with Bonferroni Correction, $\alpha = 0.05$, one-sided greater). The LC_{50} at test termination was not determined by statistical analysis as no mortality above 50% occurred.

The software used to perform the statistical analysis was ToxRat Professional, Version 3.3.0, ToxRat® Solutions GmbH.

II. Results and Discussion

Validity criteria according to the OECD 226 guideline (2016) were met.

- Mean adult female mortality should not exceed 20% at the end of the test (actual: 5% in control, 5% in solvent control)
- The mean number of juveniles per replicate (with 10 adult females introduced) should be at least 50 at the end of the test (actual: 174 - 217 in control, 194 - 233 in solvent control)
- The coefficient of variation calculated for the number of juvenile mites per replicate should not be higher than 30% at the end of the definitive test (actual: 7.0% in control, 6.6% in solvent control)

Mortality of *Hypoaspis aculeifer* in the test item treated groups ranged from 0% to 10%. The values were not statistically significantly different compared to the pooled control where 5% mortality was observed (Chi² Test, $\alpha = 0.05$, one-sided greater).

Table CA 8.4.2.1/14-1 Mortality data observed after 14 days exposure

Treatment group (mg/kg)	Mean mortality (%)	Standard deviation (%)	Significance[1]
Control	5	± 5%	-
Solvent control	5	± 9%	n.s.[1]
Pooled control	5	± 7%	
62.5	0	± 0%	n.s.[2]
125	0	± 0%	n.s.[2]
250	8	± 10%	n.s.[2]
500	5	± 6%	n.s.[2]
1000	10	± 8%	n.s.[2]

n.s.[1] not statistically significantly different compared to the control, Fisher's Exact Test, two-sided, $\alpha = 0.05$
n.s.[2] not statistically significantly different compared to the pooled control, Chi² Test, one-sided greater, $\alpha = 0.05$
- not applicable

There were no statistically significant effects on reproduction of *Hypoaspis aculeifer* up to and including the highest test concentration of 1000 mg/kg (Dunnett's t-test, $\alpha = 0.05$, one-sided smaller).

Table CA 8.4.2.1/14-2 Reproduction data observed after 14 days exposure

Treatment group (mg/kg)	Mean	Standard deviation	% of control	% of solvent control	Significance
Control	215	± 15	-	-	-
Solvent control	198	± 13	92	-	*[1]

Treatment group (mg/kg)	Mean	Standard deviation	% of control	% of solvent control	Significance
62.5	197	± 14	-	99	n.s.[2]
125	191	± 17	-	96	n.s.[2]
250	184	± 17	-	93	n.s.[2]
500	197	± 7	-	99	n.s.[2]
1000	208	± 19	-	105	n.s.[2]

*[1] significantly different compared to the control, Student t-test, α = 0.05, two-sided
n.s.[2] not significantly different compared to the solvent control, Dunnett's t-test, α = 0.05, one-sided smaller
- not applicable

To verify the sensitivity of the test system, the reference item (dimethoate) was tested at concentrations of 1.54, 2.23, 3.23, 4.69 and 6.80 mg a.s./kg soil dry weight in a separate study. In the most recent GLP study, an EC_{50} of 3.18 mg a.s./kg soil dry weight was determined for juvenile production. The effects on the reduction of reproduction showed that the test system was sensitive and reflected the expected toxicity as given in the OECD 226 test guideline (EC_{50} for reproduction should be between 3.0 and 7.0 mg a.s./kg soil dry weight).

III. Conclusion

KWG 4168-carboxylic acid caused no statistically significant effects on mortality or reproduction of *Hypoaspis aculeifer* up to and including the highest test concentration of 1000 mg/kg soil.

Therefore, the NOEC for mortality was determined to be ≥1000 mg/kg and the LOEC was estimated to be >1000 mg/kg. The NOEC for reproduction was determined to be ≥1000 mg/kg and the LOEC was estimated to be >1000 mg/kg.

The LC_{50} was estimated to be >1000 mg/kg. Due to the lack of a concentration-response relationship, no reliable EC_x-calculation was possible. The EC_{10}, EC_{20} and EC_{50} values were estimated to be >1000 mg/kg soil as there were no effects >10%, >20% or >50% in reproduction observed at any test item concentration.

Assessment and conclusion by applicant:

This is a new study that has not been previously submitted or evaluated.

Validity criteria according to the OECD 226 guideline (2016) were met.

- Mean adult female mortality should not exceed 20% at the end of the test (actual: 5% in control, 5% in solvent control).
- The mean number of juveniles per replicate (with 10 adult females introduced) should be at least 50 at the end of the test (actual: 174 - 217 in control, 194 – 233 in solvent control)
- The coefficient of variation calculated for the number of juvenile mites per replicate should not be higher than 30% at the end of the definitive test (actual: 7.0% in control, 6.6% in solvent control)

The reference item also demonstrated sufficient sensitivity of the test organisms. The study is therefore considered acceptable.

The NOEC for reproduction was determined to be 1000 mg/kg soil dry weight.

Relevant literature on earthworms and other soil macro-organisms

No relevant scientifically peer-reviewed open literature could be found on spiroxamine or its major metabolites, from an ecotoxicological perspective, on earthworms and other soil meso- and macro-organisms. Details of the literature search undertaken can be found in the M-CA Section 9 of the current submission.

CA 8.5 Effects on nitrogen transformation

The available data for the metabolites of spiroxamine with soil micro-organisms are presented in the table below. No data are available for spiroxamine technical but studies are available using Spiroxamine EC 500 (please refer to Document M-CP Section 10 for Spiroxamine EC 500).

Table CA 8.5-1 Summary of nitrogen transformation studies with metabolites of spiroxamine

Test item	Test type	Endpoints		Reference
KWG 4168-desethyl (M01)	Nitrogen transformation	<25% effect after 28 days at 4.53 mg/kg soil	EU	M-282056-01-1
KWG 4168-despropyl (M02)	Nitrogen transformation	<25% effect after 70 days at 5.0 mg/kg soil	NEW	M-680757-01-1
KWG 4168-N-oxide (M03)	Nitrogen transformation	<25% effect after 56 days at 6.9 mg/kg soil	NEW	M-680759-01-1
KWG 4168-acid (M06)	Nitrogen transformation	<25% effect after 28 days at 5.0 mg/kg soil	NEW	M-688317-01-1

EU: previously evaluated as part of the original EU review and listed in EFSA conclusion and DAR
NEW: new study or data generated since the previous EU review or previously not submitted

No nitrogen transformation data using spiroxamine technical are available. However, data are available using the representative formulations and full summaries have been provided in Document M-CP Section 10.

Metabolites

KWG 4168-desethyl (M01)

Data Point:	KCA 8.5/01
Report Author:	[redacted]
Report Year:	2007
Report Title:	Metabolite KWG 4168-desethyl: Determination of effects on nitrogen transformation in soil
Report No:	LRT-N-81/07
Document No:	M-382056-01-1
Guideline(s) followed in study:	OECD/OECD Guideline No. 216, Adopted: 21st January 2000, OECD Guideline for the Testing of Chemicals, Soil Micro organisms: Nitrogen Transformation Test
Deviations from current test guideline:	Yes OECD 216 (2000) Only one concentration tested instead of the recommended two test concentrations.
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

A silty sand soil was exposed to KWG 4168-desethyl for 28 days to assess the effects on soil nitrogen transformation.

KWG 4168-desethyl was added to soil at a concentration of 4.53 mg test item/kg soil dry weight and the rate of nitrogen transformation was observed.

After 28 days, no statistically significant difference from the control was observed in the rate of nitrogen transformation at a concentration of 4.53 mg/kg soil dry weight.

