Document Title

Tier 2 Summary of the Ecotoxicological Studies on the Active Substance for BYI 02960 (Flupyradifurone)

Data Requirements

Regulation (EC) No 1107/2009

Regulatory Directive 2003-01/Canada/PMRA
OPPTS guidelines/US/EPA

Annex IIA
Section 6, Point 8
Document M

According to OECD format guidance for industry data submissions on plant protection products and their active substances

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OWNERSHIP STATEMENT

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IIA 8 Ecotoxicological Studies on the Active Substance

IIA 8.1 Avian toxicity

Avian toxicity testing was performed following the requirements in various regulatory regions and countries.

The lowest acute oral LD_{50} of 232 mg a.i. /kg b.w. was observed in the standard testing species Northern Bobwhite quail (*Colinus virginianus*). Additional acute oral toxicity testing in the Canary (*Serinus canaria*) and the hen (*Gallus gallus domesticus*) resulted in higher LD_{50} values.

Short term dietary toxicity studies were conducted with chicks of the Northern Bobwhite quail and of the Mallard duck (*Anas platyrhynchos*). In these studies, no mortality occurred up to the nominal concentration of 5000 ppm, corresponding to an achieved daily dietary doses of 825 mg a.i./kg b.w./d in the ducklings and 470 mg a.i. /kg b.w./d in the quail chicks.

Based on a significant reduction of the 8-d bodyweight at 2075 ppm (262 mg a.i./kg b.w./d), the Bobwhite quail was considered the more sensitive species also in short term dietary testing. It is worth noting, that the toxicity of BYI 02960 from repeated dietary exposure of that most sensitive species was found to be much less pronounced than toxicity from single acute oral dosing.

Reproduction toxicity testing was conducted in the most sensitive species (Northern Bobwhite quail) and in the Mallard duck.

In the Mallard duck, the NOAEL for both parental and reproductive toxicity endpoints was found at the top test level of 845 ppm (achieved daily dietary dose 81 mg a.i./kg b.w./d).

Exposure of Northern Bobwhite Quail to BYI 02960 prior to and during their reproduction resulted in clear effects on parental survival, body weight and health at the top dose level of 1000 ppm. The number of eggs laid by the birds at this top dose level was significantly reduced, resulting in a reduced number of hatchlings and 14-d survivors. No treatment related, statistically and biologically relevant effects were observed at the lower treatment levels (111 and 333 ppm).

Therefore, the NOAEL in the most sensitive species for both parental and reproductive toxicity endpoints was established at the test level of 333 ppm, corresponding to an achieved daily dietary dose of 40 mg a.i. /kg b.w./d.

IIA 8.1.1 Acute oral toxicity to quail species, mallard duck or other bird

Report:	**KIIA 8.1.1/01; [redacted], [redacted] & [redacted], T.L. (2010)**
Title:	Toxicity of BYI 2960 Technical During an Acute Oral LD_{50} with the Northern Bobwhite Quail (*Colinus virginianus*)
Report No:	EBRVP022
Document No:	M-386036-01-1
Guidelines:	**OPPTS 850.2100** **OECD Guideline 223**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** Some data (screening of feed and corn-oil for contaminants) were not performed according to GLP, as described in the study report

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2% w/w) to Northern Bobwhite Quail (*Colinus virginianus*).

Nineteen-week old adults were orally dosed via gelatine capsules at 0, 25, 50, 100, 200 and 400 mg a.i./kg body weight, respectively, and subsequently monitored for a period of 14 days. Mortality, signs of intoxication, food consumption, body weight and gross necropsy results were evaluated to determine the endpoints.

There were no mortalities in the control, 25, 50 and 100 mg a.i./kg b.w. treatment groups. Mortality of 40 and 90% was observed at 200 and 400 mg a.i./kg b.w., respectively.

No clinical signs of toxicity were observed in the control, 25 or 50 mg a.i./kg b.w. treatment groups. Hypo-reactivity to stimuli was observed in the 100, 200 and 400 mg a.i./kg b.w. treatment groups. Post-mortem examinations were generally unremarkable, with the exception that several birds were found with fluid in their gastrointestinal tracts.

Body weight on DAT 7 was significantly reduced at the 200 mg a.i./kg b.w. treatment level compared to the control birds. The NOAEL was considered to be 100 mg a.i./kg b.w.

The acute oral LD_{50} for BYI 02960 technical in northern bobwhite quail was 232 mg a.i./kg body weight (95% CL = 173 to 313 mg a.i./kg body weight).

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Stability of test compound:	Expiry date: 16.01.2011, when stored at +25 ± 5°C

2. Test organisms

Species:	*Colinus virginianus*
Common name:	Northern Bobwhite Quail
Source:	[redacted]
Age at study initiation:	19 weeks old (adult birds)
Maintenance prior to test:	
Period of acclimation to test conditions:	14 days

B. Study design and methods

1. In life dates: August 18 to September 01, 2009

2. Experimental treatments

Colinus virginianus (19 weeks old) were administered orally with BYI 02960; (purity 96.2% w/w). After the birds had received 25, 50, 100, 200 and 400 mg a.i./kg body weight via gelatine capsule the birds were observed over a period of 14 days. Each cage served as one treatment level containing 5 (males and females separately) birds.

3. Observation and measurements

Mortality and signs of intoxications were assessed. Body weight measurements were conducted at days -1, 7 and 14. The food consumption was calculated from weighing the residual food at 0 - 7, 7 - 14 and 0 - 14 days after treatment. Gross necropsies were conducted after test termination.

4. Statistical analysis

Mortality data were analyzed with a multi-method program (CT-Tox) that can determine the LC_{50} and 95% confidence interval using non-linear interpolation, Binomial, Moving Average, Probit, and Spearman-Karber methods.

For body weight and growth, normality and homogeneity of variance of the data were tested using the Shapiro-Wilk's test ($\alpha = 0.01$) and the Levene's test ($\alpha = 0.05$), respectively. Normally distributed data were subjected to standard one-way ANOVA followed by Dunnett's test or Bonferroni t-test.

RESULTS AND DISCUSSION

A. Environmental Conditions

Birds were kept under conditions which are summarized as follows:

Test temperature:	Mean 21°C
Relative humidity:	Mean 54%
Photoperiod:	10 hours light / 14 hours dark
Light source	Natural daylight
Air change:	15 changes per hour (average)

B. Biological Findings

Bird mortality: No mortalities occurred during the study in the control and at the dose levels of 25, 50 and 100 mg a.i./kg b.w., respectively. At 200 and 400 mg a.i./kg b.w., respectively, 40 and 90% mortality occurred. The LD_{50} of 232 mg a.i./kg b.w. (95% CL: 173 to 313 mg a.i./kg bw), slope 5.9 (95% CL 2.5-9.3), was estimated by probit analysis.

Body weight and food consumption: Body weight data were normally distributed and variances were homogeneous; therefore, parametric statistical procedures were conducted with a Bonferroni t-test ($\alpha = 0.05$). Male and female body weights in the 200 mg a.i./kg b.w. level were significantly lower as compared to the control for Day 7.

Based on empirical analyses, there was a reduction in female feed consumption among treatment groups compared to the control group. However the male feed consumption among the treatment groups was greater than the control group throughout the study period.

Clinical observations: No symptoms of toxicity were observed within the control, 25 or 50 mg a.i./kg body weight treatment groups, respectively. All birds appeared normal following dosing with no effects of regurgitation observed. Hypo-reactivity to stimuli was observed in the 100, 200 and 400 mg a.i./kg body weight treatment groups.

Post-Mortem examinations: Necropsy revealed no pathomorphological changes with the exception that several birds at 400 mg a.i./kg b.w. were found with fluid in their gastrointestinal tracts.

Table: Effect of BYI 02960 on mortality, body weight and food consumption of *Colinus virginianus*

Treatment level [mg a.i./kg b.w.]	Sex	% mortality Day 14	Mean body weight [g]			Mean daily feed consumption [g/bird/day]
			Day 0	Day 7	Day 14	
untreated control	male	0	266.6 ± 7.5	224.6 ± 8.6	228.2 ± 9.7	20.6 ± 4.6
	female	0	225.2 ± 5.4	222.6 ± 5.3	225.2 ± 5.5	27.8 ± 10.2
25	male	0	225.8 ± 7.9	223.2 ± 6.3	229.8 ± 7.6	25.3 ± 8.3
	female	0	225.6 ± 5.3	220.6 ± 8.7	225.8 ± 10.9	22.5 ± 7.4
50	male	0	226.0 ± 5.8	222.8 ± 7.6	228.0 ± 8.0	23.0 ± 5.9
	female	0	225.2 ± 6.1	215.8 ± 4.2	223.6 ± 8.4	19.9 ± 5.6
100	male	0	226.8 ± 6.1	223.8 ± 9.6	228.8 ± 9.9	26.5 ± 13.8
	female	0	225.8 ± 5.3	216.8 ± 4.2	220.8 ± 5.5	18.8 ± 7.1
200	male	40	226.6 ± 6.4	209.0 ± 4.6 [a]	219.7 ± 1.5	29.9 ± 17.5
	female	40	224.4 ± 5.9	206.7 ± 4.0 [a]	216.0 ± 1.7	19.2 ± 11.7
400	male	100	227.2 ± 6.0	-	-	n.d.
	female	80	224.6 ± 6.0	184 [b]	197 [b]	16.6 ± 9.7

[a] Statistically different from the control group
[b] excluded from statistical comparisons due to sample size (n=1)
n.d. not determined

C. Validity Criteria

The validity criterion of control mortality less than 10% was fulfilled.

D. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

LD_{50}:	232 mg a.i./kg b.w. (95% confidence limits: 173 to 313 mg a.i./kg bw)
Lowest lethal dose (LLD)	200 mg a.i./kg b.w.

CONCLUSION

The acute oral LD_{50} for BYI 02960 technical in Northern Bobwhite quail (*Colinus virginianus*) was 232 mg a.i./kg b.w. (95%o CL = 173 to 313 mg a.i./kg b.w.).

Report:	**KIIA 8.1.1/02; [redacted], [redacted] and [redacted], [redacted] (2011)**
Title:	Toxicity of BYI 02960 Technical During an Acute Oral LD_{50} with the Canary (*Serinus canaria*)
Report No:	EBRVP036
Document No:	M-408514-01-1
Guidelines:	**OPPTS 850.2100** **OECD Guideline 223**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** Some data (contaminant screening of bird feed, determination of sex of canaries, screening of corn-oil and tap water) was not collected in accordance with GLP, the details are given in the study report

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2% w/w) to Canary (*Serinus canaria*).

Adult *Serinus canaria* were orally dosed via gelatine capsules at 0, 44, 88, 175, 350 and 700 mg a.i./kg body weight, and subsequently monitored for a period of 14 days. Mortality, signs of intoxication, food consumption, body weight and gross necropsy results were evaluated to determine the endpoints.

There were no mortalities in the control, 44 and 88 mg a.i./kg b.w. treatment groups. Mortality in the 175, 350 and 700 mg a.i./kg b.w. treatment groups amounted to 50, 50 and 70%, respectively. Ataxia (loss of muscular coordination), hypo-reactivity to stimuli, and immobility were observed in the 44, 88, 175, 350 and 700 mg a.i./kg b.w. treatment groups. Severity and prevalence of clinical observations were primarily dose dependent, however several birds at each treatment level had minimal to no observed adverse effects. By 36 hours after dosing all birds recovered from observed symptoms or died.

Body weight and food consumption were not significantly different from the control treatment.

The acute oral LD_{50} was determined to be 330 mg a.i./kg b.w. (95% confidence limits: 215 to 625 mg a.i./kg b.w.).

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Storage conditions	Ambient temperature
Application via…:	Gelatine capsule
Negative control:	Deionised water

2. Test organisms

Species:	*Serinus canaria*
Common name:	Canary
Source:	[redacted]
Feeding during test:	ABBA® 1700 Canary Diet and Living World® Premium Canary Food
Body weight at test start:	17.4 to 23.5 g
Maintenance prior to test:	
Food:	ABBA® 1700 Canary Diet and Living World® Premium Canary Food
Drinking water:	Tap water, *ad libitum*
Period of acclimation to test conditions:	14 days
Starvation period prior to test start:	15 hours
Mortality during acclimation period:	One bird died of unknown causes after 2 weeks

B. Study design and methods

1. In life dates August 31 to September 14, 2010

2. Experimental treatments

Serinus canaria were administered orally with BYI 02960; (purity 96.2% w/w). After the birds had received 44, 88, 175, 350 and 700 mg a.i./kg b.w, via gelatine capsule, the birds were observed over a period of 14 days. In addition, deionised water was tested as negative control. Each cage contained 1 bird. The test was conducted with 10 birds (5 males and 5 females) per treatment level.

3. Observation and measurements

Mortality and signs of intoxications were assessed. Body weight measurements were conducted at days -1, 7 and 14. The food consumption was calculated from weighing the residual food at 1 – 7 days and 7 – 14 days after treatment. Gross necropsies were conducted after test termination.

4. Statistical analysis

Mortality data were analyzed with a multi-method program (CT-Tox) that can determine the LC_{50} and 95% confidence interval using non-linear interpolation, Binomial, Moving Average, Probit, and Spearman-Karber methods.

For body weight and growth, normality and homogeneity of variance of the data were tested using the Chi-Square-Test ($\alpha = 0.01$) and the Levene's test ($\alpha = 0.05$), respectively. Normally distributed data were subjected to standard one-way ANOVA followed by Dunnett's test or Bonferroni t-test.

RESULTS AND DISCUSSION

A. Environmental Conditions

Birds were kept under conditions which are summarized as follows:

Test temperature:	24 °C (mean)
Relative humidity:	48% (mean)
Photoperiod:	10 hours light/ 14 hours dark
Light intensity:	267 lux
Air changes:	16 changes per hour (average)

B. Biological Findings

Dose-dependent mortality was observed at 175 mg a.i./kg b.w. and above. The acute oral LD_{50} was 330 mg a.i./kg body weight (95% CL = 215 to 625 mg a.i./kg body weight).The slope of the dose-response curve was 2.3 (95% CL = 1.1 to 3.5).

No statistically significant effects were observed on food consumption and body weight.

Table: Effect of BYI 02960 on mortality, food consumption and body weight of *Serinus canaria*

Treatment level [mg a.i./kg bw]	Sex	Mortality day 14 [%]	Body weight (g) (mean ± SD [a])			Daily feed consumption (mean ± SD) [g/bird/day]
			day 0	day 7	day 14	
untreated control	male	0	21.2 ± 1.3	21.3 ± 1.1	21.4 ± 1.6	3.4 ± 0.4
	female	0	19.9 ± 1.1	20.2 ± 1.3	20.8 ± 2.0	3.8 ± 0.4
44	male	0	20.8 ± 2.0	21.8 ± 1.9	20.7 ± 2.3	3.8 ± 0.7
	female	0	19.5 ± 1.2	19.7± 1.1	19.6 ± 2.5	3.1 ± 0.3
88	male	0	20.7 ± 1.9	20.8 ± 1.6	20.9 ± 2.3	3.2 ± 0.7
	female	0	19.5 ± 1.0	20.7 ± 1.6	20.6 ± 1.1	3.6 ± 1.0
175	male	40	20.8 ± 1.8	20.1 ± 1.9	20.7 ± 1.8	3.5 ± 0.4
	female	60	19.4 ± 1.2	(20.6–21.9) [b]	(20.9–21.9) [b]	(3.0 – 3.7) [b]
350	male	60	21.0 ± 1.7	(20.7–22.0) [b]	(20.7–23.6) [b]	(3.0 – 3.8) [b]
	female	40	19.6 ± 1.2	19.4 ± 0.9	19.1 ± 1.5	3.1 ± 0.1
700	male	80	21.1 ± 1.8	19.2 [b]	19.6 [b]	3.8[a]
	female	60	19.8 ± 1.1	(19.4–20.3) [b]	(19.6–20.5) [b]	(2.6 – 4.1) [b]

[a] SD = standard deviation

[b] Range reported when only 2 surviving birds present. Value reported when only 1 surviving bird present

Clinical observations: Severity of clinical observations was primarily dose dependent, however several birds at each treatment level had minimal to no observed adverse effects. On day 0, at 44 mg a.i./kg bw and 88 mg a.i./kg bw, four birds were observed with a loss of muscular coordination (ataxia - still able to balance on perch), one and two birds, respectively, showed hyporeactivity and one and three birds, respectively, were immobile. All birds recovered on the same day. At 175 mg a.i./kg bw and 350 mg a.i./kg bw, all birds showed symptoms of toxicity, five died and five recovered within < 31 hours after dosing. At 700 mg a.i./kg bw, seven birds died and the remaining three recovered from toxicity symptoms (ataxia, immobility) with 24 hours

C. Validity Criteria

The validity criterion of control mortality less than 10% was fulfilled.

D. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

LD_{50}:	330 mg a.i./kg body weight (95% confidence limits:215 to 625 mg a.i./kg body weight)
Lowest lethal dose (LLD):	175 mg a.i./kg b.w.

CONCLUSION

The acute oral LD_{50} for BYI 02960 technical in Canary (*Serinus canaria*) was 330 mg a.i./kg b.w. (95% CL = 215 to 625 mg a.i./kg b.w.).

Report:	**KIIA 8.1.1/03; Barfknecht, R., Wilkens, S. (2011)**
Title:	Acute oral toxicity of chicken (*Gallus gallus domesticus*) with BYI 2960 (tech.), according to OECD 223 - Limit test -
Report No:	BAR/LD 141
Document No:	M-420519-01-2
Guidelines:	**OECD Guideline 223**
Deviations:	**The observation period was prolonged to be in compliance with some national requirements.**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2% w/w) to hens (*Gallus gallus domesticus*) in a limit test, and was conducted in order to satisfy national data requirements in individual countries.

Five adult hens (treatment group) were orally administered with a single dose of 2000 mg a.i./kg bw via gelatine capsules and subsequently monitored for a period of 28 days. In addition, 10 control birds were kept under the same conditions from the beginning of the test until day 21 of the study (only 5 control birds from day 21 to day 28).

Mortality, signs of intoxication, food consumption, body weight and gross necropsy results were evaluated to determine the endpoints. Body weights were recorded prior to test initiation (day -1), on study day 3, 7, day 14, 21 and at test termination (day 28). Food consumption was measured daily until day 3, then for the periods 3-7 and 7-14, 14-21 and 21-28.

The most noticeable effect after the application was an almost complete reduction of food consumption with all dosed birds. All further observed symptoms were consequences of this starvation: excretion of uric acid, fluffed feather or reduced vigilance. One bird started to feed in the 2nd week after application, another one in the 3rd week after application. Both birds showed soft excrements or diarrhoea as indication of digestion distress. A third bird behaved similarly in the 4th week after application.

Two dosed birds did not resume feeding at all after application of the test substance. They were sacrificed for humane reasons on day 28. Since all other birds were free of symptoms or showed clear indications of recovery by that time point, the test was terminated on day 28. Due to the reduced food

consumption all dosed birds showed severe body weight losses. Two birds started to regain body weight in the last week of the test.

The acute oral LD_{50} was determined to be > 2000 mg a.i./kg body weight..

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Storage conditions	Ambient temperature
Application via…:	Gelatine capsule
Negative control:	Deionised water

2. Test organisms

Species:	*Gallus gallus domesticus*
Common name:	Hen
Age:	18 weeks
Source:	[redacted]
Feeding during test:	Standard rearing diet for quails (ssniff Spezialdiäten GmbH, Ferdinand-Gabriel-Weg 16, D-59494 Soest)
Body weight at test start:	1170 to 1452 g (range of both control and treatment group)
Maintenance prior to test:	
Food:	Standard rearing diet for quails (ssniff Spezialdiäten GmbH, Ferdinand-Gabriel-Weg 16, D-59494 Soest)
Drinking water:	Tap water, *ad libitum*
Period of acclimation to test conditions:	14 days
Starvation period prior to test start:	16 hours
Mortality during acclimation period:	None

B. Study design and methods

1. In life dates September 13 to October 25, 2011

2. Experimental treatments

Five birds (treatment group) were orally administered with gelatine capsules containing 2000 mg a.i./kg b.w. and were observed for a period of 28 days. In addition, 10 control birds were kept under the same circumstances from test start until day 21, where 5 control birds were sacrificed. From day 21 to 28, only 5 birds remained in the control group.