I. Materials and Methods

A. Materials

Test Material	KWG 4168-desethyl, a metabolite of KWG 4168
Lot/Batch #:	921103ELB02
Purity:	98%
Description:	Clear brown oily liquid
Stability of test compound:	Not reported
Reanalysis/Expiry date:	7 December 2009
Density:	Not reported
Treatments	
Test rates:	4.53 mg metabolite/kg dry weight soil (3.4 kg metabolite/ha)
Solvent/vehicle:	Quartz sand
Analysis of test concentrations:	No
Test design	
Test vessel:	500 ml brown glass bottles and these were closed with Para film
Replication:	3 per treatment
Duration of test:	28 days
Environmental test conditions	
Temperature:	20 ± 2°C
Photoperiod:	Darkness

B. Study Design

This study was conducted in order to assess the effects of KWG 4168-desethyl, a KWG 4168 metabolite, on soil nitrogen transformation over 28 days.

The soil used was from Bayer CropScience AG's experimental farm, Laacherhof, Germany. Plant protection chemicals had not been used on this field since 1981.

Sieved soil (2 mm) was treated with either a control or a test item mixture. The control mixture was 10 g ground quartz sand/kg dry weight soil and the test item mixture was quartz sand and KWG 4168-desethyl at a concentration of 4.53 mg metabolite/kg dry weight soil. This is equivalent to 3.4 kg KWG 4168-desethyl/ha.

The samples were mixed with pulverized Lucerne-grass-green meal (5 g/kg dry weight soil) to stimulate nitrogen transformation. The samples were added to 3L polyethylene containers and mixed by rolling on a gyro wheel mixer for 15 min at 50 rpm.

After mixing, soil samples equivalent to 300 g dry weight were poured into 500 mL brown glass bottles and these were closed with Para film. Three replicates were prepared per treatment. Soil particles were removed by filtration and the extracts analysed for their content of ammonium-N, nitrite-N and nitrate-N plus nitrite-N on a Bran + Lübbe Auto analyzer 3.

The soil was kept in darkness at 20 ± 2°C and samples were taken after 9, 7, 14 and 28 days.

II. Results and Discussion

Validity criteria according to the OECD 216 guideline (2000) were met.

- Variation between replicate control samples ≤ 15% (actual: max 14%)

The difference in rate of nitrogen transformation between control and treatment group was 10%, which was not significantly different to the control.

During the 28-day test, KWG 4168-desethyl caused a temporary stimulation of the daily nitrate rate (7-14 days after treatment). At the end of the test (28 days after treatment), the effects were < 25 % and met the trigger values recommended by the guideline for termination of the study. Under field conditions, this metabolite should not have an impact on nitrogen transformation in soils.

Table CA 8.5/01-1 Nitrogen transformation in soil treated with KWG 4168-desethyl

	Days after treatment	Ammonium-N (mg /kg soil dry weight)		Nitrate-N (mg /kg soil dry weight)	
		Mean	CV (%)	Mean	CV (%)
Control	0	4.40	2	24.14	1
	7	2.07	3	11.47	10
	14	1.98	14	16.00	8
	28	1.85	8	34.31	1
4.53 mg metabolite/kg dry weight soil	0	4.30		24.55	-
	7	2.32	-	9.87	-
	14	1.95	-	16.13	-
	28	2.04	-	32.63	-

Table CA 8.5/01-2 Rate of nitrogen transformation per time interval in soil treated with KWG 4168-desethyl

	Days after treatment	Mean (mg N/kg dry weight soil)	SD (mg N/kg dry weight soil)
Control	0-7	-12.66	0.98
	7-14	4.53	1.74
	14-28	18.31	1.31
4.53 mg metabolite/kg dry weight soil	0-7	-14.68	3.62
	7-14	6.26	3.09
	14-28	16.51	1.71

Table CA 8.5/01-3 Rate of nitrogen transformation per day in soil treated with KWG 4168-desethyl

	Days after treatment	Mean (mg N/kg dry weight soil)	SD (mg N/kg dry weight soil)	% Difference from control
Control	0-7	-1.81	0.14	-
	7-14	0.65	0.25	-
	14-28	1.31	0.09	-
4.53 mg metabolite/kg dry weight soil	0-7	-2.10	0.52	16
	7-14	0.89	0.44	38
	14-28	1.18	0.12	10

III. Conclusion

During the 28-day test, KWG 4168-desethyl caused a temporary stimulation of the daily nitrate rate (7-14 days after treatment). At the end of the study (28 days after treatment) the effects were < 25 % at the rate of 4.53 mg/kg soil dry weight.

Assessment and conclusion by applicant:

Validity criteria according to the current OECD 216 guideline (2000) were met:

- Variation between replicate control samples to be less than ±15% (actual: max. 14%)

It is noted that the OECD 216 test guideline states that two concentrations should be tested for agrochemicals whereas this study tested only a single concentration. However, the concentration tested was sufficiently high to cover the predicted soil concentration of this metabolite and clearly shows that there were <25% effects after 28 days. As such the results are considered suitable for use in the risk assessment of spiroxamine.

The study is therefore considered acceptable.

There were <25 % effects after 28 days at the rate of 4.53 mg/kg soil dry weight.

KWG 4168-despropyl (M02)

Data Point:	KCA 8.5/02
Report Author:	[redacted]
Report Year:	2020
Report Title:	KWG4168-despropyl: Effects on the activity of the soil microflora in the laboratory (nitrogen transformation)
Report No:	143071080
Document No:	M-680757-01-1
Guideline(s) followed in study:	OECD Guideline 216 (2000)
Deviations from current test guideline:	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 purpose of this study was to assess the effects of the test item on the activity (nitrogen transformation) of the soil microflora in the laboratory.

KWG 4168-despropyl was tested at concentrations of 1 and 5 mg test item/kg soil dry weight.

After 70 days, the test item KWG 4168-despropyl had no long-term impact on nitrogen transformation (nitrate content, mineral nitrogen content and nitrate formation rates) of soil microorganisms when applied at 1 and 5 mg test item/kg soil dry weight.

I. Materials and Methods

A. Materials

Test Material KWG 4168-despropyl

Lot/Batch #: AE 1344303-PU-01

Purity: 99.1% w/w

Description:	Colourless liquid
Stability of test compound:	Sufficient based on expiration date
Reanalysis/Expiry date:	13 May 2022
Density:	Not reported
Treatments	
Test rates:	1 and 5 mg test item/kg soil dry weight
Solvent/vehicle:	Acetone
Analysis of test concentrations:	No
Test design	
Test vessel:	Disposable plastic boxes, approximately 0.5 L in volume
Test substrate:	According to the guideline, taken from fallow grassland (location: "In der Speyerer Hohl", No. 977). No pesticides or organic or mineral fertiliser had been used on the soil for at least four years prior to test initiation
Replication:	Three per treatment group and control
Duration of test:	70 days
Environmental test conditions	
Temperature:	20 ± 2°C
pH:	7.3 – 7.5
Photoperiod:	Darkness
Water content:	49 to 51% of WHC

B. Study Design

The purpose of this study was to assess the effects of the test item on the activity (nitrogen transformation) of soil microflora in the laboratory.

Test rates were 1 and 5 mg test item/kg soil dry weight. There were three replicates per treatment and control group.

Test units were disposable plastic boxes with 300 g soil (dry weight), the box volume was approximately 0.5 L and the dimensions were 0.10 m width x 0.10 m depth x 0.065 m height.

Incubation was at 18 to 22°C in the dark. Test conditions (temperature) were recorded continuously using suitable instruments, documented in the raw data and reported in the final report.

KWG 4168-despropyl was soluble in acetone; Therefore a stock solution in acetone was prepared by dissolving 75 mg KWG 4168-despropyl in 25 mL acetone and applied onto quartz sand. After evaporation of the acetone, the quartz sand was mixed into the soil by means of a laboratory mixer. Throughout the application the soil was ventilated and the soil water content was adjusted to 50% of WHC.

For the determination of nitrogen content, soil samples were taken within 6 hours after application and afterwards on each sampling date (7, 14, 28, 42, 56 and 70 days). The nitrogen content was determined in each sample of treated and control soils.

II. Results and Discussion

Validity criteria according to the OECD 216 guideline were met:

- Variation between replicate control samples to be less than ±15% (actual: max. 4.90%)

No adverse effects of the test item on nitrate content in soil were observed at days 28, 42, 56 and 70. At day 28, differences to the control were 14.82% and -21.96% in the 1 mg and 5 mg test item/kg soil dry weight treatment, respectively. At test end at day 70, differences to the control were 7.53% and -7.52% in the 1 mg and 5 mg test item/kg soil dry weight treatment, respectively.

At sampling points day 28, 42, 56 and 70, the differences were statistically significant compared to the control for both test rates (Student t-test, α = 0.05).

Very low nitrite and ammonium contents below 1.7 mg/kg dry weight were measured at days 28, 42, 56 and 70 in the control and the test item treatments.

At day 28, differences in mineral nitrogen content of test soil to the control were 13.39% and -21.60% in the 1 mg and 5 mg test item/kg soil dry weight treatment, respectively. At day 70, differences to the control were 7.40% and -7.40% in the 1 mg and 5 mg test item/kg soil dry weight treatment, respectively.

At sampling points day 28, 42, 56 and 70, the differences were statistically significant compared to the control for both test rates (Student t-test, α = 0.05).

Table CA 8.5/02-1 Nitrogen transformation test: effects of the test item on ammonium- (mean values)

	Control		1 mg KWG 4168-despropyl/kg soil dw		5 mg KWG 4168-despropyl/kg soil dw	
Day	NH_4-N mg/kg dry weight	CV[1] %	NH_4-N mg/kg dry weight	Dev. %[2]	NH_4-N mg/kg dry weight	Dev. %[2]
0	7.520	2.29	7.657	1.82	7.257	-3.50
7	1.914	3.03	1.916	0.10	1.710	-10.66*
14	1.603	4.12	1.295	-19.21*	1.621	1.12
28	1.650	0.97	1.339	-18.85*	1.335	-19.09*
42	≤0.701	0.00	≤0.701	0.00	≤0.701	0.00
56	≤0.701	0.00	≤0.701	0.00	≤0.701	0.00
70	≤0.701	0.00	≤0.701	0.00	≤0.701	0.00

[1]: CV, coefficient of variation; [2]Dev., Deviation from control: dw: dry weight;
*: statistically significant (according to Student t-test, two-sided, α=0.05)

Table CA 8.5/02-2 Nitrogen transformation test: effects of the test item on nitrite- (mean values)

	Control		1 mg KWG 4168-despropyl/kg soil dw		5 mg KWG 4168-despropyl/kg soil dw	
Day	NO_2-N mg/kg dry weight	CV[1] %	NO_2-N mg/kg dry weight	Dev. %[2]	NO_2-N mg/kg dry weight	Dev. %[2]
0	0.431	8.82	0.399	-7.42	0.399	-7.42
7	0.399	0.00	0.399	0.00	0.399	0.00
14	0.420	2.14	0.416	-0.95	0.405	-3.57*
28	0.407	0.25	0.421	3.44*	0.424	4.18*
42	≤0.399	0.00	≤0.399	0.00	≤0.399	0.00
56	≤0.399	0.00	≤0.399	0.00	≤0.399	0.00
70	≤0.399	0.00	≤0.399	0.00	≤0.399	0.00

[1]: CV, coefficient of variation;
[2]:Dev., Deviation from control: dw: dry weight;
*: statistically significant (according to Student t-test, two-sided, α=0.05)

Table CA 8.5/02-3 Nitrogen transformation test: effects of the test item on nitrate- (mean values)

	Control		1 mg KWG 4168-despropyl/kg soil dw		5 mg KWG 4168-despropyl/kg soil dw	
Day	**NO_3-N mg/kg dry weight**	**CV[1] %**	**NO_3-N mg/kg dry weight**	**Dev. %[2]**	**NO_3-N mg/kg dry weight**	**Dev. %[2]**
0	24.860	0.65	26.004	4.60	25.048	0.76
7	18.250	4.90	21.144	15.86*	10.950	-40.00*
14	25.675	2.73	29.041	13.11*	15.340	-40.25*
28	40.047	2.47	45.980	14.82*	31.251	-21.96*
42	45.265	3.65	50.710	12.03*	39.075	-13.68*
56	52.842	3.29	58.565	10.83*	49.682	-5.98*
70	62.846	2.33	67.578	7.53*	58.117	-7.52*

[1]: CV, coefficient of variation;
[2]:Dev., Deviation from control: dw: dry weight;
*: statistically significant (according to Student t-test, two-sided, α=0.05)

Table CA 8.5/02-4 Nitrogen transformation test: effects of the test item on N_{min}-content (mean values)

	Control		1 mg KWG 4168-despropyl/kg soil dw		5 mg KWG 4168-despropyl/kg soil dw	
Day	**N_{min}-N mg/kg dry weight**	**CV[1] %**	**N_{min}-N mg/kg dry weight**	**Dev. %[2]**	**N_{min}-N mg/kg dry weight**	**Dev. %[2]**
0	32.812	0.91	34.060	3.80	32.704	-0.33
7	20.563	4.09	23.459	14.08*	13.059	-36.49*
14	27.699	2.60	30.751	11.02*	17.366	-37.30*
28	42.104	2.32	47.740	13.39*	33.009	-21.60*
42	46.365	3.56	51.810	11.74*	40.175	-13.35*
56	53.942	3.23	59.665	10.61*	50.782	-5.86*
70	63.946	2.29	68.678	7.40*	59.217	-7.40*

[1]: CV, coefficient of variation;
[2]:Dev., Deviation from control: dw: dry weight;
*: statistically significant (according to Student t-test, two-sided, α=0.05)

The cumulative soil nitrate formation rates did exceed the trigger range of ± 25% set by OECD guideline 216 at the 0 - 28 day determination for both test rates. Differences to the control were 31.73% and -59.04% in the 1 mg and 5 mg test item/kg soil dry weight treatment, respectively. Therefore the study was prolonged for 14 days.

The cumulative soil nitrate formation rates did exceed the trigger range of ± 25% set by OECD guideline 216 at the 0 - 42 day determination for the high test rate. Differences to the control were 20.99% and -31.28% in the 1 mg and 5 mg test item/kg soil dry weight treatment, respectively. The study was therefore prolonged for a further 14 days.

The cumulative soil nitrate formation rates did not exceed the trigger range of ± 25% set by OECD guideline 216 at the 0 - 56 day determination and the 0 - 70 day determination for both test rates. At test end at the 0 - 70 day determination, differences to the control were 9.93% and -13.08% in the 1 mg and 5 mg test item/kg soil dry weight treatment, respectively.

The incremental soil nitrate formation rates did not exceed the trigger range of ± 25% set by OECD guideline 216 at the 14 - 28 day determination. Differences to the control were 17.82% and 10.71% in the 1 mg and 5 mg test item/kg soil dry weight treatment, respectively.

The incremental soil nitrate formation rates did exceed the trigger range of ± 25% set by OECD guideline 216 at the 28 - 42 day determination and the 42 – 56 day determination for the high test rate. Therefore the study was prolonged to day 70. In the last incremental nitrate formation rate interval day 56 – 70, the differences to the control were -9.93% and -15.80% in the 1 mg and 5 mg test item/kg soil dry weight treatment, respectively.