Birds were housed individually, in stainless steel wire racks which were placed indoors.

3. Observation and measurements

Observations on mortality and signs of intoxication were made continuously during the first 2 hours and then approximately hourly on the day of dosing, afterwards at least once work-daily until test termination.

Due to the findings on day 21, the test was prolonged to 28 days.

Body weights were recorded prior to test initiation (day -1), on study day 3, 7, 14, 21 and 28 (test termination).

Food consumption was measured daily until day 3, then for the periods 3-7, 7-14, 14-21 and 21-28.

On study days 1, 2, 3, 7, 14 and 21 all remaining food was replaced by fresh food after cleaning. At the end of the study all surviving birds were sacrificed by CO_2 asphyxiation. Gross necropsies were carried out on all survivors at the end of the study.

4. Statistical analysis

Summarizing of raw data as well as pre-calculations (mean and standard deviation) was performed by using "Excel 2003 for Windows©" of the Microsoft Corporation / USA.

RESULTS AND DISCUSSION

A. Environmental Conditions

Birds were kept under conditions which are summarised as follows:

Test temperature:	21 °C (mean)
Relative humidity:	53.9% (mean)
Photoperiod:	14 hours light / 10 hours dark
Light intensity:	Not stated
Air changes:	Not stated

B. Biological Findings

Mortality and behaviour:

The most noticeable effect after the application was an almost complete reduction of food consumption with all dosed birds. All further observed symptoms were consequences of this starvation, e.g. excretion of uric acid, fluffed feather, or altered behaviour like reduced vigilance.

One bird started to feed in the 2nd week after application, another one in the 3rd week after application. Both birds showed soft excrements or diarrhoea as indication of digestion distress. A third bird behaved similarly in the 4th week after application.

Two birds dosed did not resume feeding at all after application of the test substance. They were sacrificed for human reasons on day 28. These birds may be considered as treatment related mortalities. Since all other birds were free of symptoms or showed clear indications of recovery by that time point, the test was terminated on day 28.

Body weight development:

Due to the reduced food consumption all dosed birds showed severe body weight losses. Two birds started to regain body weight in the last week of the test.

Pathological findings at necropsy:

All birds were emaciated.

C. Validity Criteria

The validity criterion of control mortality less than 10% was fulfilled.

D. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

LD_{50}: > 2000 mg a.i./kg body weight

CONCLUSION

The acute oral LD_{50} for BYI 02960 technical in hens (*Gallus gallus domesticus*) was > 2000 mg a.i./kg body weight.

IIA 8.1.2 Avian dietary toxicity (5-day) test in quail species or mallard duck

Report:	**KIIA 8.1.2/01; [redacted], T.B., [redacted] C.V. & [redacted], T.L. (2010)**
Title:	Toxicity of BYI 02960 Technical During an Acute Dietary LC_{50} with the Mallard Duck (*Anas platyrhynchos*)
Report No:	EBRVP020
Document No:	M-388718-01-1
Guidelines:	**OECD Guideline No. 205** **OPPTS 850.2200**
Deviations:	**None**
GLP:	**yes (certified laboratory)** Some data (screening of diet and water for contaminants) was not performed in accordance with GLP as described in the study report

EXECUTIVE SUMMARY

The aim of the study was to determine the short-term effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2%) to Mallard Duck (*Anas platyrhynchos*).

Anas platyrhynchos (10 days old) were exposed to treated feed during a period of 5 days and observed thereafter for another 3 days while fed with untreated feed. Nominal concentrations in feed were 313, 625, 1250, 2500 and 5000 ppm (respective mean measured concentrations: 294, 581, 1175, 2238 and 4741 ppm) which corresponded to daily uptake doses of 66, 129, 272, 459 and 825 mg a.i./kg body weight/day, respectively. In addition, untreated diet was tested as negative control.

Mortality, signs of intoxication, food consumption, body weight and gross necropsy results were evaluated to determine the endpoints. No clinical signs of toxicity or mortalities were noted at any treatment level. Post-mortem examinations revealed no gross lesions or unusual observations. There was a statistically significant reduction in Day 5 body weight and Day 0 to 5 growth at the 2238 and 4741 ppm levels, respectively, and on Day 8 for body weight at the 4741 ppm level compared to the control group. By empirical comparisons, feed consumption was less than the control group for the

4741 ppm level, and intermediate at the 2238 ppm level. Since the reduction in body weight at 2238 ppm was recovered by Day 8 the NOAEL based on body weight was 2238 ppm and the LOAEL was 4741 ppm.

The LC_{50} was determined to be > 4741 ppm (= mg a.i./kg feed), corresponding to a LDD_{50} > 825 mg a.i./kg body weight/ day.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Storage conditions:	Ambient temperature

2. Test organisms

Species:	*Anas platyrhynchos*
Common name:	Mallard Duck
Source:	Dan and Imagene Nichols, Hartville, Missouri
Age at start of the exposure phase:	10 days
Maintenance prior to test (chick rearing):	
Temperature:	32 to 38°C
Photoperiod:	14 hours light / 10 hours dark
Food:	Teklad Bayer Starter Ration
Drinking water:	Local tap water
Mortality:	Three mortalities in the hatchling population
Remarks:	Only birds that appeared healthy were used in the study

B. Study design and methods

1. In life dates October 22 to 30, 2009

2. Experimental treatments

Following a pre-exposure period of 6 days, *Anas platyrhynchos* (10 days old) were offered feed treated with BYI 02960 (purity 96.2%) for 5 days. In addition, untreated diet as negative control was tested. Thereafter the chicks were fed with untreated feed and observed for another 3 days. Each cage (galvanized steel brooders) served as one treatment level containing ten individually marked chicks.

Per treatment level a total amount of 11 kg feed was prepared. The test substance was rinsed into a beaker with an appropriate amount of solvent and stirred until it dissolved. The dissolved test substance was added to the corn oil and stirred. The untreated feed (i.e. raw feed) was weighed into the mixing bowl and placed on the mixer. The corn oil containing the test substance, as well as the additional

solvent rinse, was applied to the feed using a separatory funnel while the feed was mixed for a total of fifteen minutes.

3. Observation and measurements

Mortality and signs of intoxications were assessed approximately 1, 2 and 4 hours after diet administration on day 0, twice daily during the remainder of the study, once daily on weekends and on day 8 (study termination). Body weight measurements were conducted at day -3, day 0, day 5 and day 8. The feed consumption for each level (control and treatment birds) was recorded daily during the entire period. Gross necropsies were conducted after test termination on all birds at the control and the highest test level, and on 40% of the birds in the remaining treatment levels (randomly selected).

Food was analysed in order to verify the concentrations of the test item. In order to prove the stability of the test item in the brooder and in the freezer, food samples were analysed for the highest and lowest treatment level (313 and 5000 ppm).

4. Statistical analysis

No LC_{50} (median lethal concentration) was calculated as no treatment related mortalities occurred at any treatment level. For body weight and growth, normality and homogeneity of variance of the data were tested using the Chi-Square-Test ($\alpha = 0.01$) and the Levene's test ($\alpha = 0.05$), respectively. Normally distributed data were subjected to standard one-way ANOVA followed by Dunnett's test or Bonferroni t-test.

RESULTS AND DISCUSSION

A. Environmental Conditions

Chicks were kept under conditions which are summarized as follows:

Room temperature during test:	22 °C
Brooder temperature during test:	24 to 37°C
Relative humidity:	57%
Photoperiod:	14 hours light / 10 hours dark
Light intensity:	280 lux
Ventilation of test facility:	16 changes per hour

B. Biological Findings

No mortality was observed during the test.

No sublethal signs of intoxications were observed.

Post-mortem examinations revealed no treatment related gross lesions or unusual observations.

Body weight at 2238 and 4741 ppm was statistically significantly different (lower) than the control on day 5. By day 8, full recovery of the bodyweight reduction was observed at 2238 ppm and partial recovery was observed at 4761 ppm.

Table: Effect of BYI 02960 on mortality, intoxication symptoms and necropsy findings of *Anas platyrhynchos*

Mean measured concentration [ppm]	% mortality Day 5	% mortality Day 8	Intoxication symptoms		# Necropsy evaluations	Necropsy findings
			Exposure	Post-exposure		
Control	0	0	no	no	10	none
294	0	0	no	no	4	none
581	0	0	no	no	4	none
1175	0	0	no	no	4	none
2238	0	0	no	no	4	none
4741	0	0	no	no	10	none

Table: Effect of BYI 02960 on body weight and growth of *Anas platyrhynchos*

Mean measured concentration [ppm]	Mean body weight [g] (mean ± SD)			Growth [g] (mean ± S.D.)	
	Day 0	Day 5	Day 8	Exposure [a]	Post-exposure [b]
Control	141.0 ± 5.8	261.0 ± 18.7	290.2 ± 26.7	120.0 ± 16.4	29.2 ± 22.9
294	136.9 ± 9.0	258.3 ± 18.6	290.1 ± 26.4	121.5 ± 10.9	31.7 ± 29.6
581	139.6 ± 7.2	255.8 ± 19.7	315.1 ± 18.6	116.2 ± 14.4	59.3 ± 23.9
1175	136.9 ± 6.2	254.1 ± 15.8	315.4 ± 24.2	117.2 ± 10.7	61.3 ± 14.3
2238	139.5 ± 11.6	240.3 ± 20.6[c]	307.6 ± 24.8	100.8 ± 22.4[c]	67.3 ± 27.8
4741	135.6 ± 8.0	166.9 ± 16.3[c]	260.5 ± 30.3[c]	31.3 ± 21.2[c]	93.6 ± 36.1

[a] The difference between Day 5 and initiation body weights.
[b] The difference between termination and Day 5 body weights.
[c] Statistically significant difference compared to the control group.

Table: Effect of BYI 02960 on food consumption (g per bird per day) of *Anas platyrhynchos*

Mean measured concentration [ppm]	Pre-exposure [g/bird/day] (mean ± S.D.)	Exposure[a] [g/bird/day] (mean ± S.D.)	Post-exposure [g/bird/day] (mean ± S.D.)
	day -3 to -1	day 0 to 4	day 5 to 7
Control	25.2 ± 7.5	46.0 ± 13.5	37.6 ± 7.9
294	22.9 ± 7.6	44.4 ± 11.5	40.9 ± 11.1
581	24.6 ± 8.1	43.9 ± 12.0	45.0 ± 1.4
1175	23.0 ± 12.4	45.2 ± 13.0	48.7 ± 2.5
2238	22.8 ± 7.0	39.0 ± 10.0	45.3 ± 5.1
4741	23.1 ± 7.6	26.3 ± 10.0 [b]	57.1 ± 8.1

[a] Day 0 = first 24 hours of feed consumption; Day 1 = second 24 hours of feed consumption; etc.
[b] Reduction in feed consumption as compared to the controls based on empirical analysis

Table: Calculation of daily dietary dose intake of BYI 02960 by *Anas platyrhynchos*

Mean measured concentration [ppm = mg a.i./kg feed]	Mean b.w. [kg b.w. /bird]	kg feed/bird/day	Daily dietary dose
	day 0 to 5	day 0 to 5	[mg a.i./kg bw/day]
294	0.1976	0.0444	66
581	0.1977	0.0439	129
1175	0.1955	0.0452	272
2238	0.1899	0.0390	459
4741	0.1512	0.0263	825

C. Analytical Findings

Results from analytical measurements are given in the following table:

Table: Nominal and measured diet concentrations of BYI 02960

Nominal treatment level [ppm]	Verification samples	
	Measured concentration [ppm = mg a.i./kg feed]	% of nominal
Control	< 10	-
313	294	94
625	581	93
1250	1175	94
2500	2238	90
5000	4741	95

D. Validity Criteria

The control mortality was less than 10%. Measured concentrations of test item in the feed were above 80% of nominal.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

No LC_{50} (median lethal concentration) was calculated, as no treatment related mortalities occurred at any treatment level. Since the reduction in body weight for the 2238 ppm treatment level was recovered by Day 8 the NOAEL based on body weight was 2238 ppm and the LOAEL was 4741 ppm.

as concentration in feed:	(measured concentration)
LC_{50}:	> 4741 ppm (mg a.i./kg feed)
NOAEL	2238 ppm (mg a.i./kg feed)
as daily dietary dose:	(based on measured concentration)
LDD_{50}:	> 825 mg a.i./kg body weight/day
NOAEL	459 mg a.i./kg body weight/day

CONCLUSION

The short-term effect of BYI 02960 on Mallard Duck (*Anas platyrhynchos*) after dietary uptake can be quantified as an LC_{50} of > 4741 ppm (= mg a.i./kg feed) which corresponds to > 825 mg a.i./kg body weight/day.

Report:	**KIIA 8.1.2/02; [redacted], T.L. & [redacted] C.V. (2010)**
Title:	Toxicity of BYI 02960 Technical During an Acute Dietary LC_{50} with the Northern Bobwhite Quail (*Colinus virginianus*)
Report No:	EBRVP021
Document No:	M-394535-01-1
Guidelines:	**OECD Guideline No. 205** **OPPTS 850.2200**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** Some data (screening of diet and water for contaminants) was not performed in accordance with GLP as described in the study report

EXECUTIVE SUMMARY

The aim of the study was to determine the short-term effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2%) to Northern Bobwhite Quail (*Colinus virginianus*).

Colinus virginianus (13 days old) were exposed to treated feed during a period of 5 days and observed thereafter for another 3 days while fed with untreated feed. Nominal concentrations in feed were 313, 625, 1250, 2500 and 5000 ppm (= mg a.i./kg feed). Mean measured concentrations in feed were 278, 607, 1133, 2075 and 4876 ppm, which corresponded to daily uptake doses of 48, 99, 170, 262 and 470 mg a.i./kg body weight/day, respectively. In addition, untreated diet was tested as negative control.

Mortality, signs of intoxication, food consumption, body weight and gross necropsy were used to determine the endpoints.

No mortalities occurred during the study. No symptoms of toxicity were noted at any time point in any birds in the control group or at 278, 607, 1133 and 2075 ppm, during this study. Observations of hyporeactivity, loss of righting reflex, and wing drop were noted at 4876 ppm. Post-mortem examinations revealed no gross lesions or unusual observations.

There was a statistically significant reduction in Day 5 body weight, Day 8 body weight and Day 0 to 8 growth at 2075 and 4876 ppm, respectively, compared to the control group. There was also a statistically significant reduction in Day 0 to 5 growth at 1133, 2075 and 4876 ppm compared to the control group. By empirical comparisons, feed consumption was less than the control group during the exposure period for the 2075 and 4876 ppm treatment levels, and intermediate for the 1133 ppm treatment level. Since the reduction in body weight for the 1133 ppm treatment level was recovered by Day 8, the NOAEC based on body weight was 1133 ppm and the LOAEC was 2075 ppm.

The LC_{50} was determined to be > 4876 ppm (= mg a.i./kg feed), corresponding to a LDD_{50} > 470 mg a.i./kg body weight/day.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Storage conditions:	Ambient temperature

2. Test organisms

Species:	*Colinus virginianus*
Common name:	Northern Bobwhite Quail
Age at start of the exposure phase:	13 days
Source:	[redacted]
Hatching date:	04 SEP 2009
Maintenance prior to test (chick rearing):	
Temperature:	32 to 38°C
Photoperiod:	14 hours light / 10 hours dark
Food:	Teklad Bayer Starter Ration
Drinking water:	Local tap water
Remarks:	Only birds that appeared healthy were used on the study

B. Study design and methods

1. In life dates September 17 to 25, 2009

2. Experimental treatments

Following a pre-exposure period of 3 days, *Colinus virginianus* (13 days old) were offered feed treated with BYI 02960 (purity 96.2%) for 5 days. In addition, untreated diet was tested as negative control. Thereafter, the chicks were fed with untreated feed and observed for another 3 days. Each cage served as one treatment level containing 10 individually marked chicks each.

Per treatment level a total amount of 11 kg feed was prepared. The test substance was rinsed into a beaker with 100 mL solvent and stirred until it dissolved. 110 g corn oil was weighed into a separate beaker. The dissolved test substance was added to the corn oil and stirred using a magnetic stir plate to keep it in solution. The untreated feed (i.e. raw feed) was weighed into the mixing bowl in order to obtain 11 kg treated feed per treatment level in total. Corn oil (1%) was used as a vehicle.

3. Observation and measurements

Mortality and signs of intoxications were assessed approximately 1, 2 and 4 hours after diet administration on day 0, twice daily during the remainder of the study, once daily on weekends and on day 8 (study termination). Body weight measurements were conducted at day -3, day 0, day 5 and day 8. The feed consumption for each level (control and treatment birds) was recorded daily. All surviving birds in the control group and the high test level were necropsied. No treatment related gross lesions or unusual observations were found at the high level, therefore, only 40% of the birds in the remaining treatment levels were necropsied.

Food was analysed in order to verify the concentrations of the test item. In order to prove the stability of the test item in the brooder food samples were analysed which had been in the brooder for the highest and lowest treatment level (one day): 97 to 99%. In order to prove the stability of the test item in the freezer, food samples were analysed which had been stored in the freezer for at highest and lowest treatment level (seven days): 92 to 100%.

4. Statistical analysis

No LC_{50} (median lethal concentration) was calculated as no treatment related mortalities occurred at any treatment level. For body weight and growth, normality and homogeneity of variance of the data were tested using the Chi-Square-Test ($\alpha = 0.01$) and the Levene's test ($\alpha = 0.05$), respectively. Normally distributed data were subjected to standard one-way ANOVA followed by Dunnett's test or Bonferroni t-test.

RESULTS AND DISCUSSION

A. Environmental Conditions

Chicks were kept under conditions which are summarized as follows:

Room temperature during test:	22 °C average
Brooder temperature during test:	32 to 38°C
Relative humidity:	55% average
Photoperiod:	14 hours light / 10 hours dark
Light intensity:	315 lux
Ventilation of test facility:	16 changes per hour (average)

B. Biological Findings

No mortality was observed during the test.

Hyporeactivity, loss of righting reflex and wing drop were noted in the 4876 ppm level.