Table CA 8.5/02-5 Nitrogen transformation test: effects of the test item on nitrate formation rates (mean values)

	Control		1 mg KWG 4168-despropyl/kg soil dw		5 mg KWG 4168-despropyl/kg soil dw	
Day	**Mean mg NO_3-N/kg soil dry weight per day[3]**					
	mg/day	**CV %[1]**	**mg/day**	**Dev. %[2]**	**mg/day**	**Dev. %[2]**
0 - 7	-0.944	-12.08	-0.694	-26.48	-2.014	113.35*
0 - 14	0.058	79.31	0.217	274.14*	-0.694	-1296.55*
0 - 28	0.542	6.09	0.714	31.73*	0.222	-59.04*
0 - 42	0.486	7.61	0.588	20.99*	0.334	-31.28*
0 - 56	0.500	6.20	0.581	16.20*	0.440	-12.00
0 - 70	0.543	3.87	0.594	9.39	0.472	-13.08*
	Control		**1 mg KWG 4168-despropyl/kg soil dw**		**5 mg KWG 4168-despropyl/kg soil dw**	
Day	**Mean mg NO_3-N/kg soil dry weight per day[4]**					
	mg/day	**CV %[1]**	**mg/day**	**Dev. %[2]**	**mg/day**	**Dev. %[2]**
0 - 7	-0.944	-12.08	-0.694	-26.48	-2.014	113.35*
7 - 14	1.061	3.77	1.128	6.31	0.627	-40.90*
14 - 28	1.027	2.24	1.210	17.82*	1.137	10.71*
28 - 42	0.373	12.87	0.338	-9.38	0.559	49.87*
42 - 56	0.541	9.06	0.561	3.70	0.758	40.11*
56 - 70	0.715	4.20	0.644	-9.93	0.602	-15.80

[1]: CV, coefficient of variation;
[2]:Dev., Deviation from control; dw: dry weight;
[3]:Calculated from the mean values of NO3-N content between the sampling date and day 0;
[4]:Calculated from the mean values of NO3-N content between each sampling date;
*: statistically significant (according to Student t-test, two-sided, α=0.05)

III. Conclusion

After 70 days, exposure to the test item KWG 4168-despropyl had no long-term impact on nitrogen transformation (nitrate content, mineral nitrogen content and nitrate formation rates) of soil microorganisms when applied at 1 and 5 mg test item/kg soil dry weight.

Assessment and conclusion by applicant:

This is a new study that has not been previously evaluated.

Validity criteria according to the current OECD 216 guideline (2000) were met:

- Variation between replicate control samples to be less than ±15% (actual: max. 4.90%)

The study is therefore considered acceptable.

There were <25 % effects after 70 days at rates up to 5.0 mg/kg soil dry weight.

KWG 4168-N-oxide (M03)

Data Point:	KCA 8.5/03
Report Author:	[redacted]
Report Year:	2020
Report Title:	KWG4168-N-oxide: Effects on the activity of the soil microflora in the laboratory (nitrogen transformation)
Report No:	143081080
Document No:	M-680759-01-1
Guideline(s) followed in study:	OECD Guideline 216 (2000)
Deviations from current test guideline:	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 purpose of this study was to assess the effects of the test item on the activity (nitrogen transformation) of the soil microflora in the laboratory.

KWG 4168-N-oxide was tested at concentrations of 1.4 and 6.9 mg test item/kg soil dry weight.

After 56 days, the test item had no impact on nitrogen transformation (nitrate content, mineral nitrogen content and nitrate formation rates) of soil microorganisms when applied at 1.4 mg and 6.9 mg test item/kg soil dry weight.

I. Materials and Methods

A. Materials

Test Material KWG 4168-N-oxide

Lot/Batch #: AE 1344305 00 1C74 0001

Content: 72.9% w/w

Description: Light yellow liquid

Stability of test compound: Sufficient based on expiration date

Reanalysis/Expiry date: 28 November 2022

Density: Not reported

Treatments

Test rates: 1.4 and 6.9 mg test item/kg soil dry weight

Solvent/vehicle: Acetone

Analysis of test concentrations: No

Test design

Test vessel: Disposable plastic boxes, approximately 0.5 L in volume

Test substrate:	According to the guideline, taken from fallow grassland (location: "In der Speyerer Hohl ", No. 977). No pesticides or organic or mineral fertiliser had been used on the soil for at least four yers prior to test initiation
Replication:	Three per treatment group and control
Duration of test:	56 days
Environmental test conditions	
Temperature:	20 ± 2°C
pH:	7.3 – 7.5
Photoperiod:	Darkness
Water content:	49 to 52% WHC

B. Study Design

The purpose of this study was to assess the effects of the test item on the activity (nitrogen transformation) of the soil microflora in the laboratory.

Test rates were 1.4 and 6.9 mg test item/kg soil dry weight. There were three replicates per treatment and control group.

Test units were disposable plastic boxes with 300 g soil (dry weight), the box volume was approximately 0.5 L and the dimensions were 0.10 x 0.10 x 0.065 m.

Incubation was at 18 to 22°C in the dark. Test conditions (temperature) were recorded continuously using suitable instruments, documented in the raw data and reported in the final report. (Short-term deviations (<2 hours) from the recommended temperature range do normally not result in major disturbances of the test performance and were not reported.

KWG 4168-despropyl was soluble in acetone; therefore a stock solution in acetone was prepared by dissolving 90 mg KWG 4168-N-oxide in 25 ml acetone and applied onto quartz sand. After evaporation of the acetone, the quartz sand was mixed into the soil by means of a laboratory mixer.

Throughout the application the soil was ventilated and the soil water content was adjusted to 50% of WHC.

For the determination of nitrogen content, soil samples were taken within 6 hours after application and afterwards on each sampling date (7, 14, 28, 42 and 56 days). The nitrogen content was determined in each sample of treated and control soils.

II. Results and Discussion

Validity criteria according to the OECD 216 guideline were met:

- Variation between replicate control samples to be less than ±15% (actual: max. 4.90%)

No adverse effects of the test item on nitrate content in soil were observed at day 28. At day 28, differences to the control were -16.43% and 9.73% in the 1.4 mg and 6.9 mg test item/kg soil dry weight treatment, respectively.

No adverse effects of the test item on nitrate content in soil were observed at day 42. At day 42, differences to the control were -9.84% and 8.57% in the 1.4 mg and 6.9 mg test item/kg soil dry weight treatment, respectively.

No adverse effects of the test item on nitrate content in soil were observed at day 56. At day 56, differences to the control were -7.30% and 9.83% in the 1.4 mg and 6.9 mg test item/kg soil dry weight treatment, respectively.

At days 28, 42 and 56, all differences were statistically significant compared to the control for both test rates (Student t-test, α = 0.05).

Very low nitrite and ammonium contents below 1.0 mg/kg dry weight were measured at days 28, 42 and 56 in control and the test item treatments.

The mineral nitrogen contents in soil were within the trigger range of ± 25% at day 28. At day 28, differences to the control were -16.31% and 8.52% in the 1.4 mg and 6.9 mg test item/kg soil dry weight treatment, respectively. The mineral nitrogen contents in soil were within the trigger range of ± 25% at day 42. At day 42, differences to the control were -9.61% and 8.36% in the 1.4 mg and 6.9 mg test item/kg soil dry weight treatment, respectively.

The mineral nitrogen contents in soil were within the trigger range of ± 25% at day 56. At day 56, differences to the control were -6.77% and 9.63% in the 1.4 mg and 6.9 mg test item/kg soil dry weight treatment, respectively.

At days 28, 42 and 56, all differences were statistically significant compared to the control for both test rates (Student t-test, α = 0.05), except the low test rate at day 56.

Table CA 8.5/03-1 Nitrogen transformation test: effects of the test item on ammonium- (mean values)

	Control		1.4 mg KWG 4168-despropyl/kg soil dw		6.9 mg KWG 4168-despropyl/kg soil dw	
Day	**NH_4-N mg/kg dry weight**	**CV[1] %**	**NH_4-N mg/kg dry weight**	**Dev. %[2]**	**NH_4-N mg/kg dry weight**	**Dev. %[2]**
0	7.520	2.29	7.561	0.55	7.829	-3.07
7	1.914	3.03	1.841	-3.81	1.736	-9.30*
14	1.603	4.12	1.670	4.18	1.627	1.50
28	1.650	0.97	1.347	-18.36*	1.319	-20.06*
42	0.701	0.00	0.701	0.00	0.701	0.00
56	0.701	0.00	0.905	29.10	0.701	0.00

[1]: CV, coefficient of variation;

[2]:Dev., Deviation from control: dw: dry weight;

*: statistically significant (according to Student t-test, two-sided, α=0.05)

Table CA 8.5/03-2 Nitrogen transformation test: effects of the test item on nitrite- (mean values)

	Control		1.4 mg KWG 4168-despropyl/kg soil dw		6.9 mg KWG 4168-despropyl/kg soil dw	
Day	**NH_4-N mg/kg dry weight**	**CV[1] %**	**NH_4-N mg/kg dry weight**	**Dev. %[2]**	**NH_4-N mg/kg dry weight**	**Dev. %[2]**
0	0.431	8.82	0.570	32.25*	0.434	0.70
7	0.399	0.00	0.399	0.00	0.399	0.00
14	0.420	2.14	0.412	-1.90	0.414	-1.43
28	0.407	0.25	0.425	4.42*	0.428	5.16*
42	0.399	0.00	0.399	0.00	0.399	0.00
56	0.399	0.00	0.399	0.00	0.399	0.00

[1]: CV, coefficient of variation;

[2]:Dev., Deviation from control: dw: dry weight;

	Control	1.4 mg KWG 4168-despropyl/kg soil dw	6.9 mg KWG 4168-despropyl/kg soil dw

*: statistically significant (according to Student t-test, two-sided, α=0.05)

Table CA 8.5/03-3 Nitrogen transformation test: effects of the test item on nitrate- (mean values)

	Control		1.4 mg KWG 4168-despropyl/kg soil dw		6.9 mg KWG 4168-despropyl/kg soil dw	
Day	**NH_4-N mg/kg dry weight**	**CV[1] %**	**NH_4-N mg/kg dry weight**	**Dev. %[2]**	**NH_4-N mg/kg dry weight**	**Dev. %[2]**
0	24.860	0.65	25.042	0.73	26.247	5.58*
7	18.280	4.90	12.950	-29.04*	21.980	20.44*
14	25.675	2.73	18.349	-28.53*	28.815	12.23*
28	40.047	2.47	33.466	-16.43*	43.945	9.73*
42	45.265	3.65	40.810	-9.84*	49.142	8.57*
56	52.842	3.29	48.987	-7.30*	58.035	9.83*

[1]: CV, coefficient of variation;

[2]:Dev., Deviation from control: dw: dry weight;

*: statistically significant (according to Student t-test, two-sided, α=0.05)

Table CA 8.5/03-4 Nitrogen transformation test: effects of the test item on N_{min}-content (mean values)

	Control		1.4 mg KWG 4168-despropyl/kg soil dw		6.9 mg KWG 4168-despropyl/kg soil dw	
Day	**NH_4-N mg/kg dry weight**	**CV[1] %**	**NH_4-N mg/kg dry weight**	**Dev. %[2]**	**NH_4-N mg/kg dry weight**	**Dev. %[2]**
0	32.812	0.91	33.174	1.10	33.970	3.53
7	20.563	4.09	15.191	-26.12*	24.115	17.27*
14	27.699	2.60	20.431	-16.24*	30.855	11.39*
28	42.104	2.38	35.238	-16.31*	45.692	8.52*
42	46.365	3.56	41.910	-9.61*	50.242	8.36*
56	53.942	3.23	50.291	-6.77	59.135	9.63*

[1]: CV, coefficient of variation;

[2]:Dev., Deviation from control: dw: dry weight;

*: statistically significant (according to Student t-test, two-sided, α=0.05)

The cumulative soil nitrate formation rates did exceed the trigger range of ± 25% set by OECD guideline 216 at the 0 - 28 day determination for the low test rate. Therefore, the study was prolonged for 14 days. Differences to the control were -44.46% and 16.61% in the 1.4 mg and 6.9 mg test item/kg soil dry weight treatment, respectively, for the 0 – 28 day determination. The differences were statistically significant for the 0 – 28 day determination for both test rates (Student t-test, α = 0.05).

The cumulative soil nitrate formation rates did not exceed the trigger range of ± 25% set by OECD guideline 216 at the 0 - 42 day determination. Differences to the control were -22.84% and 12.14% in the 1.4 mg and 6.9 mg test item/kg soil dry weight treatment, respectively, for the 0 - 42 day determination. The difference was statistically significant for the 0 – 42 day determination for the low test rate (Student ttest, α = 0.05).

The cumulative soil nitrate formation rates did not exceed the trigger range of ± 25% set by OECD guideline 216 at the 0 - 56 day determination. Differences to the control were -14.40% and 13.60% in the 1.4 mg and 6.9 mg test item/kg soil dry weight treatment, respectively, for the 0 – 56 day determination. The differences were not statistically significant for the 0 – 56 day determination for both test rates (Student t-test, α = 0.05).

The incremental soil nitrate formation rates did not exceed the trigger range of ± 25% set by OECD guideline 216 at the 14 - 28 day determination. Differences to the control were 5.16% and 5.26% in the 1.4 mg and 6.9 mg test item/kg soil dry weight treatment, respectively, for the 14 – 28 day determination. The differences were not statistically significant for the 14 – 28 day determination for both test rates (Student t-test, α = 0.05).

The incremental soil nitrate formation rates did exceed the trigger range of ± 25% set by OECD guideline 216 at the 28 - 42 day determination for the low test rate. Therefore, the study was prolonged for 14 days. Differences to the control were 40.48% and -0.54% in the 1.4 mg and 6.9 mg test item/kg soil dry weight treatment, respectively, for the 28 – 42 day determination. The difference was statistically significant for the 28 – 42 day determination for the low test rate (Student t-test, α = 0.05).

The incremental soil nitrate formation rates did not exceed the trigger range of ± 25% set by OECD guideline 216 at the 42 - 56 day determination. Differences to the control were 7.95% and 17.38% in the 1.4 mg and 6.9 mg test item/kg soil dry weight treatment, respectively, for the 42 – 56 day determination. The differences were not statistically significant for the 42 – 56 day determination for both test rates (Student t-test, α = 0.05).

Table CA 8.5/03-5 Nitrogen transformation test: effects of the test item on nitrate formation rates (mean values)

	Control		**1.4 mg KWG 4168-despropyl/kg soil dw**		**6.9 mg KWG 4168-despropyl/kg soil dw**	
Day	**Mean mg NO_3-N/kg soil dry weight per day**					
	mg/day	**CV %[1]**	**mg/day**	**Dev. %[2]**	**mg/day**	**Dev. %[2]**
0 - 7	-0.944	-12.08	-1.728	83.05*	-0.610	-35.38*
0 - 14	0.058	79.31	-0.478	-924.14*	0.183	215.52
0 - 28	0.542	6.09	0.301	-44.46*	0.632	16.61*
0 - 42	0.486	7.61	0.375	-22.84*	0.545	12.14
0 - 56	0.500	6.20	0.428	-14.4	0.568	13.60
	Control		**1.4 mg KWG 4168-despropyl/kg soil dw**		**6.9 mg KWG 4168-despropyl/kg soil dw**	
Day	**Mean mg NO_3-N/kg soil dry weight per day[4]**					
	mg/day	**CV %[1]**	**mg/day**	**Dev. %[2]**	**mg/day**	**Dev. %[2]**
0 - 7	-0.944	-12.08	-1.728	83.05*	-0.610	-35.38*
7 - 14	1.061	3.77	0.771	-27.33*	0.977	-7.92
14 - 28	1.027	2.24	1.080	5.16	1.081	5.26
28 - 42	0.373	12.87	0.524	40.48*	0.371	-0.54
42 - 56	0.541	9.06	0.584	7.95	0.635	17.38

[1]: CV, coefficient of variation;
[2]:Dev., Deviation from control: dw: dry weight;
[3]:Calculated from the mean values of NO_3-N content between the sampling date and day 0;
[4]:Calculated from the mean values of NO_3-N content between each sampling date;
*: statistically significant (according to Student t-test, two-sided, α=0.05)

III. Conclusion

After 56 days, the test item had no impact on nitrogen transformation (nitrate content, mineral nitrogen content and nitrate formation rates) of soil microorganisms when applied at 1.4 mg and 6.9 mg test item/kg soil dry weight.