Table: Effect of BYI 02960 on mortality, intoxication symptoms and necropsy findings of *Colinus virginianus*

Mean measured concentration [ppm]	% Mortality day 5	% Mortality day 8	Intoxication symptoms		# Necropsy evaluations	Necropsy findings
			Exposure	Post-exposure		
Untreated control	0	0	no	no	10	none
278	0	0	no	no	4	none
607	0	0	no	no	4	none
1133	0	0	no	no	4	none
2075	0	0	no	no	4	none
4876	0	0	yes	no	10	none

Table: Effect of BYI 02960 on body weight and growth of *Colinus virginianus*

Mean measured concentration [ppm]	Body weight [g] (mean ±SD)			Growth [g] (mean ± S.D.)	
	day 0	day 5	day 8	Exposure period [a]	Post-exposure [b]
Control	30.8 ± 1.1	48.6 ± 2.0	59.1 ± 2.6	17.9 ± 1.3	10.4 ± 1.1
278	32.1 ± 1.5	49.4 ± 2.6	60.5 ± 3.5	17.2 ± 1.7	11.2 ± 1.6
607	31.0 ± 1.9	47.2 ± 3.1	59.8 ± 4.9	16.2 ± 1.5	12.7 ± 2.1
1133	32.1 ± 0.8	45.7 ± 3.2c	57.5 ± 2.5	13.6 ± 3.5[c]	11.8 ± 2.0
2075	32.1 ± 1.3	36.6 ± 3.2[c]	53.7 ± 5.9[c]	4.5 ± 2.9[c]	17.1 ± 7.2[c]
4876	32.0 ± 1.5	27.3 ± 2.6[c]	42.3 ± 3.9[c]	-4.7 ± 2.9[c]	15.0 ± 2.4[c]

[a] The difference between Day 5 and initiation body weights
[b] The difference between termination and Day 5 body weights
[c] Statistically significant difference compared to the control group

Table: Effect of BYI 02960 on food consumption (g per bird per day) of *Colinus virginianus*

Mean measured concentration [ppm]	Feed consumption [g/bird/d] (mean ±SD)		
	Pre-exposure day -3 to -1	Exposure [a] day 0 to 4	Post-exposure day 5 to 7
Control	4.4 ± 0.8	6.9 ± 0.9	7.4 ± 0.2
278	4.6 ± 0.5	7.0 ± 0.8	7.4 ± 0.8
607	4.4 ± 0.7	6.3 ± 0.8	7.5 ± 0.5
1133	4.7 ± 0.7	5.8 ± 1.1	7.5 ± 1.0
2075	4.9 ± 0.7	4.3 ± 1.0[b]	7.8 ± 1.1
4876	4.6 ± 0.6	2.9 ± 0.8[b]	6.9 ± 0.5

[a] Day 0 = first 24 hours of feed consumption; Day 1 = second 24 hours of feed consumption; etc.
[b] Reduction in feed consumption as compared to the controls based on empirical analysis

Table: Calculation of daily food intake of BYI 02960 by *Colinus virginianus*

Mean measured concentration [ppm]	Daily dietary dose calculation		
	Mean b.w. day 0 to 5 [kg b.w. /bird]	kg food/bird/day day 0 to 5	Daily dietary dose [mg a.i./kg b.w./day]
278	0.0408	0.007	48
607	0.0391	0.0063	99
1133	0.0389	0.0058	170
2075	0.0343	0.0043	262
4876	0.0296	0.0029	470

No LC_{50} (median lethal concentration) was calculated as no treatment related mortalities occurred at any treatment level.

C. Analytical Findings

Results from analytical measurements are given in the following table:

Table: Measured diet concentrations of BYI 02960

Nominal dietary concentration [mg a.i./kg feed = ppm]	Verification samples	
	Measured dietary concentration [mg a.i./kg feed = ppm]	% of nominal
Control	< 10	-
313	278	89
625	607	97
1250	1133	91
2500	2075	83
5000	4876	98

D. Validity Criteria

The control mortality was less than 10%. Measured concentrations of test item in the feed were above 80% of nominal.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

No LC_{50} (median lethal concentration) was calculated, as no treatment related mortalities occurred at any treatment level. Since the reduction in body weight for the 1133 ppm treatment level was recovered by Day 8 the NOAEL based on body weight was 1133 ppm and the LOAEL was 2075 ppm.

as concentration in feed:	(measured concentration)
LC_{50}:	> 4876 ppm (mg a.i./kg feed)
NOAEL	1133 ppm (mg a.i./kg feed)
as daily dietary dose:	(based on measured concentration)
LDD_{50}:	> 470 mg a.i./kg body weight/day
NOAEL	170 mg a.i./kg body weight/day

CONCLUSION

The short-term effect of BYI 02960 on Northern Bobwhite Quail (*Colinus virginianus*) after dietary uptake can be quantified as an LC_{50} of > 4876 ppm (= mg a.i./kg feed) which corresponds to > 470 mg a.i./kg body weight/day.

IIA 8.1.3 Avian dietary toxicity (5-day) test in a second unrelated species

Two avian dietary toxicity tests have been conducted; one with mallard duck, another with northern bobwhite quail. Both studies are presented under Annex point IIA 8.1.2 above.

IIA 8.1.4 Subchronic and reproductive toxicity to birds

Report:	**KIIA 8.1.4/01; [redacted], T.L., [redacted], [redacted] & [redacted] C.V. (2011)**
Title:	Toxicity of BYI 02960 Technical on Reproduction to the Mallard Duck (*Anas platyrhynchos*)
Report No:	EBRVP018
Document No:	M-412917-02-1
Guidelines:	**OECD Guideline No. 206** **OPPTS 850.2300** **FIFRA Guideline 71-4**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** Some data (screening of diet and water for contaminants) was not performed in accordance with GLP as described in the study report

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of dietary exposure to BYI 02960 technical 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2%) on the health and reproductive capacity of Mallard Ducks (*Anas platyrhynchos*).

Fifteen pairs per treatment level of *Anas platyrhynchos* (19 weeks old) were exposed to treated feed during a period of approximately 20 weeks. Nominal concentrations in feed were 111, 333 and 1000 mg a.i./kg feed (= ppm) which corresponded with mean measured concentrations of 91, 298 and 845 ppm (achieved daily doses: 9, 28 and 81 mg a.i./kg body weight per day). In addition, untreated diet was tested as negative control.

Mortality, abnormal behaviour and signs of intoxication, food consumption, body weight and gross necropsy were used to determine the endpoints for the adults. Reproductive parameters included egg number, egg shell quality, embryo viability and hatchling number, weight, growth and survival.

No treatment related adverse effects occurred in any of the endpoints under evaluation.

The No Observed Adverse Effect Level (NOAEL) for both parental toxicity and reproduction endpoints of Mallard ducks exposed to BYI 02960 over a 20-week period was ≥845 mg a.i./kg feed (mean measured concentration), which corresponds to a daily dietary dose of ≥81 mg a.i./kg b.w./day.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Storage conditions:	Ambient temperature

2. Vehicle and /or positive control

Vehicle	None
Positive Control	None

3. Test organisms

Species:	*Anas platyrhynchos*
Common name:	Mallard Duck
Age at start of the exposure phase:	19 weeks
Source	[redacted]
Maintenance prior to test (chick rearing):	
Housing:	Adult birds were housed indoors in a single room for the acclimation and the study period. The adult mallard cages measured approximately 79(L) x 61(W) x 55(H) cm were constructed of stainless steel wire grid and stainless steel sheeting. Cage floors were constructed of plastic coated steel wire and slopped to accommodate egg collection. Each cage was equipped with a bin feeder which was filled with sufficient feed on a weekly basis.
Food:	Teklad Bayer Game Bird Ration
Drinking water:	Local tap water
Mortality:	No bird mortality occurred during the acclimation period

4. Study design

Replicates:	15 breeding pairs per dose level; one male and one female per cage
Acclimation period:	Approx. 7 weeks prior to experimental start
Temperature:	Adults: 21.3°C (mean) Hatchlings: 21.9°C (mean)
Relative humidity:	Adults: 54.7 % (mean) Hatchlings: 55.9 % and 57.8 % (mean)
Photoperiod:	14 h light : 10 h dark
Light intensity:	8.8 to 23.2 footcandles (95 to 250 lux)

B. Study design and methods

1. In life dates December 1, 2009 to June 1, 2010

2. Experimental treatments

Following an acclimation period of 7 weeks, *Anas platyrhynchos* (19 weeks old) were offered feed treated with BYI 02960; (purity 96.2%) for 20 weeks.

The dietary ingredient concentrations and quantities for the study are given in the following table:

Test level Dietary Mix				
Treatment level [ppm = mg a.i./kg feed]	**Total batch weight [kg]**	**Pre-mix concentration [mg/kg]**	**4000 mg/kg pre-mix added [kg]**	**Control pre-mix added [kg]**
Control	20	0	0	5
111	20	4000	0.56	0
333	20	4000	1.67	0
1000	20	4000	5	0

The exposure period was divided into a pre-photostimulation period of 8 weeks, a pre-egglaying period of 2 weeks and an egglaying period of 10 weeks. Survival and body weight of the F1-generation were observed up to two weeks after hatch. Each cage contained 1 male and 1 female. The test was conducted with 15 replicates per treatment level.

3. Observation and measurements

Adult body weights for the mallard reproduction study were measured at randomization, on weeks 3, 5, 7 and 9, and prior to adult termination. No adult body weights were taken during the egg production phase for the study. Mortality, feed consumption and signs of intoxications were assessed daily. The food consumption was calculated from weighing the residual food. Food was analyzed in order to verify the homogeneity and the concentrations of the test item and its stability in the feeder.

Egg collection, candling, storage and incubation: Eggs were collected twice daily except for weekends and holidays where eggs were collected only once per day. All eggs were labeled according to parental cage and date. Eggs were treated to prevent pathogen contamination and then stored in an Egg Cooler. Any eggs that were not able to be set ("set" = transfer into the incubator) were recorded and discarded prior to egg set. Only eggs that were in good condition were used for eggshell strength and thickness measurements.

Egg incubation was initiated weekly (after start of reproduction). On Day 23 of incubation, the eggs were allowed to hatch.

Hatchling body weights were measured and recorded on the day they were removed from the hatcher and on Day 14. Hatchlings were observed once daily throughout the 14-day period for signs of toxicity, injuries, illness, and mortality. Mortalities that occurred prior to the end of the 14-day period were recorded and discarded. Hatchlings that survived the 14-day observation period were sacrificed by CO_2 asphyxiation, weighed, and discarded.

4. Endpoints

Adult Body Weight: The adult body weight change from initiation of dosing to adult termination, analyzed on a per cage basis by sex.

Adult Feed Consumption: The means were calculated as grams/bird/day, analyzed on a per cage basis.

Eggs Laid per hen: Analyzed as the total egg production, on a per hen basis.

Eggs Cracked: Cracked eggs as determined by candling prior to incubation, analyzed on a per hen basis.

Eggs Not Cracked of Laid (%): Eggs laid that were not cracked as a percentage of eggs laid on a per hen basis.

Eggshell Strength: The force needed to penetrate the shell and membrane measured at one point on the waist of the egg.

Eggshell Thickness: The thickness of the shell plus the membrane measured at three points around the waist of the egg.

Eggs Set: Eggs placed under incubation are "set", analyzed as total number on a per hen basis.

Viable Embryos (mean): Fertility is determined by candling on the 11th or 12th day of incubation. Fertility analyzed as total fertile eggs.

Live Embryos (mean): Live embryos are determined by candling on the 18th or 19th day of incubation.

Eggs Set of Laid (%): Eggs set as a percentage of eggs laid on a per hen basis.

Viable Embryos of Eggs Set (%): Live embryos as a percentage of eggs set on a per hen basis.

Live Embryos of Viable Embryos (%): Live embryos as a percentage of viable embryos on a per hen basis.

Number Hatched (mean): Live hatchlings that had liberated themselves from their eggs by day 28 of incubation. Analyzed as the total number of normal hatchlings on a per hen basis.

Number Hatched of Live Embryos (%): Hatchlings as a percentage of live embryos on a per hen basis.

14-day-old Survivors (mean): Live chicks at 14-days post hatch.

14-day-old Survivors of Eggs Set (%): Live chicks at 14-days post hatch as a percentage of eggs set on a per hen basis.

Number Hatched of Eggs Laid (%): Hatchlings as a percentage of eggs laid on a per hen basis.

Number Hatched of Eggs Set (%): Hatchlings as a percentage of eggs set on a per hen basis.

14-day-old Survivors of Number Hatched (%): Live chicks at 14-days post hatch as a percentage of hatchlings on a per hen basis.

Hatchling Body Weight: Individual weights of the live hatchlings taken upon removal from the hatcher, analyzed on a per hen basis.

14-day-old Survivor Body Weight: Individual weights of the 14-day-old offspring taken at sacrifice, analyzed on a per hen basis.

5. Statistical analysis

For avian reproduction data, normality and homogeneity of variance of the data were tested using the Shapiro-Wilk's Test ($\alpha = 0.01$) and the Levene's test ($\alpha = 0.05$), respectively. Normally distributed data were subjected to standard one-way ANOVA followed by Dunnett's test or William's-test. Non-parametric analyses were conducted using the Jonckheere or Mann-Whitney procedures.

Analysis of adult termination bodyweight data was conducted by subjecting data to a Chi-Square Test for normality and a Bartlett's Test for equal variances. The analysis of variance was conducted followed by a Dunnett's Test for unequal replicate size.

RESULTS AND DISCUSSION

A. Environmental Conditions

Test animals were kept under conditions which are summarised as follows:

Room temperature during test:	21.3 °C
Relative humidity:	54.7%
Photoperiod:	
Acclimation and pre-photostimulation period:	7 hours light/17 hours dark
Reproduction period:	17 hours light/7 hours dark
Light source	Fluorescent lamp
Light intensity:	95 to 250 lux
Ventilation of test facility:	15 air changes per hour
Brooder:	
Temperature:	36.9 °C
Air humidity:	59.8%
Rotation of eggs:	Approximately every two hours
Hatching compartment:	
Temperature:	36.8 °C
Air humidity:	69.3%
Pen (for hatchlings):	
Photoperiod:	14 hours light / 10 hours dark

B. Biological Findings

No adult mortality or other effects on parental health occurred at any treatment level. Several adult birds were observed in the control and treatment levels with feather loss and skin abrasions/lacerations as a result of normal cage wear for laboratory birds. No statistically or biologically significant effects were observed in the reproductive endpoints.

Table: Summary of reproductive performance of *Anas platyrhynchos* treated with BYI 02960

	Control	111 ppm	333 ppm	1000 ppm
Number of replicates	15	15	15	15
Total eggs laid	61.6	56.5	53.7	61.3
Eggs cracked	0.20	0.27	0.33	0.21
Eggs set	55.9	50.3	47.5	55.4
Viable Embryos	51.5	48.5	42.8	51.4
Live 3-Week Embryos	51.2	48.0	42.5	51.1
Hatchlings	47.5	43.3	37.7	46.3
Eggs Not Cracked/Eggs Laid (%)	99.7	99.3	99.5	99.7
Viable Embryos / Eggs Set (%)	92.7	96.7	87.3	93
Live 3-Week Embryos/Viable Embryos (%)	99.5	98.0	99.2	99.3
Hatchlings/Live 3-Week Embryos (%)	93	90.3	88.8	89.4
14-Day Old Survivors/Hatchlings (%)	99.4	99.5	99.7	99.5
Hatchlings/Eggs Set (%)	77.3	75.4	66.9	74.1
14-Day Old Survivors/Eggs Set (%)	85.2	85.2	75.7	82

Table: Egg shell thickness of *Anas platyrhynchos* treated with BYI 02960

Nominal treatment level [ppm]	Shell thickness (mean ± SD) [mm]	Shell strength (mean ± SD) [kg]
control	0.363 ± 0.018	2.66 ± 0.28
111	0.355 ± 0.020	2.72 ± 0.39
300	0.359 ± 0.018	2.55 ± 0.30
1000	0.369 ± 0.014	2.82 ± 0.27

Table: Body weight of hatchlings of *Anas platyrhynchos* treated with BYI 02960

Nominal treatment level [ppm]	Hatchlings		14 day old survivors		% Mortality
	Number	Body weight (mean ± SD) [g]	Number	Body weight (mean ± SD) [g]	
control	712	36.3 ± 2.1	708	269.8 ± 17.5	4
111	649	35.3 ± 1.9	646	268.9 ± 13.8	3
300	566	36.3 ± 2.8	564	273.4 ± 14.0	2
1000	648	35.0 ± 1.7	646	266.4 ± 15.4	2

C. Analytical Findings

Analysis of test diet: The nominal amounts of BYI 02960 technical in the feed were 0 (control), 111, 333 and 1000 ppm (= mg a.i./kg feed), respectively. The average measured amounts of BYI 02960 technical for week 1, 5, 10, 15 and 20 were determined as 0 (control), 91, 298 and 845 ppm, respectively, representing percent of nominal values of 82, 90 and 85%, respectively.

Homogeneity of mixing was verified with the 111 and 1000 ppm treatment levels. Triplicate samples were obtained from the top, middle and bottom of the mixing vessel. The average measured amounts of BYI 02960 technical for the nominal amounts of 111 and 1000 ppm were determined as 108 ppm (RSD = 13%) and 1001 ppm (RSD = 8%), respectively.

Freezer stability samples were stored in a freezer for approximately five weeks and analyzed for BYI 02960 for the nominal treatments of 111 and 1000 ppm. The measured amounts of BYI 02960 for the treatments were 86 and 932 ppm, respectively. The 5 week values were compared to the initial Week 1 measured analysis and percent initial values were determined as 81 and 93% for the 111 and 1000 ppm treatments, respectively.

Room stability samples remained for seven days under study room conditions and were analyzed for BYI 02960 for the nominal treatments of 111 and 1000 ppm. The measured amounts of BYI 02960 for the treatments were 82 and 930 ppm, respectively. The 7 day stability sample values were compared to the initial Week 1 measured analysis and percent initial values were determined as 78 and 93% for the 111 and 1000 ppm treatments, respectively

Results from analytical measurements are summarised as follows:

Table: Measured diet concentrations of BYI 02960

Nominal dietary concentration	Week 1	Week 5	Week 10	Week 15	Week 20	Mean Measured Values (±SD)	Percent of Nominal
Control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	n.d.
111 ppm	106	93	63	89	106	91.4 (17.7)	82
333 ppm	270	278	269	332	343	298.4 (36.1)	90
1000 ppm	999	730	779	999	718	845.0 (142.4)	85

Table: Calculation of the daily dietary dose of BYI 02960 based on 22-week food consumption

Measured dietary concentration	Body weight at randomization [kg]	Body weight at termination [kg]	Mean body weight [kg]	Food consumption [kg feed/bird/day]	Daily dietary dose [mg a.i./kg b.w./day]
91 ppm	1.04	1.156	1.098	0.105	9
298 ppm	1.043	1.168	1.106	0.103	28
845 ppm	1.017	1.11	1.064	0.102	81

Table: Homogeneity and analytical verification of concentrations of BYI 02960

Nominal dietary concentration	Homogeneity in diet		Verification of concentrations in diet (% recovery)				
	mean	% cv	Week 1	Week 5	Week 10	Week 15	Week 20
111 ppm	108	13%	95	83	60	80	96
333 ppm	-*	-*	81	84	81	100	103
1000 ppm	1001	8%	100	73	78	100	72

* Homogeneity was tested only for nominal concentrations of 111 and 1000 ppm, respectively

Table: 7-day ambient and freezer stability of BYI 02960

Nominal dietary concentration	7-day room stability		Freezer stability	
	Mean measured [ppm a.i.]	Mean percent of nominal	Mean measured [ppm a.i.]	Mean percent of day 0
111 ppm	82.3	78	86.3	81
1000 ppm	930	93	932	93

D. Validity Criteria

The control mortality was less than 10%. Measured concentrations of test item in the feed were above 80% of nominal. The shell thickness of eggs from the controls was above the species-specific threshold. The average number of 14-day-old survivors per hen in the controls was above the species-specific threshold.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

as concentration in feed:	(nominal concentrations)
NOAEL	≥ 1000 ppm (mg a.i./kg feed
LOAEL	> 1000 ppm (mg a.i./kg feed)
as concentration in feed:	(based on measured concentrations)
NOAEL	≥ 845 ppm (mg a.i./kg feed)
LOAEL	> 845 ppm (mg a.i./kg feed)
as daily dietary dose:	(based on measured concentrations)
NOAEL	≥ 81 mg a.i./kg bw/day
LOAEL	> 81 mg a.i./kg bw/day

CONCLUSION

The effect of BYI 02960 on the reproduction of Mallard Duck (*Anas platyrhynchos*) after dietary uptake can be quantified as a NOAEL of ≥ 845 mg a.i./kg feed which corresponds to ≥ 81 mg a.i./kg b.w./day.