Assessment and conclusion by applicant:

This is a new study that has not been previously evaluated.

Validity criteria according to the current OECD 216 guideline (2000) were met:

- Variation between replicate control samples to be less than ±15% (actual: max. 4.90%)

The study is therefore considered acceptable.

There were <25 % effects after 56 days at rates up to 6.9 mg/kg soil dry weight.

KWG 4168-carboxylic acid (M06)

Data Point:	KCA 8.5/04
Report Author:	[redacted]
Report Year:	2020
Report Title:	KWG 4168-carboxylic acid: Effects on the activity of the soil microflora in the laboratory (nitrogen transformation)
Report No:	152521080
Document No:	M-688317-01-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 (2009) OECD-Guideline for the Testing of Chemicals, Soil Microorganisms: Nitrogen Transformation Test, Guideline 216, January 21, 2000
Deviations from current test guideline:	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 purpose of this study was to assess the effects of the test item on the activity (nitrogen transformation) of the soil microflora in the laboratory.

KWG 4168-carboxylic acid was tested at concentrations of 0.5 and 5.0 mg test item/kg soil dry weight.

After 28 days, the test item KWG 4168-carboxylic acid had no long-term impact on nitrogen transformation (nitrate content, mineral nitrogen content and nitrate formation rates) of soil microorganisms when applied at 0.5 mg and 5 mg test item/kg soil dry weight treatment.

I. Materials and Methods

A. Materials

Test Material KWG 4168-carboxycylic acid

Lot/Batch #: AE 1344313-01-03

Content: 2-(2-{[ethyl(propyl)amino]methyl}-1,4-dioxaspiro[4.5]dec-8-yl)-2-methylpropionic acid; 90.6% w/w

Description: Turbid brown liquid

Stability of test compound: Sufficient based on expiration date

Reanalysis/Expiry date: 13 march 2021

Density: Not reported

Treatments

Test rates: 0.5 and 5.0 mg test item/kg soil dry weight

Solvent/vehicle: Acetone

Analysis of test concentrations:	No
Test design	
Test vessel:	Disposable plastic boxes, approximately 0.5 L in volume
Test substrate:	According to the guideline, taken from fallow grassland (location: "In der Speyerer Hohl ", No. 977). No pesticides or organic or mineral fertiliser had been used on the soil for at least four years prior to test initiation
Replication:	Three per treatment group and control
Duration of test:	28 days
Environmental test conditions	
Temperature:	20 ± 2°C
pH:	7.3 – 7.4
Photoperiod:	Darkness
Water content:	49 to 50% WHC

B. Study Design

The purpose of this study was to assess the effects of the test item on the activity (nitrogen transformation) of the soil microflora in the laboratory.

Test rates were 0.5 and 5.0 mg test item/kg soil dry weight. There were three replicates per treatment and control group.

Test units were disposable plastic boxes with 300 g soil (dry weight), the box volume was approximately 0.5 L and the dimensions were 0.10 x 0.10 x 0.065 m.

Incubation was at 18 to 22°C in the dark. Test conditions (temperature) were recorded continuously using suitable instruments, documented in the raw data and reported in the final report. Short-term deviations (<2 hours) from the recommended temperature range do normally not result in major disturbances of the test performance and were not reported.

KWG 4168-carboxylic acid was soluble in acetone; therefore, a stock solution in acetone was prepared by dissolving 50.0 mg KWG 4168-carboxylic acid in 20 mL acetone and appropriate amounts were applied onto quartz sand. After evaporation of the acetone overnight, the quartz sand and additionally 0.5% lucerne meal (based on soil dry weight) was mixed into the soil by means of a laboratory mixer.

Throughout the application the soil was ventilated and the soil water content was adjusted to 49% of WHC.

To the control, acetone treated quartz sand (evaporated) and additionally 0.5% lucerne meal (based on soil dry weight) was mixed into the soil.

The soil water content was adjusted to 49% of WHC. The soil water content was determined in one replicate of each treatment group at each sampling.

For the determination of nitrogen content, soil samples were taken within 6 hours after application and afterwards on each sampling date (7, 14 and 28 days). The nitrogen content was determined in each sample of treated and control soils.

II. Results and Discussion

Validity criteria according to the OECD 216 guideline were met:

- Variation between replicate control samples to be less than ±15% (actual: max. 1.90%)

The study is therefore considered acceptable.

No adverse effects of the test item on nitrate content in soil were observed at day 28. At day 28, differences to the control were 0.75% and -4.12% in the 0.5 mg and 5 mg test item/kg soil dry weight treatment, respectively.

At sampling point day 28, the difference was statistically significantly different compared to the control for the high test rate (Student t-test, α = 0.05).

Very low nitrite and ammonium contents below 0.6 mg/kg dry weight were measured at day 28 in the control and the test item treatments.

The mineral nitrogen contents in soil were within the trigger range of ± 25% set by EPPO and SETAC guidelines at day 28. At day 28, differences to the control were 0.73% and - 4.03% in the 0.5 mg and 5 mg test item/kg soil dry weight treatment, respectively.

At sampling point day 28, the difference was statistically significantly different compared to the control for the high test rate (Student t-test, α = 0.05).

The variation between replicate control samples was below the validity criterion of 15% of the OECD test guideline 216.

Table CA 8.5/04-1 Nitrogen transformation test: effects of the test item on ammonium- (mean values)

	Control		0.5 mg KWG 4168-carboxycylic acid/kg soil dw		5.0 mg KWG 4168-carboxycylic acid/kg soil dw	
Day	**NH_4-N mg/kg dry weight**	**CV[1] %**	**NH_4-N mg/kg dry weight**	**Dev. %[2]**	**NH_4-N mg/kg dry weight**	**Dev. %[2]**
0	5.532	4.95	5.281	-4.54	5.723	3.45
7	0.548	0.00	0.548	0.00	0.548	0.00
14	0.548	0.00	0.548	0.00	0.548	0.00
28	0.548	0.00	0.548	0.00	0.548	0.00

[1]: CV, coefficient of variation;
[2]:Dev., Deviation from control; dw: dry weight;
*: statistically significant (according to Student t-test, two-sided, α=0.05)

Table CA 8.5/04-2 Nitrogen transformation test: effects of the test item on nitrite- (mean values)

	Control		0.5 mg KWG 4168-carboxycylic acid/kg soil dw		5.0 mg KWG 4168-carboxycylic acid/kg soil dw	
Day	**NO_2-N mg/kg dry weight**	**CV[1] %**	**NO_2-N mg/kg dry weight**	**Dev. %[2]**	**NO_2-N mg/kg dry weight**	**Dev. %[2]**
0	0.404	12.13	0.485	20.05*	0.476	17.82*
7	0.347	0.00	0.347	0.00	0.347	0.00
14	0.347	0.00	0.347	0.00	0.347	0.00
28	0.347	0.00	0.347	0.00	0.347	0.00

[1]: CV, coefficient of variation;
[2]:Dev., Deviation from control; dw: dry weight;
*: statistically significant (according to Student t-test, two-sided, α=0.05)

Table CA 8.5/04-3 Nitrogen transformation test: effects of the test item on nitrate- (mean values)