Report:	**KIIA 8.1.4/02; [redacted], T.L., [redacted], [redacted] & [redacted] C.V. (2012)**
Title:	Toxicity of BYI 02960 Technical on Reproduction to the Northern Bobwhite Quail (*Colinus virginanus*)
Report No:	EBRVP019
Document No:	M-424704-01-1
Guidelines:	**OECD Guideline No. 206** **OPPTS 850.2300** **FIFRA Guideline 71-4**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of dietary exposure to BYI 02960 technical (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2%) on the health and reproductive capacity of Northern Bobwhite Quail (*Colinus virginanus*).

Eighteen pairs of birds per treatment of *Colinus virginianus* (18 weeks old) were exposed to treated feed during a period of 23 weeks. Nominal concentrations in feed were 111, 333 and 1000 mg a.i./kg feed (=ppm) which corresponded to mean measured concentrations of 107, 302 and 999 ppm and achieved daily doses of 14, 40 and 154 mg a.i./kg body weight per day, respectively.

Birds were observed for mortality, abnormal behavior and signs of toxicity; adult body weight and feed consumption were measured; gross pathology was conducted; reproductive parameters, as well as hatchling health, growth and survival, were examined.

At 1000 ppm, there were statistically and biologically significant effects on parental survival, health and body weight, and subsequently on several reproductive parameters. At 300 ppm, there were statistically significant but small effects on several parameters that were not considered biologically relevant.

Therefore, the No Observed Adverse Effect Level (NOAEL) for both parental toxicity and reproduction endpoints of bobwhite quail exposed to BYI 02960 over a 23-week period was 302 mg a.i./kg feed (ppm), which corresponded to 40 mg a.i./kg b.w./day.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Storage conditions:	Approved until 2012-1-11 when stored at ambient temperature
Vehicle and/or positive control:	None

2. Test organisms

Species:	*Colinus virginianus*
Common name:	Northern Bobwhite Quail
Age at start of the exposure phase:	18 weeks
Source	[redacted]
Maintenance prior to test (chick rearing):	
Housing:	Adult birds were housed indoors in a single room for the acclimation and the study period. The adult quail cages measured approximately 56(L) x 28(W) x 27(H) cm were constructed of stainless steel wire grid and stainless steel sheeting. During the feeding period, additional feed was weighed and added to the bin feeders as needed.
Food:	Teklad Bayer Game Bird Ration
Drinking water:	Local tap water
Mortality:	No bird mortality occurred during the acclimation period

3. Study design

Replicates:	18 breeding pairs per dose level; one male and one female per cage
Acclimation period:	Approx. 4 weeks prior to experimental start
Temperature:	Adults: 21.3°C (mean) Hatchlings: 22.2°C (mean)
Relative humidity:	Adults: 55.3 % (mean) Hatchlings: 57.5 % (mean)
Photoperiod:	Adults: 7 h light : 17 h dark (acclimation and short day length phase (8 weeks)) 17 h light : 7 h dark (remainder of the study) Hatchlings: 14 h light : 10 h dark
Light intensity:	Adults: 64 to 194 lux Hatchlings: 252 lux

B. Study design and methods

1. In life dates April 15 to November 01, 2010

2. Experimental treatments

Following an acclimation period of 4 weeks, *Colinus virginianus* (18 weeks old) were offered feed treated with BYI 02960; (purity 96.2%) for 23 weeks.

The dietary ingredients and quantities for the study are given in the following table:

Test Level Dietary Mixing					
Nominal Treatment Concentration [ppm]	Total Batch Weight [kg]	Pre-Mix Concentration [mg/kg]	2000 mg/kg Pre-Mix added [kg]	Control Pre-Mix added [kg]	Amount of Raw Feed [kg]
Control	11	0	0	5.5	5.5
111	11	2000	0.61	0	10.39
333	11	2000	1.83	0	9.17
1000	11	2000	5.5	0	5.5

Eggs were collected twice daily from the parental birds during the exposure phase. The exposure period was divided into a short day length period of 8 weeks and a long day length period of 15 weeks. Effects on adult survival, health, body weight, and feed consumption were evaluated. In addition, the effects of adult exposure to BYI 02960 technical on the number of eggs laid, fertility, embryo viability, hatchability, offspring survival, and eggshell quality (strength and thickness) were evaluated. Each cage served as one replicate containing 1 male and 1 female. The test was conducted with 18 replicates per dose.

3. Observation and measurements

Adult body weights and feed consumption for the quail reproduction study were measured at randomization, on weeks 3, 5, 7 and 9, and prior to adult termination. No adult body weights were taken during the egg production phase for the study. Adult feed consumption was measured weekly by cage throughout the study and calculated from weighing the residual food. Food was analysed in order to verify the homogeneity and the concentrations of the test item and its stability in the feeder.

Egg collection, candling, eggshell evaluation, storage and incubation: Eggs were collected twice daily except for weekends and holidays where eggs were collected only once per day. All eggs were labeled according to parental cage and date. Eggs were treated to prevent pathogen contamination and then stored in an Egg Cooler. Any eggs that were not able to be set ("set" =transfer into the incubator) were recorded and discarded prior to egg set.

Eggshell strength and thickness was measured each week during the egg laying phase. One egg was collected from each of the odd numbered cages during odd numbered weeks (i.e. 1, 3, 5, 7, and 9) and one egg was collected from each of the even numbered cages during even numbered weeks (i.e. 2, 4, 6, 8, and 10). Only eggs that were in good condition were used for eggshell strength and thickness measurements.

Egg incubation was initiated weekly (after start of reproduction). On Day 21 of incubation, the eggs were allowed to hatch.

Hatchling body weights were measured and recorded on the day the hatchlings were removed from the hatcher and on Day 14. Feed consumption for the offspring was not monitored. Hatchlings were observed once daily throughout the 14-day period for signs of toxicity, injuries, illness, and mortality. A record was maintained of all observations. Mortalities that occurred prior to the end of the 14-day period were recorded and discarded. Hatchlings that survived the 14-day observation period were sacrificed by CO_2 asphyxiation, weighed, and discarded.

Food was analysed in order to verify the homogeneity and the concentrations of the test item and its stability in the feeder.

4. Endpoints

Adult Body Weight: The adult body weight change from initiation of dosing to adult termination. Analyzed on a per cage basis by sex.

Adult Feed Consumption: The means were calculated as grams/bird/day, analyzed on a per cage basis.

Eggs Laid per Hen: Analyzed as the total egg production, on a per hen basis.

Eggs Cracked of Eggs Laid: Cracked eggs are determined by candling prior to incubation, analyzed on a per hen basis.

Eggs Not Cracked of Laid (%): Eggs laid that were not cracked as a percentage of eggs laid on a per hen basis.

Eggshell Strength: The force needed to penetrate the shell and membrane measured at one point on the waist of the egg.

Eggshell Thickness: The thickness of the shell plus the membrane measured at three points around the waist of the egg.

Eggs Set: Eggs placed under incubation are "set", analyzed as total number on a per hen basis.

Viable Embryos (mean): Fertility is determined by candling on the 11^{th} or 12^{th} day of incubation. Fertility analyzed as total fertile eggs.

Live Embryos (mean): Live embryos are determined by candling on the 18^{th} or 19^{th} day of incubation.

Eggs Set of Laid (%): Eggs set as a percentage of eggs laid on a per hen basis.

Viable Embryos of Eggs Set (%): Live embryos as a percentage of eggs set on a per hen basis.

Live Embryos of Viable Embryos (%): Live embryos as a percentage of viable embryos on a per hen basis.

Number Hatched (mean): Live hatchlings, which had liberated themselves from their eggs by day 28 of incubation. Analyzed as the total number of normal hatchlings on a per hen basis

Number Hatched of Live Embryos (%): Hatchlings as a percentage of live embryos on a per hen basis.

14-day-old Survivors (mean): Live chicks at 14-days post hatch.

14-day-old Survivors of Eggs Set (%): Live chicks at 14-days post hatch as a percentage of eggs set on a per hen basis.

Number Hatched of Eggs Laid (%): Hatchlings as a percentage of eggs laid on a per hen basis.

Number Hatched of Eggs Set (%): Hatchlings as a percentage of eggs set on a per hen basis.

14-day-old Survivors of Number Hatched (%): Live chicks at 14-days post hatch as a percentage of hatchlings on a per hen basis.

Hatchling Body Weight: Individual weights of the live hatchlings taken upon removal from the hatcher, analyzed on a per hen basis.

14-day-old Survivor Body Weight: Individual weights of the 14-day-old offspring taken at sacrifice, analyzed on a per hen basis.

5. Statistical analysis

The no observed adverse effect level (NOAEL) and lowest observed adverse effect level (LOAEL) was identified for each parameter using hypothesis testing methodology. All hypotheses testing were performed with a specialized statistical program designed to analyze avian reproduction data (EFED). All data was analyzed independently according to each end-point. Data from treatment groups were compared to controls using the Shapiro-Wilk's test for normality and Levene's test of equal variance to determine if dose groups had unequal variances. If assumption of normality ($p \leq 0.01$) and homogeneity of variance ($p \leq 0.05$) were met, then parametric analyses were conducted using analysis of variance (ANOVA) followed by Dunnett's test or William's test. If variances were unequal, then the non-parametric analyses were conducted using the Jonckheere or Mann-Whitney procedures. Statistical analyses were performed using SAS® statistical software for personal computers with conclusions of statistical significance at the $\alpha = 0.05$ (95% confidence level).

RESULTS AND DISCUSSION

A. Environmental Conditions

Test animals were kept under conditions which are summarised as follows:

Room temperature during test:	21.3 °C
Relative humidity:	55.3%
Photoperiod:	
Acclimation and pre-photostimulation period:	7 hours light/17 hours dark
Reproduction period:	17 hours light/7 hours dark
Light source	Fluorescent lamp
Light intensity:	64 to 194 lux
Ventilation of test facility:	13.6 air changes per hour
Brooder:	
Temperature:	37.0 °C
Air humidity:	58.9%
Rotation of eggs:	Approximately every two hours
Hatching compartment:	
Temperature:	37.3 °C
Air humidity:	68.4%
Pen (for hatchlings):	
Photoperiod:	14 hours light / 10 hours dark

B. Biological Findings

Adult mortality

Nine adult mortalities occurred during the test with two birds in the control group, one bird in the 111 ppm level, and six birds at 1000 ppm. No adult mortalities occurred in the 333 ppm treatment level.

Table: Adult mortality of *Colinus virginianus* treated with BYI 02960

Adult Bird Mortality Summary					
Cage No.	Bird No.	Bird Sex	Mortality Date	Final Bird Weight [g]	Observation
Control					
002	218	Female	05-Sep-10	251	Feather loss on head
008	831	Male	26-May-10	193	None
111 ppm					
103	734	Male	14-Sep-10	150	Feather loss on head, abrasions on feet
1000 ppm					
303	814	Male	04-Jul-10	139	Lacerations on feet, emaciated
307	302	Female	15-Sep-10	124	Laceration on foot, feather loss on breast, emaciated
308	273	Female	10-Sep-10	121	Feather loss on back, laceration on foot and lower back, emaciated
310	330	Female	10-Sep-10	141	Feather loss on back, wing, and neck, lacerations on back and leg, emaciated
313	277	Female	06-Sep-10	106	Feather loss on neck, back, and leg, laceration on foot, toes, and head
315	271	Female	07-Sep-10	107	Feather loss on breast, lacerations on feet, emaciated

The mortalities at 1000 ppm were considered treatment related following necropsy observations. Four female birds in the 1000 ppm treatment level were observed as being emaciated with all five female birds either having regressed ovaries, no maturing follicles, or reduced egg-laying.

Thus, the LOAEL for parental survival can be considered at the top dose level (1000 ppm). The related NOAEL is 333 ppm.

Adult bird feed consumption

There were no statistically or biologically significant differences in the feed consumption to the control at any treatment level. Thus, the LOAEL for adult feed consumption can be considered at above the top dose level (> 1000 ppm). The related NOAEL is 1000 ppm.

Table: Adult feed consumption of *Colinus virginianus* treated with BYI 02960

Nominal Treatment [ppm]	**Adult feed consumption (g/bird/day) (mean ± S.D.)**	**% reduction**
Control	27.2 ± 3.6	-
111	26.2 ± 6.1	3.40
333	28.3 5.1	-4.16
1000	25.7 ± 3.4	5.43

Adult body weight and body weight gain

There was a statistically significant effect for the adult male body weight gain at the 333 ppm test level, however the magnitude of the difference to the control bodyweight remained < 10%, and no dose-response was apparent, so that the biological significance was considered to be low. There was a statistically significant effect on the adult female body weight gain at 1000 ppm that was related to a biologically significant difference in body weight. No effects were observed on adult male and female body weight in any other test levels. Thus, the LOAEL for parental body weight can be considered at the top dose level (1000 ppm). The related NOAEL is 333 ppm.

Table: Adult male body weight and bodyweight gain of *Colinus virginianus* treated with BYI 02960

Nominal Treatment [ppm]	Bodyweight [g] (mean ± S.D.) Week 1	Termination	Bodyweight gain [g] (mean ± S.D.)
Control	202 ± 11.0	222 ± 20.3	19.0 ± 12.7
111	197 ± 9.4	207 ±16.4	10.4 ± 14.5
333	200 ± 9.6	209 ± 20.7	5.9 ± 20.0 *
1000	195 ± 11.3	203 ± 18.5	9.6 ± 10.8

* Statistically significant [B] Biologically significant

Table: Adult female body weight and bodyweight gain of *Colinus virginianus* treated with BYI 02960

Nominal Treatment [ppm]	Bodyweight [g] (mean ± S.D.) Week 1	Termination	Bodyweight gain [g] (mean ± S.D.)
Control	199 ± 7.2	234 ± 37.6	35.0 ± 34.0
111	199 ± 7.5	233 ± 27.3	37.0 ± 25.9
333	199 ± 7.2	239 ± 18.5	42.1 ± 13.6
1000	199 ± 7.0	204 ± 29.7 [B]	10.7 ± 26.9 *

* Statistically significant [B] Biologically significant

Adult bird necropsy

Necropsy of all adult birds at study termination showed an apparent treatment effect at the 1000 ppm level. Adult female birds in the 1000 ppm treatment level were observed with the following:

8 birds with regressed ovaries which were higher as opposed to other treatment levels; 10 birds with a lower number of maturing follicles than in the other treatment levels; and 5 eggs found in the oviduct which were lower than the other treatment levels.

In addition, a higher number of birds in the 1000 ppm treatment level skin lesions/abrasions and emaciated birds as compared to the other treatment levels. Feather loss was noted for birds in all treatment levels due to normal cage wear for laboratory reared quail.

No other effects attributable to treatment with BYI 02960 were observed at any other treatment level. Thus, the LOAEL for parental health based on necropsy results can be considered at the top dose level (1000 ppm). The related NOAEL is 333 ppm.

Egg endpoints

Egg number: there were statistically and biologically significant differences at the 1000 ppm treatment level as compared to the control for the number of eggs laid and the number of eggs set. There was a statistically significant difference at the 333 and 1000 ppm treatment levels as compared to the control for the percent eggs set of eggs laid, however these differences were not considered biologically significant as the % reduction was 1.89% and 3.62%, respectively.

The LOAEL for the egg endpoints was determined at the top dose level (1000 ppm). The related NOAEL is 333 ppm.

Table: Egg number of *Colinus virginianus* treated with BYI 02960

Nominal Treatment [ppm]	No. of eggs laid		No. of eggs set		% Eggs set of eggs laid	
	mean ± SD	% reduction	mean ± SD	% reduction	mean ± SD	% reduction
Control	55.6 ± 16.5	-	50.1 ± 16.6	-	88.7± 7.5	
111	51.1 ± 13.0	8.15	44.3 ± 12.5	11.62	86.3 ± 5.3	2.78
333	60.8 ± 9.2	-9.42	53.1 ± 8.9	- 5.87	87.1 ± 3.5	1.89*
1000	30.9 ± 16.4 *[B]	44.4	26.6 ± 14.7 *[B]	46.85	85.5 ± 3.1	3.62*

* Statistically significant [B] Biologically significant

Eggshell quality

There were no statistically significant differences at any treatment level as compared to the control for eggshell thickness or eggshell strength. The LOAEL for the eggshell quality end-points was considered at above the top dose level (> 1000 ppm). The related NOAEL is ≥ 1000 ppm.

Table: Egg shell quality of *Colinus virginianus* treated with BYI 02960

Nominal Treatment [ppm]	Shell thickness (mean ± SD) [mm]	Shell strength (mean ± SD) [kg]
control	0.21 ± 0.01	0.84 ± 0.14
111	0.22 ± 0.01	0.90 ± 0.17
300	0.21 ± 0.01	0.79 ± 0.16
1000	0.22 ± 0.01	0.79 ± 0.13

Embryo endpoints

There were statistically and biologically significant differences at the 1000 ppm treatment level as compared to the control for the number of viable embryos and the number of live embryos.

There were no statistically significant differences at any treatment level as compared to the control for percent viable embryos of eggs set and live embryos of viable embryos. The LOAEL for the embryo endpoints was determined at the top dose level (1000 ppm) but the effect is primarily due to a lower number of eggs laid or set, rather than to reduced embryo survival. The related NOAEL is 333 ppm.

Table: Embryo endpoints of *Colinus virginianus* treated with BYI 02960 (absolute)

Nominal Treatment [ppm]	No. of viable embryos		No. of live embryos	
	mean ± SD	% reduction	mean ± SD	% reduction
Control	44.8 ± 17.9	-	44.3 ± 17.7	
111	42.9 ± 12.5	4.20	42.5 ± 12.6	3.98
333	49.3 ± 10.0	-10.12	48.9 ± 10.1	-10.36
1000	24.8 ± 14.9*[B]	44.56	24.7 ± 14.7*[B]	44.17

* Statistically significant [B] Biologically significant

Table: Embryo endpoints of *Colinus virginianus* treated with BYI 02960 (relative)

Nominal Treatment [ppm]	Viable embryos of eggs set [%]		Live embryos of viable embryos [%]	
	mean ± SD	% reduction	mean ±SD	% reduction
Control	91.4 ± 21.1	-	99.0	-
111	96.6 ± 5.0	-5.65	99.0	0.02
333	94.2 ± 15.8	-3.07	99.0	-0.05
1000	92.6 ± 17.0	-1.33	99.8	-0.87

Hatchling Number & Survival

Statistically and biologically significant differences occurred at the 1000 ppm treatment level for the following end-points: number hatched, percent number hatched of eggs laid, percent number hatched of live embryos, and 14-day survivors. A small but statistically significant difference in the percent number hatched of live embryos at 333 ppm was not considered biologically significant. No statistically significant differences occurred for the following end-points: percent number hatched of eggs set, percent 14-day survivors of eggs set, and percent 14-day survivors of number hatched.

The LOAEL for the hatchling number and survival was determined at the top dose level (1000 ppm) but the effect is primarily due to a lower number of eggs laid or set, rather than to reduced hatch success or hatchling survival. The related NOAEL is 333 ppm.

Table: Hatchling number and survival of *Colinus virginianus* treated with BYI 02960 (absolute)

Nominal Treatment [ppm]	No. of hatchlings		Hatchlings of eggs set [%]		Hatchlings of live embryos [%]	
	mean ± SD	% reduction	mean ± SD	% reduction	mean ±SD	% reduction
Control	42.1 ± 16.2	-	86.6 ± 20.3	-	95.9 ± 3.7	-
111	39.8 ± 11.6	5.45	89.8 ± 7.0	-3.72	94.0 ± 5.4	2.05
333	45.0 ± 10.0	-6.99	85.8 ± 15.9	0.85	91.9 ± 6.0*	4.16
1000	21.4 ± 12.1*[B]	49.21	81.4 ± 17.1	5.98	88.2 ± 10.3*[B]	8.03

* Statistically significant [B] Biologically significant

Hatchling Body Weight

There was a statistically and biologically significant difference at the 1000 ppm treatment level as compared to the control for initial hatchling body weight and for 14-d survivor body weight.

The LOAEL for the hatchling body weight was determined at the top dose level (1000 ppm). The related NOAEL is 333 ppm.

Table: Hatchling body weight of *Colinus virginianus* treated with BYI 02960 (absolute)

Nominal Treatment [ppm]	Initial Hatchling body weight [g]		14-d survivor body weight [g]	
	mean ± SD	% reduction	mean ± SD	% reduction
Control	6.9 ± 0.4	-	34.5 ± 2.1	-
111	6.7 ± 0.3	3.91	33.2 ± 1.4	3.87
333	6.8 0.5	2.21	33.6 ± 2.6	2.59
1000	6.0 ± 0.4	13.60*[B]	30.8 ± 2.9*[B]	10.79

* Statistically significant [B] Biologically significant

C. Analytical Findings

Analysis of test diet: The nominal amounts of BYI 02960 technical in the feed were levels of 0 (control), 111, 333, and 1000 mg a.i./kg feed (= ppm). The average measured amounts of BYI 02960 technical for week 1, 5, 10, 15, and 20 were determined as 0, 107, 302 and 999 ppm representing percent nominal values of 96, 91 and 100%, respectively. These values correspond to daily dietary dose levels of 0, 14, 40 and 154 mg a.i./kg b.w./day, respectively. Homogeneity of mixing was verified with the 111 and 1000 ppm treatment levels. Duplicate samples were obtained from the top, middle, and bottom of the mixing vessel from the week 1, 10 and 20 feed mixes.

Freezer stability samples were stored in a freezer for approximately eight weeks and analyzed for BYI 02960 for the nominal treatments of 111 and 1000 ppm. The measured amounts of BYI 02960 for the treatments were 90.8 and 940 ppm, respectively. The freezer stability values were compared to the Week 1 analysis on 03-Jun-10 and percent initial values were determined as 80 % and 108 % for the 111- and 1000- ppm treatments, respectively.

Room stability samples remained for seven days under study room conditions and were analyzed for BYI 02960 for the nominal treatments of 111 and 1000 ppm. The measured amounts of BYI 02960 for the treatments were 130 and 916 ppm, respectively. The stability sample values were compared to the Week 1 analysis on 03-Jun-10 and percent initial values were determined as 114 % and 106 % for the 111 and 1000ppm treatments, respectively.

Results from analytical measurements are summarised as follows:

Table: Measured diet concentrations of BYI 02960

Nominal Treatment [ppm]	**Week 1**	**Week 5**	**Week 10**	**Week 15**	**Week 20**	**Mean Measured Values (±SD)**	**Percent of Nominal**
Control	<LOQ [a]	<LOQ [a]	<LOQ [a]	<LOQ [a]	<LOQ [a]	--	--
111	114	106	90.6	117	109	107 (10.3)	96%
333	290	283	334	331	272	302 (28.6)	91%
1000	868	997	1142	1008	979	999 (97.6)	100%

[a] Limit Of Quantitation (LOQ) = 10 ppm

Table: Calculation of the daily dietary dose of BYI 02960 based on 23-week food consumption

Measured concentration [ppm]	**Body weight at randomization** [g]	**Body weight at termination** [g]	**Mean body weight** [kg]	**Food consumption** [kg feed/bird/day]	**Daily dietary dose** [mg a.i./kg b.w./day]
107	198	208	0.203	0.0262	14
302	199	224	0.212	0.0283	40
999	197	136	0.167	0.0257	154

Table: Homogeneity and analytical verification of concentrations of BYI 02960

Nominal Treatment [ppm]	Homogeneity in diet		Verification of concentrations in diet (% recovery)				
	mean	% cv	week 1	week 5	week 10	week 15	week 20
111	97.1	10	102	96	82	106	98
333			87	85	100	99	82
1000	855	8	87	100	114	101	98

D. Validity Criteria

The control mortality was less than 10%. Measured concentrations of the test item in the feed were above 80% of nominal. The shell thickness of eggs from the controls was above the species-specific threshold. The average number of 14-day-old survivors per hen in the controls was above the species-specific threshold.

E. Biological Endpoints Derived

Exposure of Northern Bobwhite Quail (*Colinus virginianus*) to BYI 02960 prior to and during their reproduction resulted in clear effects on parental survival, body weight and health at the top dose level of 1000 ppm. The number of eggs laid by the birds at this top dose level was significantly reduced, resulting in a reduced number of hatchlings and 14-d survivors. No treatment related, statistically and biologically relevant effects were observed at the low (111 ppm) and the mid dose (333 ppm) treatment levels. Therefore, the following biological endpoints can be derived from the study:

as concentration in feed:	(nominal concentrations)
NOAEL	333 ppm (mg a.i./kg feed
LOAEL	1000 ppm (mg a.i./kg feed)
as concentration in feed:	(based on measured concentrations)
NOAEL	302 ppm (mg a.i./kg feed)
LOAEL	999 ppm (mg a.i./kg feed)
as daily dietary dose:	(based on measured concentrations)
NOAEL	40 mg a.i./kg bw/day
LOAEL	154 mg a.i./kg bw/day

CONCLUSION

The effect of BYI 02960 on the reproduction of Northern Bobwhite Quail (*Colinus virginianus*) after dietary exposure can be quantified as a NOAEL of 302 mg a.i./kg feed (ppm) which corresponds to an achieved daily dietary dose of 40 mg a.i./kg bw/day.

IIA 8.2 Fish toxicity

Fish testing was performed following the recommendations given in the EU Guidance Document on Aquatic Ecotoxicology (Sanco/3268/2001 Oct. 2002) and to fulfil US, Canadian and other country specific data requirements.

In order to profile BYI 02960 for its acute toxicity, and to address requirements in different regions, rainbow trout, fathead minnow and carp were tested. A saltwater fish, sheepshead minnow, was also evaluated for its sensitivity against BYI 02960 fulfilling the data requirements for US and Canada for plant protection products.

Rainbow trout was selected for further testing of metabolites and the formulation BYI 02960 SL 200 G. Although BYI 02960 is practically non-toxic to fish on the acute basis, rainbow trout was selected due to the known intrinsic sensitivity of this fish species. However, every effort was undertaken to limit testing on the vertebrates for metabolites by taking into consideration the information available on other taxa, particularly within the group of invertebrates.

BYI 02960 degrades in soil to the major metabolite 6-chloronicotinic acid (6-CNA maximum formation ca. 17%) and difluoro acetic acid (DFA, maximum formation ca. 34%). Additionally DFA was formed in water/sediment systems (in the dark) at maximum amounts of ca. 7%. In aquatic systems, under the influence of photolysis, BYI 02960 degrades to form two major degradates BYI 02960-succinamide (maximum formation ca. 40%) and BYI 02960-azabicyclosuccinamide (maximum formation ca. 26%).

The testing strategy for the metabolites was therefore as described below

The aquatic photo-degradates BYI 02960-succinamide and BYI 02960-azabicyclosuccinamide reflect a similar level of structural complexity to the parent substance and were therefore considered to be biologically similar to BYI 02960. Hence, the testing of the photodegradates was focussed on the aquatic invertebrates since BYI 02960 was not acutely toxic to fish. BYI 02960 succinamide was tested on daphnia, chironomids and algae, considering the results of the testing of BYI 02960-succinamide, testing of BYI 02960-azasuccinamide was limited to the most sensitive species *Chironomus riparius.* The toxicity of these metabolites was then compared within the biological profile established for the parent substance.

For the soil metabolites DFA and 6-CNA a different rationale was applied. Both metabolites are a result of a reduction in molecule size and complexity due to loss of the pyradifurone moiety and hence may show different toxicity to the parent BYI 02960.

For the metabolite 6-CNA, information was already available from aquatic insects, daphnia, and algae demonstrating no relevant biological activity and testing on fish was therefore not considered necessary.

DFA has been shown to have no insecticidal activity. DFA is a very strong acid that in aquatic testing cannot be easily handled and was therefore tested as the sodium salt, knowing that dissociation will occur in the aqueous environment. DFA was tested on fish at a limit concentration of 10 mg/L to establish a satisfactory level of no concern. Additionally DFA was tested on aquatic insects, daphnia and algae.

Long-term/chronic studies are required by directive 1107/2009 based upon a half-life trigger value of 2 days in water-sediment studies. The half-life of BYI 02960 is above the given trigger and therefore information on the long-term toxicity to fish is required. An Early-Life-Stage (ELS) Test, required by US EPA, addresses relevant and sensitive chronic endpoints. Because BYI 02960, based upon its low log Pow, should not accumulate in aquatic organisms, is not expected to be highly persistent in the aquatic environment and is practically non-toxic to all fish species tested in acute studies, it was considered that a Fish-Full-Life-Cycle (FFLC) test would not add additional relevant information in addition to that provided by the an Early-Life-Stage Test. It is considered that the ELS addresses potentially sensitive endpoints for fish under conditions that are worst-case for the expected exposure to BYI 02960 under field conditions of use.

IIA 8.2.1 Acute toxicity of the active substance to fish

IIA 8.2.1.1 Rainbow trout (Oncorhynchus mykiss)

Report:	**KIIA 8.2.1.1/01; Matlock, D. & Lam, C.V. (2010)**
Title:	Acute Toxicity of BYI 02960 Technical to the Rainbow Trout (*Oncorhynchus mykiss*) Under Static Conditions
Report No:	EBRVP041
Document No:	M-390611-01-1
Guidelines:	**OECD Test Guideline 203** **EPA-FIFRA § 72-1** **OPPTS 850.1075**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** **Some data (screening of water for contaminants) was not performed under GLP as stated in study report**

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of BYI 02960 technical (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2%) to rainbow trout (*Oncorhynchus mykiss*).

Rainbow trout (groups of 10 per treatment level) were exposed under static conditions over a period of 96 hours. There was one replicate of 10 fish each in the controls and the toxicant levels. The following nominal (mean measured) concentrations were included in the study: control, solvent control, 5.00 (3.52), 10.0 (8.31), 20.0 (19.0), 40.0 (35.1) and 80.0 (74.2) mg a.i./L.

Mean measured recoveries ranged from 70 to 95% of nominal values. Results are based on mean measured test concentrations.

Survival (mortality) and sublethal behavioural effects (wet weight, biomass loading, length) were used to determine the endpoints.

Following 96 hours of exposure there were no mortalities or sublethal effects observed at any test concentration or the controls. Therefore, the 96-hr-LC_{50} was determined to be > 74.2 mg a.i./L (practical limit of solubility) and the 96-hr-NOEC was 74.2 mg a.i./L. Therefore, the 96-hr-LC_{50} was determined to be > 70.5 mg a.i./L (practical limit of solubility) and the 96-hr-NOEC was 70.5 mg a.i./L.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difluoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Storage conditions:	Room temperature
Water solubility:	Approximately 80 mg a.i./L under test conditions

2. Test solutions

Vehicle:	Dimethylformamide (DMF)
Concentration of vehicle:	0.1 mL/L
Method of preparation:	Sonicated for 3 hours and 15 minutes
Controls:	Water control and solvent control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Oncorhynchus mykiss*
Common name:	Rainbow trout
Lot Nr.:	TL111209
Source:	Troutlodge, Sumner, WA
Feeding during test:	None
Length at test start:	47.9 ± 3.2 mm (range: 43.5 to 53.5 mm)
Weight at test start:	0.79 ± 0.15 g (range: 0.58 to 1.01 g)
Static loading:	0.26 g fish/L
Maintenance of culture:	
Temperature:	11.7 to 12.7°C
Photoperiod:	16/8 hour light/dark photoperiod
Food:	Trout Chow
Period of maintenance prior to study initiation:	At least 14 days
Mortality during acclimation period:	No mortalities during 48 hours prior to testing, no treatments for disease

B. Study design and methods

1. In life dates December 7 to 11, 2009

2. Design of biological test

Oncorhynchus mykiss were exposed to BYI 02960; (purity 96.2%) in a static system over a period of 96 hours. Nominal concentrations were 5.00, 10.0, 20.0, 40.0 and 80.0 mg a.i./L. In addition a water control and a solvent control were tested. Each vessel (glass aquaria; 38 L (49.5 x 25.4 x 30.5 cm)) filled with 30 L soft processed water (reverse osmosis water blended with spring water) served as one replicate, 10 fish were used per replicate. Length of fish at test start was 47.9 ± 3.2 mm (range: 43.5 to

53.5 mm). Body weight of fish at test start was 0.79 ± 0.15 g (range: 0.58 to 1.01 g). The static biological loading was 0.26 g fish/L. The test was conducted with one replicate per treatment level.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0 and 4 from all concentrations. LC-MS/MS was used as analytical method. The limit of quantification (LOQ) was 0.62 mg a.i./L. The range of linearity was 0.001 to 0.5 mg/L.

4. Observation and measurements

Mortality of fishes, intoxication symptoms and physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

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

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	11.5 to 12.5 °C (mean: 11.6 °C)
pH:	7.6 to 8.2
Dissolved oxygen (mg/L):	9.5 to 10.7
Dissolved oxygen (% saturation):	86 to 99
Photoperiod:	16 hours light / 8 hours dark
Light source	Cool white fluorescents
Light/dark transition period:	30 minutes
Light intensity:	564 to 1045 lux (mean: 859 lux)
Hardness:	48 to 60 mg $CaCO_3$/L (mean: 53 mg $CaCO_3$/L)
Alkalinity:	45 to 47 mg/L (mean: 46 mg/L)
Conductivity:	151 to 156 µmhos/cm (mean: 153 µmhos/cm)

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of 3.52, 8.31, 19.0, 35.1 and 74.2 mg a.i./L. Mean measured recoveries ranged from 70 to 95% of nominal values. Although the recovery was below 80% of nominal at the lowest test level (range: 69% -72%, mean: 70%), the variance in the measured concentrations was appropriate and reliable. This had no impact on the outcome of the study. Results are based on mean measured test concentrations. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960

Nominal Test Concentration (mg a.i./L)	Day 0 (New)		Day 4 (Old)		Mean Measured (mg a.i./L)	Mean SD	Mean % Nominal
	Measured (mg a.i./L)	% Nominal	Measured (mg a.i./L)	% Nominal			
Control	<0.62	NA	<0.62	NA	< 0.62	NA	NA
Solvent Control	<0.62	NA	<0.62	NA	< 0.62	NA	NA
5	3.58	72	3.46	69	3.52	0.08	70
10	8.22	82	8.4	84	8.31	0.13	83
20	19.8	99	18.3	91	19.0	1.09	95
40	36.2	90	34.0	85	35.1	1.5	88
80	75.2	94	73.3	92	74.2	1.3	93

Limit of quantification = (0.62 mg a.i./L) NA = Not Applicable

C. Biological Findings

No mortality was observed, as presented below, and no sublethal behavioural changes were observed.

Table: Effect of BYI 02960 on mortality of *Oncorhynchus mykiss*

Exposure time	24 h		48 h		72 h		96 h	
Test level (mg a.i. / L)	no. of dead	% dead	no. of dead	% dead	no. of dead	% dead	no. of dead	% dead
Control	0	0	0	0	0	0	0	0
solvent control	0	0	0	0	0	0	0	0
3.52	0	0	0	0	0	0	0	0
8.31	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0
35.1	0	0	0	0	0	0	0	0
74.2	0	0	0	0	0	0	0	0

D. Validity Criteria

The validity criterion of control mortality less than 10% is fulfilled. The validity criterion of oxygen saturation above 60% was fulfilled.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

96-hour-figures:

LC_{50}:	> 74.2 mg a.i./L
Lowest concentration with effect (LOEC):	> 74.2 mg a.i./L
Highest concentration with no effect (NOEC):	74.2 mg a.i./L
Highest concentration with no mortality (NOLEC):	74.2 mg a.i./L

CONCLUSION

The acute effect of BYI 02960 on rainbow trout (*Oncorhynchus mykiss*) can be quantified as a 96-hour-LC_{50} of > 74.2 mg a.i./L. The highest concentration with no observed mortality and no sublethal behavioural effects can be set to 74.2 mg a.i./L, the highest concentration tested.

Effects on amphibians

Data on amphibians are not required in Europe under Regulation (EC) 1107/2009; however, for regional authorisations an acute toxicity test with the frog *Xenopus laevis* was undertaken.

Report:	**KIIA 8.2.1.1/02; [redacted], C.S. & [redacted] C.V. (2011)**
Title:	Acute Toxicity of BYI 02960 to *Xenopus laevis* Under Static Conditions
Report No.:	EBRVP187
Document No.:	M-417822-01-1
Guidelines:	**USEPA, OPPTS Guideline 850.1075** **USEPA-FIFRA, 40 CFR, Part 158, Guideline No. 72-1** **OECD Guideline 203**
Deviations:	**Guidelines adapted to tadpole testing**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of BYI 02960 (Origin Batch No: 2009-000239; Batch code: BYI 02960-01-03; TOX 08508-01; Purity 96.2% w/w) on survival of African clawed frog tadpoles (*Xenopus laevis*).

Tadpoles were exposed in a static system over a period of 48 hours to a nominal concentration of 80 mg a.i./L corresponding to a measured concentration of 74 mg a.i./L). In addition, a water control and solvent control was tested. Mortality and sublethal behavioural effects were determined. Based on analytical findings the biological endpoints are reported as nominal figures.

The 48-hour-LC_{50} was > 80 mg a.i./L.

MATERIAL AND METHODS

A. Materials

1. Test material

Tested material:	BYI 02960
Type of test material:	Active ingredient, technical
Chemical state and description:	Beige powder
Specification No.:	102000022313
Origin Batch No.:	2009-000239 (Batch code: BYI 02960-01-03)
Sample description:	TOX 08508-01
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difluoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Storage conditions:	Expiry date: 14.01.2013, when stored at
Water solubility:	Approximately 80 mg a.i./L under test conditions

2. Test solutions

Vehicle:	DMF (Dimethylformamide)
Concentration of vehicle:	0.1 mL/L
Method of preparation:	Sonicated approximately 2.5 hours and stirred for approximately 12 hours
Controls:	Water control and solvent control

3. Test organisms

Species:	*Xenopus laevis*
Common name:	African clawed frog tadpoles
Lot Nr.:	NXL040811
Source:	Nasco, Fort Atkinson, WI
Length at test start:	15.0 ± 0.97 mm (range 14.0 to 17.0 mm)
Maintenance of culture:	
Temperature:	21.5 to 21.9°C
Photoperiod:	16/8 hour light/dark photoperiod
Food:	Frog brittle (liquid suspension)
Period of maintenance prior to study initiation:	4 days
Mortality during acclimatization period:	Mortalities less than 5% during holding period, no treatments for disease

B. Study design and methods

1. In life dates April 12 to 14, 2011

2. Design of biological test

Xenopus laevis were exposed to BYI 02960 (purity 96.2%) in a static system over a period of 48 hours. Nominal concentration was 80 mg a.i./L corresponding to a measured concentration of 74 mg a.i./L. In addition a water control and solvent control was tested. Each vessel (glass aquaria; 8.4 L) served as one replicate filled with 7 L hard processed water (reverse osmosis water blended with spring water). Ten tadpoles were used per replicate. Length of the tadpoles at test start was 15.0 ± 0.97 mm (range 14.0 to 17.0 mm). The test was conducted with 3 replicates per treatment level.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0 and 2 from all concentrations. LC/MS/MS was used as analytical method. The limit of quantification (LOQ) was 8.0 mg a.i./L. The range of linearity was 0.005 to 0.5 mg/L.