	Control		0.5 mg KWG 4168-carboxycylic acid/kg soil dw		5.0 mg KWG 4168-carboxycylic acid/kg soil dw	
Day	**NO_3-N mg/kg dry weight**	**CV[1] %**	**NO_3-N mg/kg dry weight**	**Dev. %[2]**	**NO_3-N mg/kg dry weight**	**Dev. %[2]**
0	18.065	0.19	17.909	-0.86	17.960	-0.58
7	18.626	0.86	19.432	4.33	18.697	0.38
14	27.227	1.77	28.580	4.97*	27.647	1.54
28	40.779	1.90	41.084	0.75	39.098	-4.12*

	Control	0.5 mg KWG 4168-carboxycylic acid/kg soil dw	5.0 mg KWG 4168-carboxycylic acid/kg soil dw

[1]: CV, coefficient of variation;
[2]:Dev., Deviation from control: dw: dry weight;
*: statistically significant (according to Student t-test, two-sided, α=0.05)

Table CA 8.5/04-4 Nitrogen transformation test: effects of the test item on N_{min}-content (mean values)

	Control		0.5 mg KWG 4168-carboxycylic acid/kg soil dw		5.0 mg KWG 4168-carboxycylic acid/kg soil dw	
Day	N_{min}-N mg/kg dry weight	CV[1] %	Nmin-N mg/kg dry weight	Dev. %[2]	Nmin-N mg/kg dry weight	Dev. %[2]
0	24.000	1.01	23.674	-1.36	24.159	0.66
7	19.521	0.82	20.327	4.13	19.592	0.36
14	28.122	1.71	29.475	4.81	28.542	1.49
28	41.674	1.86	41.979	0.73	39.993\	-4.03*

[1]: CV, coefficient of variation;
[2]:Dev., Deviation from control: dw: dry weight;
*: statistically significant (according to Student t-test, two-sided, α=0.05)

The cumulative soil nitrate formation rates did not exceed the trigger range of ± 25% set by OECD guideline 216 at the 0 - 28 day determination for both test rates. Differences to the control were 2.10% and -6.91% in the 0.5 mg and 5 mg test item/kg soil dry weight treatment, respectively.

The difference was statistically significantly different compared to the control for the high test rate (Student t-test, α = 0.05) for the cumulative nitrate rate at the 0 - 28 day determination.

The incremental soil nitrate formation rates did not exceed the trigger range of ± 25% set by OECD guideline 216 at the 14 - 28 day determination. Differences to the control were -7.75% and -15.50% in the 0.5 mg and 5 mg test item/kg soil dry weight treatment, respectively.

The difference was statistically significantly different compared to the control for the high test rate (Student t-test, α = 0.05) for the incremental nitrate rate at the 14 - 28 day determination.

Table CA 8.5/04-5 Nitrogen transformation test: effects of the test item on nitrate formation rates (mean values)

	Control		0.5 mg KWG 4168-carboxycylic acid/kg soil dw		5.0 mg KWG 4168-carboxycylic acid/kg soil dw	
Day	Mean mg NO_3-N/kg soil dry weight per day[3]					
	mg/day	CV %[1]	mg/day	Dev. %[2]	mg/day	Dev. %[2]
0 - 7	0.080	32.50	0.218	172.50*	0.105	31.25
0 - 14	0.654	5.35	0.762	16.51*	0.692	5.81
0 - 28	0.811	3.58	0.828	2.10	0.755	-6.91*
	Control		0.5 mg KWG 4168-carboxycylic acid/kg soil dw		5.0 mg KWG 4168-carboxycylic acid/kg soil dw	
Day	Mean mg NO_3-N/kg soil dry weight per day[4]					
	mg/day	CV %[1]	mg/day	Dev. %[2]	mg/day	Dev. %[2]
0 - 7	0.080	32.50	0.218	172.50*	0.105	31.25
7 - 14	1.229	3.91	1.307	6.35	1.279	4.07
14 - 28	0.968	7.95	0.893	-7.75	0.818	-15.50*

[1]: CV, coefficient of variation;
[2]:Dev., Deviation from control: dw: dry weight;
[3]:Calculated from the mean values of NO3-N content between the sampling date and day 0;
[4]:Calculated from the mean values of NO3-N content between each sampling date;
*: statistically significant (according to Student t-test, two-sided, α=0.05)

III. Conclusion

After 28 days, the test item KWG 4168-carboxylic acid had no long-term impact on nitrogen transformation (nitrate content, mineral nitrogen content and nitrate formation rates) of soil microorganisms when applied at 0.5 mg and 5 mg test item/kg soil dry weight.

Assessment and conclusion by applicant:

This is a new study that has not been previously evaluated.

Validity criteria according to the current OECD 216 guideline (2000) were met:

- Variation between replicate control samples to be less than ±15% (actual: max. 1.90%)

The study is therefore considered acceptable.

There were <25 % effects after 28 days at rates up to 5.0 mg/kg soil dry weight.

Relevant literature on soil micro-organisms

No relevant scientifically peer-reviewed open literature could be found on spiroxamine or its major metabolites, from an ecotoxicological perspective, on soil micro-organisms. Details of the literature search undertaken can be found in the M-CA Section 9 of the current submission.

CA 8.6 Effects on terrestrial non-target higher plants

CA 8.6.1 Summary of screening data

Screening data are not available but GLP seedling emergence (OECD 208) and vegetative vigour (OECD 227) data are available using the representative formulations. Please refer to Document-M-CP Section 10 for the representative formulations.

CA 8.6.2 Testing on non-target plants

There are no data available with spiroxamine technical but seedling emergence (OECD 208) and vegetative vigour (OECD 227) data are available using the representative formulations which have been presented in Document M-CP Section 10.

Relevant literature on non-target terrestrial plants

No relevant scientifically peer-reviewed open literature could be found on spiroxamine or its major metabolites, from an ecotoxicological perspective, on non-target terrestrial plants. Details of the literature search undertaken can be found in the M-CA Section 9 of the current submission.

CA 8.7 Effects on other terrestrial organisms (flora and fauna)

No data are available on the effects of spiroxamine on other terrestrial organisms. Additional data are not considered to be necessary.

CA 8.8 Effects on biological methods for sewage treatment

An activated sludge respiration inhibition test (ASRIT) study has been conducted using spiroxamine technical. The endpoint is summarised in the table below and full details of the study are provided in the summary.

Table CA 8.8-1 Summary of studies on biological methods for sewage treatment with spiroxamine

Test item	Test type	EU endpoint		Reference
Spiroxamine	Activated sludge, respiration inhibition test	EC_{50} 191.1 mg a.s./L	EU	M-298672-01-1

EU: previously evaluated as part of the original EU review and listed in EFSA conclusion and DAR

Data Point:	KCA 8.8/01
Report Author:	[redacted]
Report Year:	2008
Report Title:	Activated sludge, respiration inhibition test with Spiroxamine, tech. substance
Report No:	2008/0013/01
Document No:	M-298672-01-1
Guideline(s) followed in study:	Council Directive 67/548/EEC; Annex V, Method C.11 Activated sludge, respiration inhibition (1988). This test method is equal to OECD Guideline 209 (1984).
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2010), RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Activated sludge was exposed to spiroxamine technical at different concentrations in a 3-hour test in order to assess effects on respiration rates and respiration inhibition.

Exposure to spiroxamine showed 90.32% respiration inhibition of activated sludge at a test item concentration of 1000 mg/L.

The EC_{50} and EC_{10} were determined to be 191.1 and 53.9 mg a.s./L, respectively.