4. Observation and measurements

Mortality of tadpoles, intoxication symptoms and physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

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

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	Mean: 21.8°C (range: 21.5 to 22.0 °C)
pH:	8.4 to 8.6
Dissolved oxygen (mg/L):	8.3 to 8.6 mg/L
Dissolved oxygen (% saturation):	91 to 95%
Photoperiod:	16 hours light / 8 hours dark
Light source	Cool white fluorescents
Light/dark transition period:	30 minutes
Light intensity:	739 to 1040 lux (mean 923 lux)
Hardness:	172 to 180 mg/L
Alkalinity:	142 to 148 mg/L
Conductivity:	456 to 482 µmhos/cm

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of mean 92% of nominal concentrations (range 81 to 104%) calculated as arithmetic mean. Biological results are reported as nominal. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960

Nominal concentration	Measured Concentration (average of 2 detections)				Percent of nominal
	on day 0		on day 2		
	mg a.i./L	% of nominal	mg a.i./L	% of nominal	
Control	< 8.0	-	< 8.0	-	-
Solvent control	< 8.0	-	< 8.0	-	-
80 mg a.i. / L	64.7	81	82.9	104	**92%**

C. Biological Findings

No mortality was observed as listed below.

Table: Effect of BYI 02960 on mortality of *Xenopus laevis*

	Mortality					
Exposure time	**6 h**		**24 h**		**48 h**	
Treatment [mg a.i./L]	**no. of dead**	**% dead**	**no. of dead**	**% dead**	**no. of dead**	**% dead**
Control	0	0	0	0	0	0
solvent control	0	0	0	0	0	0
80	0	0	0	0	0	0

No sublethal behavioural changes were observed.

D. Validity Criteria

The validity criteria according to OECD 203 for control mortality (less than 10%) and oxygen saturation (above 60%) are fulfilled.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

48-hour-figures:

LC_{50}:	**> 80 mg a.i./L**
Highest concentration with no effect (NOEC):	80 mg a.i./L
Lowest concentration with effect (LOEC):	> 80 mg a.i./L

CONCLUSION

The acute effect of BYI 02960 on African clawed frog tadpoles (*Xenopus laevis*) can be quantified as a 48-hour-LC_{50} of > 80 mg a.i./L, the highest level tested.

IIA 8.2.1.2 Warm water fish species

Report:	**KIIA 8.2.1.2/01; Matlock, D. & Lam, C.V. (2010)**
Title:	Acute Toxicity of BYI 02960 Technical to the Fathead Minnow (*Pimephales promelas*) Under Static Conditions
Report No:	EBRVP035
Document No:	M-392560-01-1
Guidelines:	**OECD Test Guideline 203** **EPA-FIFRA § 72-1** **OPPTS 850.1075**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** **Some data (screening analysis of water) was not collected in accordance with GLP as described in the study report**

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of BYI 02960 technical (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2%) to fathead minnow (*Pimephales promelas*).

Fathead minnow (groups of 10 per treatment level) were exposed under static conditions over a period of 96 hours. There was one replicate of 10 fish each in the controls and the toxicant levels. The following nominal (mean measured) concentrations were included in the study: control, solvent control, 5.00 (4.29), 10.0 (9.00), 20.0 (19.4), 40.0 (34.3) and 80.0 (70.5) mg a.i./L. Mean measured recoveries ranged from 86 to 97% of nominal values. Results are based on mean measured test concentrations.

Survival (mortality) and sublethal behavioural effects (wet weight, biomass loading, length) were used to determine the endpoints.

Following 96 hours of exposure there were no mortalities or sublethal effects observed at any test concentration or in the controls. The 96-hour-LC_{50} was > 70.5 mg a.i./L , the 96-hour-NOEC was determined to be 70.5 mg a.i./L.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difluoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Storage conditions:	Room temperature
Water solubility:	Approximately 80 mg a.i./L under test conditions

2. Test solutions

Vehicle:	Dimethylformamide (DMF)
Concentration of vehicle:	0.1 mL/L
Method of preparation:	Sonicated for 70 minutes
Controls:	Water control and solvent control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Pimephales promelas*
Common name:	Fathead minnow
Lot Nr.:	ABS101409
Source:	Aquatic Bio Systems, Inc., Fort Collins, CO
Feeding during test:	None
Length at test start:	45.7 ± 2.5 mm (range: 42.0 to 49.0 mm)
Weight at test start:	0.85 ± 0.14 g (0.68 to 1.06 g)
Static loading:	0.28 g fish/L
Maintenance of culture:	
Temperature:	21.8 to 22.9°C
Photoperiod:	16/8 hour light/dark photoperiod
Food:	Tetramin flake food and live brine shrimp
Period of maintenance prior to study initiation:	At least 14 days
Mortality during acclimation period:	No mortalities during 48 hours prior to testing, no treatments for disease

B. Study design and methods

1. In life dates November 16 to 20, 2009

2. Design of biological test

Pimephales promelas were exposed to BYI 02960 (purity 96.2%) in a static system over a period of 96 hours. Nominal concentrations were 5.00, 10.0, 20.0, 40.0 and 80.0 mg a.i./L, respectively.

In addition, a water control and solvent control was tested. Each vessel (glass aquaria; 38 L; 49.5 x 25.4 x 30.5 cm) served as one replicate filled with 30 L soft processed water (reverse osmosis water blended with spring water). 10 fish were used per replicate, one replicate was used per treatment level.

Length of fish at test start was 45.7 ± 2.5 mm (range: 42.0 to 49.0 mm). Body weight of fishes at test start was 0.85 ± 0.14 g (0.68 to 1.06 g). The static biological loading was 0.28 g fish/L.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0 and 4 from all concentrations. LC-MS/MS was used as analytical method. The limit of quantification (LOQ) was 0.62 mg a.i./L. The range of linearity was 0.001 to 0.5 mg/L.

4. Observation and measurements

Mortality of fishes, intoxication symptoms and physical-chemical water parameters were assessed.

5. Statistical analysis

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

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	22.2 to 22.6 °C (mean: 22.4 °C)
pH:	7.5 to 8.0
Dissolved oxygen (mg/L):	6.1 to 8.5
Dissolved oxygen (% saturation):	71 to 97
Photoperiod:	16 hours light / 8 hours dark
Light source	Cool white fluorescents
Light/dark transition period:	30 minutes
Light intensity:	637 to 1020 lux (mean: 829 lux)
Hardness:	mean: 52 mg/L(range: 48 to 54 mg $CaCO_3$/L)
Alkalinity:	mean 48 mg/L (range: 42 to 53 mg/L)
Conductivity:	mean 157 µmhos/cm (range: 150 to 165 µmhos/cm)

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of 4.29, 9.00, 19.4, 34.3 and 70.5 mg a.i./L, respectively; corresponding to 86 to 97% of nominal, calculated as arithmetic mean. Biological results are reported as mean measured concentrations. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960

Nominal Test Conc (mg a.i./L)	Day 0 (New)		Day 4 (Old)		Mean Measured (mg a.i./L)	Mean Standard Deviation	Mean % Nominal
	Measured (mg a.i./L)	% Nominal	Measured (mg a.i./L)	% Nominal			
Control	<0.62	NA	<0.62	NA	< 0.62	NA	NA
Solvent Control	<0.62*	NA	<0.62	NA	< 0.62	NA	NA
5	4.32	86	4.26	85	4.29	0.04	86
10	8.66	87	9.34	93	9	0.48	90
20	19.2	96	19.6	98	19.4	0.3	97
40	31.8	80	36.8	92	34.3	3.5	86
80	68.5	86	72.5	91	70.5	2.82	88

NA = Not Applicable

* Test solution analysis on day 0 showed a detection of BYI 02960 in the solvent control sample at a level of 0.96 mg a.i./L. Analysis of the same solution on Day 1 and Day 4 were below the LOQ (< 0.62 mg a.i./L). This was an acceptable result and indicated that the test vessel was not contaminated. The detection on day 0 was most likely the result of a sample handling error.

C. Biological Findings

Mortality was observed as listed below:

Table: Effect of BYI 02960 on mortality of *Pimephales promelas*

Exposure time	24 h		48 h		72 h		96 h	
Test level mg a.i. / L	no. of dead	% dead	no. of dead	% dead	no. of dead	% dead	no. of dead	% dead
Control	0	0	0	0	0	0	0	0
solvent control	0	0	0	0	0	0	0	0
4.29	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0
19.4	0	0	0	0	0	0	0	0
34.3	0	0	0	0	0	0	0	0
70.5	0	0	0	0	0	0	0	0

No sublethal behavioural changes were observed.

D. Validity Criteria

The validity criterion of control mortality less than 10% is fulfilled. The validity criterion of oxygen saturation above 60% is fulfilled.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

96-hour-figures:

LC_{50}:	> 70.5 mg a.i./L (practical limit of solubility)
Lowest concentration with effect (LOEC):	> 70.5 mg a.i./L
Highest concentration with no effect (NOEC):	70.5 mg a.i./L
Highest concentration with no mortality (NOLEC):	70.5 mg a.i./L

CONCLUSION

The acute effect of BYI 02960 on fathead minnow (*Pimephales promelas*) can be quantified as a 96-hour-LC_{50} of > 70.5 mg a.i./L. The highest concentration with no observed mortality and no sublethal behavioural effects can be set as 70.5 mg a.i./L. the highest concentration tested.

Report:	**KIIA 8.2.1.2/02; Bruns, E. (2011)**
Title:	Acute toxicity of BYI 02960 (tech.) to fish (*Cyprinus carpio*) under static conditions (limit test)
Report No:	EBRVP186
Document No:	M-420407-01-2
Guidelines:	**OECD Test Guideline No. 203; EU Directive 92/69/EEC, C.1 (1992) EPA-FIFRA § 72-1; OPPTS 850.1075 JMAFF, 12 Nousan No. 8147**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

A limit test at 100 mg a.i./L was performed in order to demonstrate that fish (*Cyprinus carpio*) were not affected by the test item BYI 02960 tech. (Sample description: TOX 08508-01 (Batch ID: 2009-000239); Specification No.: 102000022313; purity 96.2% w/w) at this test level.

Thirty fish (fifteen fish per test vessel I and II) were exposed in a limit test for 96 h under static test conditions to 100 mg a.i./L (nominal) against a water control and a solvent control with further 30 fish. During the test, fish were examined after four hours and then daily for mortalities and signs of poisoning. Within the study the pH-value, the oxygen saturation level and the temperature were measured daily with commercial measurement devices.

The analytical determination of BYI 02960 (in water by HPLC – MS / MS) revealed mean recovery values of 101% to 108% of nominal over the whole testing period of 96 hours at the limit test concentration of 100 mg a.i./L. Therefore all results are given as nominal values.

Test conditions met all validity criteria, given by the aforementioned guidelines. There were neither any sub-lethal effects nor any mortality in the control group.

The 96-hour-LC_{50} was > 100 mg a.i./L, the 96-hour-NOEC was determined to be 100 mg a.i./L.

The highest concentration which did not result in any mortality within the exposure period (NOLEC) was at 100 mg a.i./L the tested concentration.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-01
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Storage conditions:	Expiry date: 2013-01-14, when stored at +10 to +30°C

2. Test solutions

Vehicle:	Dimethylformamide (DMF)
Concentration of vehicle:	0.1 mL/L
Controls:	Water control
Evidence of undissolved material:	Yes: Test item at surface and precipitation at the bottom

3. Test organisms

Species:	*Cyprinus carpio*
Common name:	Common carp
Lot Nr.:	F 15 / 11 B
Source:	Osage Catfisheries, INC, Osage Beach, Mo, U.S.A.
Length at test start:	5.1 ± 0.6 cm
Weight at test start:	1.7 ± 0.6 g
Static loading:	0.64 g fish/L
Maintenance of culture:	
Photoperiod:	16/8 hour light/dark photoperiod
Food:	Commercial trout food (Inicio (formerly Ecostart 17), BioMar, Denmark)
Period of maintenance prior to study initiation:	At least 14 days
Mortality during acclimatization period:	In the 48 hour acclimation period before testing less than 5 percent of the fish died.
Remarks:	The fish were healthy and no treatments for disease were administered

B. Study design and methods

1. In life dates June 20 to November 3, 2011

2. Design of biological test

Cyprinus carpio were exposed to BYI 02960 (purity 96.2% w/w) in a static system over a period of 96 hours at a nominal concentration of 100 mg a.i./L (limit test). In addition, a water control was tested.

The sensitivity of fish against the reference substance Copper(II)sulfate ($CUSO_4$) was tested separately, resulting in a 96h-LC_{50} of 41.0 µg/L.

The test was conducted with 2 replicates (I and II) per treatment level; 15 fish were used per replicate.

Each vessel (glass aquarium) served as one replicate filled with 40 L reconstituted water prepared by adding salt stock solutions to de-mineralized water.

Mean body length of fish at test start was 5.1 cm. Mean body weight of fish at test start was 1.7 g. The biomass loading was 0.64 g fish/L test medium.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0, 2 and 4 from all concentrations. BYI 02960, purity 99.4% served as analytical standard. HPLC-MS/MS was used as analytical method. The limit of quantification (LOQ) was 0.2173 µg/L. The range of linearity was 0.05 µg/L to 11 µg/L.

4. Observation and measurements

Mortality of fish, intoxication symptoms and physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

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

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	21.1°C to 24.0°C
pH:	6.9 to 7.5
Dissolved oxygen (% saturation):	82 to 112 %
Photoperiod:	16 hours light / 8 hours dark
Hardness:	40 – 60 mg $CaCO_3$/L

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of 101% to 108% of nominal over the whole testing period calculated as arithmetic mean. Biological results are reported as nominal. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960 (rounded values)

Nominal concentration in test item [mg/L]	Measured Concentration in mg a.i./L				Percent of nominal
	on day 0*	on day 2*	on day 4*	Mean of measured concentrations	
Control I	< 0.000217	< 0.000217	< 0.000217	-	-
Control II	< 0.000217	< 0.000217	< 0.000217	-	-
Solvent control I	< 0.000217	< 0.000217	< 0.000217	-	-
Solvent control II	< 0.000217	< 0.000217	< 0.000217	-	-
(104) 100 I	120	104	101	108	108
(104) 100 II	101	104	96.5	101	101

* Average of 2 detections (2 aquaria at each concentration)
I = Replicate 1; II = Replicate 2

C. Biological Findings

No mortality was observed, as presented in the table below, and no sublethal behavioural changes were observed.

Table: Effect of BYI 02960 on mortality of *Cyprinus carpio*

Exposure time	4 h		24 h		48 h		72 h		96 h	
BYI 02960 [mg a.i./L]	**no. of dead**	**% dead**	**no. of dead**	**% dead**	**no. of dead**	**% dead**	**no. of dead**	**% dead**	**no. of dead**	**% dead**
Control I	0	0	0	0	0	0	0	0	**0**	**0**
Control II	0	0	0	0	0	0	0	0	**0**	**0**
Solvent control I	0	0	0	0	0	0	0	0	**0**	**0**
Solvent control II	0	0	0	0	0	0	0	0	**0**	**0**
100 I (108 mm)	0	0	0	0	0	0	0	0	**0**	**0**
100 II (101 mm)	0	0	0	0	0	0	0	0	**0**	**0**

mm = mean measured concentration (mg a.i./L)
I = Replicate 1; II = Replicate 2

D. Validity Criteria

The validity criterion of control mortality less than 10% is fulfilled. Also there was less than 5 % mortality within the 48-hour settling-in period. The validity criteria of oxygen saturation above 60% and pH variation of ≤ 1.0 units are fulfilled.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

96-hour-figures:	
LC_{50}:	**> 100 mg a.i./L** (108 mg a.i./L mean measured)
Lowest concentration with effect (LOEC):	> 100 mg a.i./L (108 mg a.i./L mean measured)
Highest concentration with no effect (NOEC):	≥ 100 mg a.i./L (108 mg a.i./L mean measured)
Highest concentration causing no mortality (NOLEC):	≥ 100 mg a.i./L (108 mg a.i./L mean measured)

CONCLUSION

A limit test at 100 mg BYI 02960/L was conducted with common carp (*Cyprinus carpio*) resulting in a 96-hour-LC_{50} of > 100 mg a.i./L. The highest concentration causing no mortality (NOLEC) and the highest concentration without toxic effects (NOEC) can both be set to ≥ 100 mg a.i./L.

IIA 8.2.1.3 Acute toxicity of metabolites to the more sensitive of fish species

For reasoning of acute toxicity tests for metabolites see introduction in point IIA 8.2 above.

Report:	**KIIA 8.2.1.3/01; Bruns, E. (2011)**
Title:	Acute toxicity of BYI 02960 – succinamide (tech.) to fish (*Oncorhynchus mykiss*) under static conditions (limit test)
Report No:	EBRVP203
Document No:	M-414293-01-2
Guidelines:	**OECD Test Guideline 203** **EU Directive 92/69/EEC, C.1 (1992)** **EPA-FIFRA § 72-1** **OPPTS 850.1075** **JMAFF, 12 Nousan No. 8147**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of the metabolite BYI 02960 – succinamide technical (Sample description: TOX 09343-00 (Batch ID: BCOO 6329-2-10); purity 97.8% w/w) to rainbow trout (*Oncorhynchus mykiss*).

Thirty fish (fifteen fish per test vessel I and II, respectively) were exposed in a limit test to a nominal concentration of 100 mg pure metabolite/L for 96 h under static test conditions against a water control and a solvent control with a further 30 fish each.

During the test, fish were examined after four hours and then daily for mortalities and signs of poisoning.

The analytical determination of BYI 02960 – succinamide (in water by HPLC - UV) revealed a mean recovery of 114% of nominal over the whole testing period of 96 hours at the limit test concentration of 100 mg pure metabolite/L. Therefore all results are given as nominal values.

No mortalities occurred in the water control. In one of the two solvent control replicates 2 fish died. The overall mortality in the solvent control is 6.7%. The overall mortality in the pooled controls is 3.3%. The observed mortalities in the controls still fulfill the validity criteria of the underlying guidelines. No behavioral changes were observed in the remaining 58 control fish.

At the limit test concentration of 100 mg pure metabolite no mortalities and no behavioral changes were observed. Therefore, the 96h-LC_{50} was > 100 mg p.m./L and the NOEC was ≥ 100 mg p.m./L, respectively.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960 – succinamide (BCS-CR74729)
Type of test material:	Substance, technical (pure metabolite)
Chemical state and description:	White powder
Batch No.:	BCOO 6329-2-10
Sample description:	TOX 09343-00
Purity:	97.8% w/w
Stability:	Expiry date: 2011-09-18, when stored at+10 to +30°C

2. Test solutions

Vehicle:	Dimethylformamide (DMF)
Concentration of vehicle:	0.1 mL/L
Controls:	Water control
Evidence of undissolved material:	Slight quantities of test item lying at the bottom

3. Test organisms

Species:	*Oncorhynchus mykiss*
Common name:	Rainbow trout
Lot Nr.:	F 16 / 11
Source:	Dr. Rosengarten, D-49124 Oesede-Georgsmarienhütte; Germany.
Length at test start:	5.2 ± 0.6 cm
Weight at test start:	1.4 ± 0.5 g
Static loading:	0.53 g fish/L
Maintenance of culture:	
Photoperiod:	16/8 hour light/dark photoperiod
Food:	Commercial trout food (Inicio; formerly Ecostart 17, BioMar, Denmark)
Period of maintenance prior to study initiation:	at least 14 days
Mortality during acclimatization period:	In the 48 hour acclimation period before testing less than 5 percent of the fish died.
Remarks:	The fish were healthy and no treatments for disease were administered

B. Study design and methods

1.In life dates July 4 to August 17, 2011

2. Design of biological test

Oncorhynchus mykiss were exposed to BYI 02960 – succinamide (purity 97.8% w/w) in a static system over a period of 96 hours. The nominal concentration was 100 mg p.m./L (limit test). In addition, a water control and a solvent control were tested.