I. Materials and Methods

A. Materials

Test Material Spiroxamine technical

Lot/Batch #: ED7H004650

Purity: 97.0%

Description: Light brown oil

Stability of test compound: Not reported

Reanalysis/Expiry date: 2 August 2009

Density: Not reported

Treatments

Test rates: 100, 180, 320, 560 and 1000 mg a.s./L

Analysis of test concentrations: None

Test design

Test vessel: 300 mL glass Erlenmeyer flasks

Test medium: Mixed population of aquatic microorganisms (activated sludge) from aeration tank of a domestic sewage treatment plant (Municipal STP Cologne-Stammheim)

Replication:	None
Duration of test:	3 hours
Environmental test conditions	
Temperature:	19.1 – 19.5°C
pH	7.6 – 7.8

B. Study Design

This study was conducted in order to assess the effects on respiration of spiroxamine technical on activated sludge in a 3-hour test. Test concentrations were selected based on the results of a preliminary range-finding test.

To measure the oxygen consumption, 250 mL of sludge with test item (or control or reference compound) was incubated for 3 hours in 300 mL closed Erlenmeyer flasks (with air inlet and outlet) and aerated through a glass tube at 50 to 100 L/h with clean oil-free air.

The test item was applied at concentrations of 100, 180, 320, 560 and 1000 mg a.s./L. The reference compound used was 3.5-Dichlorophenol and was applied at concentrations of 2.5, 5, 10, 20 and 40 mg/L. The test item concentration in physico-chemical oxygen consumption control was 1000 mg a.s./L. Two controls without test item were used, one at the start and the other at the end of the test series.

A synthetic wastewater feed was made by dissolving the following amounts of substance in 1 litre of water: 16.0 g peptone, 11.0 g meat extract, 3.0 g urea, 0.7 g NaCl, 0.4 g $CaCl_2$ x $2H_2O$, 0.2 g $MgSO_4$ x $7H_2O$, and 2.8 g K_2HPO_4.

For measurement, the content of the Erlenmeyer flasks was completely transferred to 250 mL BOD bottles and O_2-content was measured with an O_2-meter (redox electrode). Temperature and pH were measured before the use of the activated sludge as well as at the end of the test.

II. Results and Discussion

Validity criteria according to the study report were met:

- Respiratory rates of the two controls differ less than 15% (actual: 2.5%)
- EC_{50} 3.5-DCP should be between 5 mg/L to 40 mg/L (actual: 5 to 30 mg/L)

Exposure to spiroxamine technical showed 90.32% respiration inhibition of activated sludge at the highest concentration of 1000 mg a.s./L.

Respiration inhibition was observed at all concentrations of test item when compared to the control.

The respiratory rates of the two controls differ less than 15%.

Table CA 8.8/01-1 Respiratory rate and Inhibition on activated sludge exposed to spiroxamine tech. substance

Nominal concentration (mg a.s./L)	Respiratory rate test item (mg/L/h)	Inhibition (%)
Control (mean)	38.8	-
100	30.0	22.58
180	19.7	49.12
320	10.3	73.46
560	6.0	84.52
1000	3.8	90.32

III. Conclusion

Activated sludge was exposed to spiroxamine technical at different concentrations in a 3-hour test in order to assess effects on respiration rates and respiration inhibition.

The EC_{50} was determined to be 191.1 mg a.s./L of spiroxamine technical, with 95% confidence limits between 151.5 and 233.4 mg a.s./L.

The EC_{10} was determined to be 53.9 mg/L of spiroxamine technical, with 95% confidence limits between 25.5 and 79.8 mg a.s./L.

Assessment and conclusion by applicant:

This study was previously evaluated and accepted in the RAR (2010), RAR (2017).

Validity criteria according to the current OECD 209 guideline (2010) were considered to be met but one of the criteria could not be verified from the data available in the report.

- Oxygen uptake rate in the blank control ≥20 mg oxygen/g activated sludge in an hour (actual: cannot verify from data in study report)
- Coefficient of variation of oxygen uptake rate in control replicates ≤30% at the end of the test (actual: 4.6%)
- EC_{50} 3.5-DCP should be between 5 mg/L to 40 mg/L (actual: 5 to 30 mg/L)

The validity criteria used in the study report were achieved. Although it is not possible to verify the first criterion above, taken from the current OECD 209 test guideline, it is believed that the study is still valid as sufficient sensitivity was seen in the reference treatment and the control variation was within acceptable limits.

The study is therefore considered acceptable.

The EC_{50} was determined to be 191.1 mg a.s./L.

Data Point:	KCA 8.8/02
Report Author:	[redacted]
Report Year:	1989
Report Title:	Oxygen consumption test with activated sludge KWG 4168
Report No:	BA-898270
Document No:	M-009745-01-2
Guideline(s) followed in study:	ISO 8192-1986 IB (ETAD 103/OECD 209)
Deviations from current test guideline:	Yes Detailed methods unavailable to assess deviation.
Previous evaluation:	yes, evaluated and classified DAR (1997), RAR (2010), RAR (2017) As the study was not performed under GLP conditions, a new study was performed.
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

Activated sludge was treated with KWG 4168 in a 3-hour test to assess effects on oxygen inhibition and respiratory rate.

A control, a reference item and five concentrations of the test item were used in this study.

At the highest test concentration, 1000 mg/L, oxygen consumption was inhibited by 87% when compared to the control.

The EC_{50} was determined to be 159 mg a.s./L with confidence intervals between 90 and 222 mg/L.

I. Materials and Methods

A. Materials

Test Material	KWG 4168
Lot/Batch #:	17001/89
Purity:	94.2%
Description:	Not reported
Stability of test compound:	Not reported
Reanalysis/Expiry date:	Not reported
Density:	Not reported
Treatments	
Test rates:	100, 180, 320, 560 and 1000 mg/L
Analysis of test concentrations:	No
Test design	
Test vessel:	Not reported
Duration of test:	3 hours
Environmental test conditions	
Temperature:	19.6 – 20.2°C

B. Study Design

Activated sludge (6.0 g total solids/L) was treated with KWG 4168 in a 3-hour study to assess the effects on respiratory rate and oxygen consumption.

The test item was added to 8.0 mL of nutrient medium, 12.5 mL of activated sludge and 229.5 mL of water.

The test substance was applied in concentrations of 100, 180, 320, 560 and 1000 mg/L. A control was used and a the reference substance was 3,5-dichlorophenol applied at 1 mg/L.

No replication was documented in the study report.

Initial oxygen values were recorded, in mg, and after 3 hours were recorded again.

II. Results and Discussion

Validity criteria according to the OECD 209 guideline (2010) could not be assessed.

The table shows respiration rates decreasing from 20 mg/L h in the lowest test item sludge to 4 mg/L h at the highest concentration of the test item.

At the highest test concentration, 1000 mg a.s./L, oxygen consumption was inhibited by 87% when compared to the control.

Table CA 8.8/02-1 Oxygen inhibition and respiration rate in activated sludge treated with KWG 4168

Nominal concentration of test substance (mg/L)	Respiratory Rate (mg/L h)	Inhibition (%)
Control	27	-
100	20	24
180	10	63
320	6	78
560	5	82
1000	4	87
1 (Reference)	26	4
20 (Reference)	6	78

III. Conclusion

At the lowest test concentration, 100 mg a.s./L, oxygen consumption was inhibited by 24% when compared to the control. This rose to 87% at the highest test concentration, 1000 mg a.s./L.

The EC_{50} was determined to be 159 mg a.s./L with confidence intervals between 90 and 222 mg a.s./L.

Assessment and conclusion by applicant:

The study was not performed to GLP or to a recognised test guideline, although the test methodology used would appear to be consistent with current methods. The validity criteria according to the OECD 209 Guideline (2010) could not be assessed due to insufficient details in the study report.

The study is therefore considered as supporting information only but is considered to be consistent with the other available GLP data.

The EC_{50} was determined to be 159 mg a.s./L.

Relevant literature on biological methods for sewage treatment

No relevant scientifically peer-reviewed open literature could be found on spiroxamine or its major metabolites, from an ecotoxicological perspective, on biological methods for sewage treatment. Details of the literature search undertaken can be found in the M-CA Section 9 of the current submission.

CA 8.9 Monitoring data

No monitoring data on the effects of spiroxamine in the EU are available or are considered to be required.