The test was conducted with 2 replicates per treatment level; 15 fish were used per replicate. Each vessel (glass aquaria; 32 x 36 x 38 cm (l x d x h)) filled with 40 L reconstituted water (prepared by adding salt stock solutions to de-mineralized water, conductivity < 0.2 µS/cm) served as one replicate. Length of fish at test start was 5.2 ± 0.6 cm. Body weight of fish at test start was 1.4 ± 0.5 g. The static biological loading was 0.53 g fish/L test medium.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0, 2 and 4 from all concentrations, respectively. BYI 02960-succinamide (reference number MZ 393, batch BCOO 6329, purity 97.8% w/w) served as analytical standard. HPLC-UV was used as analytical method. The limit of quantification (LOQ) was 136 µg/L. The range of linearity was 11 to 2180 µg/L.

4. Observation and measurements

Mortality of fish, intoxication symptoms and physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

No statistical calculations were necessary to determine the LC_{50} for this study.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	11.5°C to 13.0°C
pH:	6.6 to 7.3
Dissolved oxygen:	82 to 98 %
Photoperiod:	16 hours light / 8 hours dark
Hardness:	40 – 60 mg $CaCO_3$/L

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of 114 mg/L calculated as arithmetic mean. Biological results are reported as nominal. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960 – succinamide (rounded values)

Nominal concentration in mg (p.m.)/L	Measured Concentration in mg p.m./L				% of nominal
	on day 0*	on day 2*	on day 4*	Mean of measured concentrations	
Control I	<0.136	<0.136	<0.136	-	-
Control II	<0.136	<0.136	<0.136	-	-
Solvent control c. I	<0.136	<0.136	<0.136	-	-
Solvent control c. II	<0.136	<0.136	<0.136	-	-
100 I	113	115	114	114	114
100 II	115	112	114	114	114

I = Replicate 1; II = Replicate 2

C. Biological Findings

Mortality was observed as listed in the table below:

Table: Effect of BYI 02960 – succinamide on mortality of *Oncorhynchus mykiss*

Exposure time	4 h		24 h		48 h		72 h		96 h	
Test level [mg p.m./L]	no. of dead	% dead	no. of dead	% dead	no. of dead	% dead	no. of dead	% dead	no. of dead	% dead
Control I	0	0	0	0	0	0	0	0	**0**	**0**
Control II	0	0	0	0	0	0	0	0	**0**	**0**
Solvent control c. I	0	0	0	0	1	6.7	2	13.3	**2**	**13.3**
Solvent control c. II	0	0	0	0	0	0	0	0	**0**	**0**
100 I	0	0	0	0	0	0	0	0	**0**	**0**
100 II	0	0	0	0	0	0	0	0	**0**	**0**

I = Replicate 1; II = Replicate 2

No sublethal behavioural changes were observed.

D. Validity Criteria

The test conditions met all validity criteria, given by the mentioned guidelines: Less than 5% mortality within the 48-hour settling-in period and ≤ 10% mortality in the control(s) (or one fish if less than ten are used).

Dissolved oxygen saturation was ≥ 60% throughout the test and pH variation was ≤ 1.0 units.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

96-hour-figures:	
LC_{50}	**> 100 mg p.m./L**
Lowest concentration with effect (LOEC):	> 100 mg p.m./L
Highest concentration with no effect (NOEC):	100 mg p.m./L
Highest concentration causing no mortality (NOLEC)	100 mg p.m./L
100 % mortality	Greater than 100 mg p.m./L

CONCLUSION

The acute effect of BYI 02960 – succinamide on rainbow trout (*Oncorhynchus mykiss*) can be quantified as a 96-hour-LC_{50} of > 100 mg p.m./L (limit test). There were no mortalities or sublethal effects noted at this concentration. The 96-hour-NOEC was ≥ 100 mg p.m./L.

Report:	**KIIA 8.2.1.3/02; Bruns, E. (2011):**
Title:	Acute toxicity of sodium difluoro acetate (BCS AB60481,tech.) to fish (*Oncorhynchus mykiss*) under static conditions (limit test)
Report No:	EBRVP080
Document No:	M-413889-01-2
Guidelines:	**OECD Test Guideline 203** **EU Directive 92/69/EEC, C.1 (1992)** **EPA-FIFRA § 72-1** **OPPTS 850.1075** **JMAFF, 12 Nousan No. 8147**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** Screening of water for contaminants was not conducted according to GLP as described in the study report

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of sodium difluoroacetate (Na-salt of DFA) (Sample description: TOX 08988-01 (Batch ID: BCOO 6092-3-1); code: BCS-AB60481; purity > 99.0% w/w) on survival of rainbow trout (Oncorhynchus mykiss).

Thirty fish (fifteen fish per test vessel I and II) were exposed to a nominal concentration of 10.0 mg test item/L in a limit test for 96 h under static test conditions. In addition a water control with a further 30 fish was tested.

During the test, fish were examined after four hours and then daily for mortalities and signs of poisoning.

The analytical determination of sodium difluoroacetate revealed mean recovery values of 103% to 104% of nominal concentrations over the whole testing period of 96 hours at the limit test concentration of 10.0 mg p.m./L. Therefore all results are given as nominal values.

In this limit test there were no mortalities or sublethal effects noted in the control group or in the dosed group. The 96h-LC50 was determined to be > 10 mg p.m./L, while the NOEC was ≥ 10 mg p.m./L..

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	Sodium difluoro acetate (Na-salt of BYI 02960-DFA)
Type of test material:	Substance, technical
Chemical state and description:	White powder
Code:	BCS-AB60481
Batch number:	BCOO 6092-3-1
Sample description:	TOX 08988-01
Purity:	> 99.0% w/w
Storage conditions:	To be stored +10 to +30°C

2. Test solutions

Vehicle:	None
Controls:	Water control
Evidence of undissolved material:	Turbidity observed at 7.5 mg product/L and above

3. Test organisms

Species:	*Oncorhynchus mykiss*
Common name:	Rainbow trout
Lot Nr.:	F 11 / 10
Source:	Dr. Rosengarten, D-49124 Oesede-Georgsmarienhütte; Germany
Length at test start:	5.0 ± 1.0 cm
Weight at test start:	1.7 ± 0.9 g
Static loading:	0.64 g fish/L
Maintenance of culture:	
Photoperiod:	16/8 hour light/dark photoperiod
Food:	Commercial trout food (Brutfutter Ecostart 17, BioMar, Denmark)
Period of maintenance prior to study initiation:	At least 14 days
Mortality during acclimation period:	In the 48 hour acclimation period before testing less than 5 percent of the fish died.
Remarks:	The fish were healthy and no treatments for disease were administered

B. Study design and methods

1. In life dates December 20, 2010 to February 17, 2011

2. Design of biological test

Oncorhynchus mykiss were exposed to sodium difluoro acetate (Na-salt of BYI 02960-DFA; code: BCS-AB60481; purity > 99.0% w/w) in a static system over a period of 96 hours.

The test was performed as a limit test at 10.0 mg p.m./L. In addition, a water control was tested.

The test was conducted with 2 replicates, consisting of 15 fish each. Each vessel (glass aquaria; 32 x 36 x 38 cm (w x l x h)) served as one replicate filled with 40 L reconstituted water prepared by adding salt stock solutions to de-mineralized water (conductivity <0.2 µS/cm).

Length of fish at test start was 5.0 ± 1.0 cm. Body weight of fish at test start was 1.7 ± 0.9 g. The static biological loading was 0.64 g fish/L test medium.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0, 2 and 4 from all test levels. HPLC-MS/MS was used as analytical method. The limit of quantification (LOQ) was 0.998 µg/L. The range of linearity was 0.11 to 11 µg/L.

4. Observation and measurements

During the test, fish were examined after four hours and then daily for mortalities and signs of poisoning.
Dissolved oxygen, water temperature and pH values were determined daily in each aquarium. Water temperature was additionally measured in the control aquarium and recorded hourly with a data logger. Analytical determinations of the pure metabolite concentrations were made in the test medium at the beginning of the test, after 48h and at test termination.

5. Statistical analysis

No statistical calculations were necessary to determine the LC_{50} for this study.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	11.8°C to 12.3°C
pH:	6.8 to 7.1
Dissolved oxygen:	84 to 99 %
Photoperiod:	16 hours light / 8 hours dark
Hardness:	40 – 60 mg $CaCO_3$/L

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of 103 to 104% of nominal calculated as arithmetic mean. Biological results are reported as nominal concentrations. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of sodium difluoro acetate (rounded values)

Nominal concentration [mg p.m./L]	Measured Concentration (mg p.m./L)				% of nominal
	on day 0*	on day 2*	on day 4*	Mean of measured concentrations	
Control I	< 0.000998	< 0.000998	< 0.000998	-	-
Control II	< 0.000998	< 0.000998	< 0.000998	-	-
10.0 mg/L II	10.4	10.4	10.0	10.3	103
10.0 mg/L I	10.3	10.5	10.4	10.4	104

* Average of 2 detections, I = Replicate 1; II = Replicate 2

C. Biological Findings

No mortality was observed, as listed below, and . n sublethal behavioural changes were observed

Table: Effect of sodium difluoro acetate on mortality of *Oncorhynchus mykiss*

Exposure time	4 h		24 h		48 h		72 h		**96 h**	
Test level	no. of dead	% dead	no. of dead	% dead	no. of dead	% dead	no. of dead	% dead	**no. of dead**	**% dead**
Control I	0	0	0	0	0	0	0	0	**0**	**0**
Control II	0	0	0	0	0	0	0	0	**0**	**0**
10.0 mg/L I	0	0	0	0	0	0	0	0	**0**	**0**
10.0 mg/L II	0	0	0	0	0	0	0	0	**0**	**0**

I = Replicate 1; II = Replicate 2

D. Validity Criteria

The test conditions met all validity criteria, given by the mentioned guidelines:

Less than 5% mortality occurred within the 48-hour settling-in period and ≤ 10% mortality in the control. Dissolved oxygen saturation was ≥ 60% throughout the test and pH variation was ≤ 1.0 units.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

96-hour-figures:

LC_{50}:	**> 10 mg p.m./L**
Highest concentration with no effect (NOEC):	≥ 10 mg p.m./L

CONCLUSION

A limit test at 10.0 mg p.m./L sodium difluoroacetate (BCS-AB60481) resulted in no mortalities to Rainbow trout (*Oncorhynchus mykiss*), therefore the 96h-LC_{50} is greater than 10.0 mg p.m./L. There were no mortalities or sublethal effects noted at this concentration. The NOEC was determined to be ≥ 10 mg p.m./L.

IIA 8.2.2 Chronic toxicity to fish

This point is covered by the points 8.2.3, 8.2.4 and 8.2.5. For explanation see also the introduction under Point 8.2, Fish toxicity .

IIA 8.2.3 Chronic toxicity (28 day exposure) to juvenile fish

Despite the fact that the acute toxicity for fish is far above the EU trigger value of 0.1 mg/L and the potential for bioconcentration in fish is very low, it was decided to perform a "chronic" fish toxicity test. In order to obtain information on longer term toxicity of BYI 02960 to fish an Early Life Stage test was performed as required by US EPA. This test was considered the optimum experiment to address chronic toxicity in fish under exposure conditions being *worst-case* as compared to BYI 02960's environmental exposure estimation, namely a duration of the test that exceeds the exposure duration at field edge and a test that addresses likely most sensitive chronic endpoints in fish such as hatching and growth of juvenile stages. Thus, in presence of the ELS test the 28 day juvenile growth test was not considered necessary.

- Chronic toxicity of metabolites to fish

The toxicity of BYI 02960 metabolites was profiled in different standard species representing different taxonomic groups. The ecotoxicity pattern obtained and the structural characteristics of the metabolites raised no concern for toxicity against fish, hence, testing was limited in order to comply with an aim to limit vertebrate testing whenever possible. In the two cases where metabolites have been subjected to an acute toxicity test in rainbow trout (DFA and BYI 02960-succinamide) there was no adverse effect seen at the maximum tested dose being significantly above the expectable exposure concentrations. Hence considering the generally low toxicity of the parent substance to fish and the observations made on the 2 tested metabolites, further chronic fish tests were not considered justifiable.

IIA 8.2.4 Fish early life stage toxicity test

Report:	**KIIA 8.2.4/01; Matlock, D. & Lam, C.V. (2011)**
Title:	Early Life Stage Toxicity of BYI 02960 Technical to the Fathead Minnow(*Pimephales promelas*) Under Flow-Through Conditions
Report No:	EBRVP033
Document No:	M-409339-01-1
Guidelines:	**OECD Guideline 210 (1992(EPA-FIFRA Guideline 72-4 (a), 1982 OPPTS 850.1400 (1996 draft)**
Deviations:	**None**
GLP	**Yes (certified laboratory)** Screening of water for contaminants was not performed under GLP.

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2%) to fathead minnow (*Pimephales promelas*).

Eggs and fry of *Pimephales promelas* were exposed in a flow-through system over a period of 35 days to nominal concentrations of 0.625, 1.25, 2.50, 5.00 and 10.0 mg a.i./L (corresponding to mean measured concentrations of 0.619, 1.11, 2.05, 4.41 and 8.40 mg a.i./L). In addition, a water control and a solvent control (0.1 mL DMF/L) were tested.

Mean measured recoveries were within the range of 82 to 99% of the nominal concentrations. Hatching rates, sublethal symptoms, fry survival and growth (length and wet and dry weight) were recorded daily. Based on analytical findings the biological endpoints are reported as mean measured figures.

The 35-day exposure to BYI 02960 technical resulted in a NOEC of 4.41 mg a.i./L and a LOEC of 8.40 mg a.i./L based on fry survival.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2%
Storage conditions:	Room temperature
Water solubility:	Approximately 80 mg a.i./L under test conditions

2. Test solutions

Vehicle:	None
Controls:	Water control and solvent control (0.1 mL DMF/L)

3. Test organisms

Species:	*Pimephales promelas*
Common name:	Fathead minnow
Source:	In-house culture
Feeding during test:	Brine shrimp (Artemia salina) starting on Day 4
Developmental stage at test start:	< 24 hours old eggs in the pre-blastula, blastula, and gastrula stages
Static loading:	0.14 g /L (mean wet weight based on controls)
Dynamic loading:	0.041 g/L/day (mean biomass based on controls)
Maintenance of culture:	
Temperature:	24.6 to 25.5°C
Photoperiod:	16 hours light, 8 hours dark
Food:	Tetramin and/or brine shrimp
Breeding tanks:	30 L with 2 males and 5 females
Remarks:	Good health, no disease treatments

B. Study design and methods

1. In life dates August 24 to September 29, 2010

2. Design of biological test

Pimephales promelas were exposed to BYI 02960; (purity 96.2% w/w) in a flow through system over a period of 35 days. Test vessels were dosed via a proportional diluter with a renewal rate of approximately 10 volume turnovers per 24 hours. Nominal concentrations were 0.625, 1.25, 2.50, 5.00 and 10.0 mg a.i./L. In addition, a water control and a solvent control (0.1 mL DMF/L) were tested. Each vessel (glass vessel; 8.4 L) served as one replicate containing one egg cup and filled with 7 L soft processed water (blended spring and reverse osmosis waters). 35 eggs at initiation, thinned to 20 alevin after hatching phase, were used per replicate. Thinning of surplus alevin took place at day 5 (when at least 90% of viable control eggs had hatched), the post-hatch phase started at day 5. The static biological loading was 0.14 g /L (mean wet weight based on controls). The dynamic biological loading

was 0.041 g/L/day (mean biomass based on controls). The test was conducted with 4 replicates per treatment level.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at experimental start, weekly (± 2 days) thereafter including experimental finish from all concentrations. BYI 02960 served as analytical standard. The limit of quantification (LOQ) was 0.06 mg a.i./L.

4. Observation and measurements

Biological data and physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

For each parameter analyzed (mortality, hatchability, growth), the following statistical tests were conducted:

1) Chi-square test to test for normality and Bartlett's test for homogeneity of variance. All data were analyzed without transformations.

2) One way Analysis of Variance (ANOVA) was used to determine if there was a significant difference between the treatment groups and the control.

If the results of the ANOVA showed significant differences (p= 0.05) then the Bonferroni t-test and the William's test (if appropriate) were conducted to identify which treatment group(s) were significantly different from the control groups. The results were used to determine the No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC).

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	24.6 to 25.0 °C (mean: 24.8 °C)
pH:	7.6 to 8.0
Dissolved oxygen (mg/L):	6.3 to 8.1 (mean 7.4)
Dissolved oxygen (% saturation):	76 to 98 (mean: 90)
Photoperiod:	16 hours light, 8 hours dark
Light source	Cool white fluorescents
Light/dark transition period:	30 min
Light intensity:	545 to 999 lux
Hardness:	46 to 54 mg/L as $CaCO_3$ (mean: 49 mg/L as $CaCO_3$)
Alkalinity:	40 to 48 mg/L as $CaCO_3$ (mean: 43 mg/L as $CaCO_3$)
Conductivity:	153 to 163 µmhos/cm (mean 158 µmhos/cm)

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of 0.619, 1.11, 2.05, 4.41 and 8.41 mg a.i./L (82% to 99% of nominal) calculated as arithmetic mean. Biological results are reported as mean measured. Detailed analytical results are presented in the following table:

Table: Nominal and mean measured concentrations of BYI 02960

Nominal concentration [mg a.i./L]	Measured Concentration in mg a.i./L 1)						Mean [mg a.i./L]	SD	% of nominal
	day 0	day 8	day 14 + 15	day 21 + 22	day 28	day 35			
Control	< 0.06	< 0.06	< 0.06	< 0.06	< 0.06	< 0.06	n.a.	n.a.	n.a.
Solvent control	< 0.06	< 0.06	< 0.06	< 0.06	< 0.06	< 0.06	n.a.	n.a.	n.a.
0.625	0.72	0.60	0.59	0.52	0.72	0.70	0.62	0.09	99
1.25	1.22	1.18	0.98	0.95	1.26	1.35	1.11	0.16	88
2.50	2.30	2.20	1.84	1.81	2.31	2.27	2.05	0.24	82
5.00	4.94	4.62	4.07	3.93	5.00	4.78	4.41	0.48	88
10.00	9.03	9.02	7.66	7.79	9.21	9.11	8.41	0.78	84

1) Values given are arithmetic mean of two replicates per day
n.a.= not applicable LOQ = < 0.06 mg a.i./L SD = Standard deviation

C. Biological Findings

Biological parameters were observed as listed below.

Table: Effect of BYI 02960 on early life stage of *Pimephales promelas*

Mean measured concentration [mg a.i./L]	Mean percent hatch		% Alevin survival	% Fry survival	Mean length [mm]	Mean dry weight [mg]
	day 4	day 5	day 5	day 35	day 35	day 35
Control	43.6	80.7	80.0	93.8	21.7	31.6
solvent control	52.1	85.0	82.9	93.8	22.4	33.1
0.619	20.0	80.0	78.6	86.3 +	22.5	34.2
1.11	27.1	83.6	83.6	95.0	22.2	33.8
2.05	65.0	85.7	83.6	97.5	22.6	34.8
4.41	21.4	80.7	80.0	88.8	22.0	33.7
8.40	38.6	83.6	82.9	87.5 *	22.5	36.1

* statistically significant effect (P= 0.05) as compared to pooled controls (William's)
+ statistically significant effect (P= 0.05) as compared to pooled controls (Bonferroni), not considered to be biologically significant

The Bonferroni t-test showed a statistically significant difference at the lowest test concentration (0.619 mg a.i./L) in comparison to pooled controls. However, the difference observed between the control and the lowest treatment group is not considered to be biologically significant because of the lack of a dose-response relationship.

D. Validity Criteria

The overall survival of fertilized eggs in the controls was greater than the species-specific limits given in the guidelines. The oxygen saturation was above 60%. The water temperature did not differ by more than ± 1.5°C between chambers or successive days. Concentrations of test item were within ± 20% of nominal.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

NOEC (overall):	**4.41 mg a.i./L**
LOEC (overall):	**8.40 mg a.i./L**
NOEC (alevin survival day 5):	8.40 mg a.i./L
NOEC (fry survival day 35):	4.41 mg a.i./L
NOEC (percent hatch):	8.40 mg a.i./L
NOEC (time to hatch):	8.40 mg a.i./L
NOEC (growth in terms of length):	8.40 mg a.i./L
NOEC (growth in terms of weight):	8.40 mg a.i./L
NOEC (morphological and behavioural effects):	8.40 mg a.i./L

CONCLUSION

The effect of (BYI 02960) on early life stages of fathead minnow (*Pimephales promelas*) can be quantified as a no observed effect concentration of 4.41 mg a.i./L (based on fry survival day 35)..

IIA 8.2.5 Fish life cycle test

Acute toxicity findings and the observed low long-term toxicity in the ELS test (8.2.4.) for BYI 02960 suggest no serious effects are expected from long term exposure of fish. BYI 02960 is only moderately persistence in water and the low potential for bioconcentration (log P_{ow} < 3.0 combined with a high water solubility of ca. 3.2 g/L) suggest long-term effects based upon accumulation in the fish are not anticipated. Therefore, a full-life-cycle (exposure) test would not be expected to provide additional data relevant for risk assessment in addition to that already established by the ELS test.,

IIA 8.2.6 Bioconcentration potential in fish

IIA 8.2.6.1 Bioconcentration potential of the active substance in fish

BYI 02960 has a log P_{ow} of 1.2, therefore no study is required according to all major worldwide regulatory requirements,

IIA 8.2.6.2 Bioconcentration potential of the metabolites, degr. & react. products

The log P_{ow} of the metabolites of BYI 02960 are all well below 3 and therefore there is no concern for bioconcentration for the metabolites.

BYI 02960 Metabolite	Maximum Log P_{ow}	Reference
BYI 02960-succinamide	0.6 at pH 5	KIIA 7.13/06
BYI 02960-azabicyclosuccinamide NA-salt	-1.3 at pH 5	KIIA 7.13/07
DFA	- 3.1 at pH5	KIIA 7.13/08
6-CNA	1.5 at pH 1.98	KIIA 7.13/09

IIA 8.2.7 Aquatic bioavailability/ biomagnification / depuration

Although BYI 02960 can be expected to be bioavailable, based upon its water solubility, it will not concentrate in biota. It should equilibrate rapidly in the exposed organisms and depuration should also be rapid ,as observed in the mammalian metabolism studies. As such, the risk for biomagnification is considered negligible.

IIA 8.3 Toxicity to aquatic species other than fish, aquatic field tests

Parent compound

BYI 02960 belongs to the chemical class of butenolides. It controls sucking insects with a good selectivity thus avoiding adverse effects on many other non-target organisms. From the pesticidal activity against insects it can be expected that aquatic invertebrates are potentially sensitive organisms in the aquatic environment. As such, all established tests for indicator species were performed to profile BYI 02960's toxicity to invertebrates. Crustaceans are represented by *Daphnia magna* and the salt water species *Americamysis bahia*, a species for which the US-EPA requires testing. The midge *Chironomus riparius* was tested representing aquatic insects, a likely sensitive group within invertebrates. For these species acute and chronic tests are available significantly reducing the uncertainty for potential chronic and population relevant effects. Although it is not a European requirement, the shell deposition test on the marine oyster *Crassostrea virginica* completes the database of BYI 02960's toxicity to aquatic invertebrates.

Metabolites

As previously discussed (see Section 8.2) the testing strategy for the metabolites considered the relationship to the parent structure in determining the testing strategy.

For the aquatic photo-degradates testing was focused on the species which were most sensitive to the parent, while for the soil metabolites DFA and 6-CNA which are less closely related to the parent structure a wider spectrum of testing was performed.

IIA 8.3.1 Acute toxicity to aquatic invertebrates

IIA 8.3.1.1 Acute toxicity (24 and 48 hour) for Daphnia preferably (Daphnia magna)

Report:	**KIIA 8.3.1.1/01; [redacted], C.S. & [redacted] C.V. (2009)**
Title:	Acute Toxicity of BYI 02960 to *Daphnia magna* Under Static Conditions
Report No:	EBRVP032
Document No:	M-357476-01-1
Guidelines:	**OECD Guideline 202** **EPA OPP 72-2** **EPA OPPTS 850.1010**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** **Routine screening of water for contaminants was not performed under GLP.**

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2%) to *Daphnia magna*.

Daphnia magna ((6 replicates of 5) <24 hour old neonates) were exposed in a static system over a period of 48 hours to nominal concentrations of 80 mg a.i./L (corresponding to analytically verified concentrations of 77.6 mg a.i./L). In addition, a water control and a solvent control were tested. There were six replicates of five daphnia each in the control, solvent control and the treatment. This study was run as a limit test at a single treatment concentration at the limit of solubility in the test system, which was determined to be approximately 80 mg a.i./L.

The mean measured recovery of the single treatment level was 97% of the nominal concentration. Results are based on the mean measured test concentration.

Immobility was defined as the inability to swim within 15 seconds after gentle agitation of the test vessel even if the organisms can still move their antennae. Sublethal and behavioral effects were also assessed during the course of the study.

Following 48 hours of exposure neither immobilisation nor sublethal effects were observed at any test level. Therefore, the 48-hour-EC_{50} was determined to be > 77.6 mg a.i./L, the 48-hour-NOEC was 77.6 mg a.i./L.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difluoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2%
Solubility:	Approximately 80 mg a.i./L under test conditions
Storage conditions:	Room temperature

2. Test solutions

Vehicle:	Dimethylformamide (DMF)
Concentration of vehicle:	0.1 mL/L
Controls:	Water control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Daphnia magna*
Common name:	Water flea
Strain:	ABS072908 (Subculture S-121)
Source:	Inhouse culture since JUL 2008; origin from Aquatic Biosystems Inc., Fort Collins, CO
Age at study initiation:	< 24 hour old neonates
Feeding during test:	None
Maintenance of culture:	
Temperature:	20 ± 2°C.
Photoperiod:	16-hour light, 8-hour dark
Food:	*Pseudokirchneriella subcapitata* and/or blended fish flake food

B. Study design and methods

1. In life dates May 12 to 14, 2009

2. Design of biological test

Daphnia magna (<24 hour old neonates) were exposed to BYI 02960 (purity 96.2% w/w) in a static system over a period of 48 hours. The nominal concentration of the limit test was 80 mg a.i./L.

In addition, a water control and a solvent control (0.1 mL DMF/L) were tested. Each vessel (glass beaker; 250 mL) served as one replicate filled with 200 mL hard water (blended spring and reverse osmosis). Biological loading rate was 40 mL/animal. The test was conducted with 6 replicates per treatment level, consisting of 5 daphnids per replicate.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at 0 hours and 48 hours from all concentrations. The limit of quantification (LOQ) was 0.62 mg a.i./L.

4. Observation and measurements

Immobilisation of daphnids, intoxication symptoms and physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

No statistical calculations were necessary to determine the EC50 for this study. The NOEC and LOEC were empirically determined based upon observation data including lethal and sublethal effects.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	Mean: 19.9°C (range: 19.6 to 20.2 °C)
pH:	8.2
Dissolved oxygen (mg/L):	8.1 to 8.3
Dissolved oxygen (% saturation at 20°C):	89 to 93
Aeration used:	No
Photoperiod:	16 hours light, 8 hours dark
Light source	Cool white fluorescents
Light/dark transition period:	30 minutes
Light intensity:	540 to 600 lux
Hardness:	Mean: 169 mg/L (range: 164 to 178 mg/L)
Alkalinity:	Mean: 136 mg/L (range: 129 to 150 mg/L)
Conductivity:	Mean: 382 µmhos/cm (range: 359 to 426 µmhos/cm)

B. Analytical Findings

Analytical verification of test solutions revealed a mean measured concentration of 77.6 mg a.i./L (97% of nominal) calculated as arithmetic mean. Biological results are reported as mean measured. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960

Nominal Concentration (mg a.i./L)	Day 0 (New)		Day 2 (Old)		Mean Measured (mg a.i./L)	Mean Percent of Nominal
	Measured (mg a.i./L)	Percent Nominal	Measured (mg a.i./L)	Percent Nominal		
80	81	102%	74	92%	77.6	97%

C. Biological Findings

Observations on immobilisation and sublethal intoxication symptoms are listed as follows:

No sublethal behavioural changes were observed.

Table: Effect of BYI 02960 on immobilisation of *Daphnia magna*

Treatment [mg/L]	No. of organisms	Observation period					
		4 hours		24 hours		48 hours	
		# immob.	% mortality	# immob.	% mortality	# immob.	% mortality
Control	30	0	0.00	0	0.00	0	0.00
Solvent control	30	0	0.00	0	0.00	0	0.00
77.6	30	0	0.00	0	0.00	0	0.00

D. Validity Criteria

The validity criterion of control mortality less than 10% is fulfilled. The validity criterion of oxygen saturation above 60% is fulfilled.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

48-hour-figures:

EC_{50}:	**> 77.6 mg a.i./L**
Highest concentration with no effect (NOEC):	77.6 mg a.i./L
Lowest Concentration With an Effect (LOEC)	> 77.6 mg a.i./L

CONCLUSION

The acute effect of BYI 02960 on *Daphnia magna* can be quantified as a 48-hour-EC_{50} of > 77.6 mg a.i./L (limit test). The highest concentration with no observed immobilisation and no sublethal behavioural effects can be set to 77.6 mg a.i./L the limit test concentration.

Report:	**KIIA 8.3.1.1/02; Bruns, E. (2011)**
Title:	Acute toxicity of BCS-AB60481 to the waterflea *Daphnia magna* in a static laboratory test system - LIMIT TEST-
Report No:	EBRVP079
Document No:	M-409326-01-2
Guidelines:	**OECD Guideline 202** **EC Council Regulation No 440/2008, Method C.2 (2008)**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** Screening of dilution water for contaminants was not performed under GLP.

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of sodium difluoroacetate (Na-salt of BYI 02960-DFA) (Sample description: TOX 08988-01; code: BCS-AB60481; purity > 99.0% w/w) on mobility of *Daphnia magna* over 48 hours under static exposure conditions. The study was performed as a limit test at a single concentration of 10 mg pure metabolite/L

Daphnia magna (<24 hour old neonates, 10 replicates of 5 individuals)) were exposed in a static system over a period of 48 hours to nominal concentrations of 10 mg/L (corresponding to analytically verified concentrations of 101% of nominal at the start and 103% of nominal at the end of the exposure period). In addition, a water control was tested.

Immobilisation and sublethal behavioural effects were recorded as endpoints. Based on analytical findings the biological endpoints are reported as nominal figures. Due to the absence of treatment

related effects up to a nominal concentration of 10 mg/L, the EC_{50} for immobilisation after 24 and 48 hours of static exposure was higher than 10 mg BCS-AB60481/L.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	Sodium difluoroacetate (Na-salt of BYI 02960-DFA, BCS-AB60481)
Type of test material:	Substance, technical (pure metabolite)
Chemical state and description:	White powder
Batch number:	BCS-AB60841-01-01
Code:	BCS-AB60481
Sample description:	TOX 08988-01
Purity:	> 99.0% w/w
Storage conditions:	+10 to +30°C

2. Test solutions

Vehicle:	None
Method of preparation:	Ultrasonicated for 3 minutes, followed by 58 minutes of stirring by a magnetic stirrer
Controls:	Water control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Daphnia magna*
Common name:	Water flea
Strain:	Clone "No. 2"; Dr. Bradley, University of Sheffield
Source:	Inhouse culture
Age at study initiation:	< 24 hour old neonates
Feeding during test:	None
Maintenance of culture:	
Temperature:	20 ± 2°C.
Photoperiod:	16-hour light, 8-hour dark
Food:	Living cells of the green alga *Desmodesmus subspicatus*

B. Study design and methods

1. In life dates January 10 to February 15, 2011

2. Design of biological test

Daphnia magna (<24 hour old neonates) were exposed to sodium difluoroacetate (Na-salt of difluoroacetic acid, DFA) (code: BCS-AB60481; purity > 99.0 % w/w) in a static system over a period of 48 hours. Nominal concentrations were 10 mg p.m./L. In addition a water control was tested. Each replicate vessel was a glass beaker (100 mL) filled with 50 mL Elendt M7. Five daphnids were used per replicate. Biological loading rate was 10 mL/animal. The test was conducted with 10 replicates per treatment level.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at 0 and 48 hours from all concentrations. HPLC-MS/MS was used as analytical method. The limit of quantification (LOQ) was 0.998 µg/L. The range of linearity was 0.11 µg/L to 11 µg/L.

4. Observation and measurements

Immobilisation of daphnids, intoxication symptoms and physical-chemical water parameters were assessed as indicated below in the result section.

. Statistical analysis

No statistical calculations were necessary to determine the EC_{50} for this study.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	20.7 to 21.1 °C
pH:	7.9 to 8.0
Dissolved oxygen (mg/L):	8.2 to 8.6
Photoperiod:	16 hours light, 8 hours dark
Light source	Cool white fluorescent bulbs
Light intensity:	Max. 1200 lux
Hardness:	249 mg/L $CaCO_3$
Alkalinity:	53 mg/L $CaCO_3$
Conductivity:	582 µS/cm
acid binding capacity:	1 mL 0.1 N HCl

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of 101% of nominal at the start and 103% of nominal at the end of the exposure period calculated as arithmetic mean. Biological results are reported as nominal. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BCS-AB60481

Nominal Concentration [mg p.m./L]	Day 0 (New)		Day 2 (Old)	
	Measured [mg p.m./L]	% of Nominal	Measured [mg p.m./L]	% of Nominal
Control	< 0.000998	NA	< 0.000998	NA
10	10.1	101	10.3	103

C. Biological Findings

Observations on immobilization and sublethal intoxication symptoms are listed as follows:

Table: Observed immobilization

Nominal Test Concentration [mg p.m./L]	Exposed Daphnids [n]	Immobilised Daphnids	
		24 h [n]	48 h [n]
Control	50	0	0
10	50	0	0

No sublethal behavioural changes were observed.

D. Validity Criteria

The validity criterion of control mortality less than 10% is fulfilled. The validity criterion of oxygen saturation above 60% is fulfilled.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

EC_{50} (48 h)	> 10 mg p.m./L

CONCLUSION

The acute effect of sodium difluoroacetate (Na-salt of difluoroacetic acid) (BCS-AB60481) on *Daphnia magna* can be quantified as EC_{50} > 10 mg p.m./L. Observations on sublethal effects revealed no abnormal behaviour of the exposed daphnids over the entire exposure period of 48 hours.

The following study was performed for the registration of acetamiprid and is the property of Nippon Soda Co. Ltd**,** access to the study has been granted. The study has been evaluated during the Annex I inclusion of acetamiprid, therefore only a very short summary of the study conclusion is repeated here. Note- the test compound IC-0 is identical to 6-CNA.

Report:	**KIIA 8.3.1.1/03; [redacted], A. ; 1997**
Title:	IC-0 - Acute toxicity (48 hours) to daphnids (*Daphnia magna*) under semi-static conditions
Report No:	C007748 (Study report number: SA 97045)
Document No:	M-196569-01-1
Guidelines:	**OECD Guideline No. 202 (1984)** **EEC Directive 92/69 - Method C.2 (1992)** **EPA / FIFRA Guideline 72-2 (1985)**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** **Routine screening of water for contaminants was not performed under GLP.**

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of 6-chloronicotinic acid (6CNA; Nisso Code IC-0; purity 99.7%) to Daphnia magna.

Daphnia magna (<24 hour old neonates) were exposed in a semi-static system over a period of 48 hours to nominal concentrations of 6.3, 12.5, 25.0, 50.0, and 100.0 mg/L (corresponding to analytically verified concentrations of 6.0, 11.9, 23.9, 47.2 and 95.1 mg/L; 94.4 to 95.6 % of nominal). In addition a water control was tested.

Immobilisation was used to determine the endpoints. Based on analytical findings the biological endpoints are reported as mean measured figures. The 48-hour-EC50 was > 95.1 mg/L, the 48-hour-NOEC was determined to be 95.1 mg/L.

IIA 8.3.1.2 Acute toxicity (24/48 h) for representative species of aquatic insects

As the toxicity of BYI 02960 to *Daphnia magna* is low obtaining additional data on a second species within the group of aquatic invertebrates was considered advisable (see also Guidance Document on Aquatic Ecotoxicology, 2002). As aquatic insects were shown to be sensitive to parent molecule, studies were also performed with the metabolites.

The acute tests were performed before the test guideline OECD 235 for the acute immobilization was finalized. As such, some minor deviations from the test guideline may be noticeable However, any minor deviations are considered unlikely to have influenced the outcome of the study.

Report:	**KIIA 8.3.1.2/01; Bruns, E. (2011)**
Title:	Acute toxicity of BYI 02960 (tech.) to larvae of *Chironomus riparius* in a 48 h static laboratory test system
Report No:	EBRVP026
Document No:	M-414739-01-2
Guidelines:	**No specified guideline; study is performed according to general aspects as quoted under OECD Guideline No. 202**
Deviations:	**According to test system**
GLP:	**Yes (certified laboratory)** **Screening of water for contaminants was not performed under GLP as described in the study report**

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2%) on larvae of Chironomus riparius.

Chironomus riparius (first instars, less than 2 to 3 days old, 40 per test concentration) were exposed in a static system over a period of 48 hours to nominal concentrations of 3.125, 6.25, 12.5, 25.0, 50.0 and 100 µg a.i./L. In addition a water control was tested. Four replicates, containing 10 animals each, were tested for each test item concentration and the control.

The analytical findings in all freshly prepared test levels on day 0 in reference to nominal concentrations ranged between 97.0 and 103 % (average 101 %). In aged test levels on day 2 analytical findings ranged between 101 and 107 % (average 104 %) of nominal. Due to the high recoveries at the beginning of the exposure and the analytical findings after 2 days, all results are based on nominal concentrations.

The concentration causing 50% immobility to larvae of Chironomus riparius (48h -EC50) was determined and occurrence of symptoms was recorded and evaluated after 48 hours of exposure.

Following 48 h of exposure 0, 0, 2.5, 2.5, 30, and 85% immobilisation was observed among daphnids exposed to the 3.125, 6.25, 125.5, 25.0, 50.0, and 100 µg a.i./L treatment levels, respectively. No immobilisation occurred in the water control.

Therefore, the 48-hour-EC50 was 61.7 µg a.i./L (95% confidence limits 41.4 to 109 µg a.i./L).

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-01
Content:	96.2% w/w
Storage conditions:	25 ± 5°C

2. Test solutions

Vehicle:	None
Controls:	Water control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Chironomus riparius*
Common name:	1st instar midge larvae
Strain:	From University of Frankfurt
Source:	Inhouse culture since 2006
Age at study initiation:	Less than 2 to 3 days old
Feeding during test:	0.01 mL of an aqueous fish food suspension (50 g Tetra Phyll/L) added at test start
Maintenance of culture:	
Temperature:	20 ± 2°C.
Photoperiod:	16-hour light, 8-hour dark
Food:	The hatched larvae are fed with green algae and an aqueous suspension of a plant material based fish food (Tetra Phyll®).

B. Study design and methods

1. In-life dates: January 14 to June 08, 2011

2. Design of biological test

Chironomus riparius (first instar, less than 2 to 3 days old) were exposed to BYI 02960 (purity 96.2% w/w) in a static system over a period of 48 hours. Nominal concentrations were 3.125, 6.25, 12.5, 25.0, 50.0 and 100 µg a.i./L, respectively. In addition, a water control was tested. Each vessel (glass beakers) served as one replicate filled with 25 mL Elendt-medium (M7). Ten larvae were used per replicate. The test was conducted with 4 replicates per treatment level.

2. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0 and day 2. HPLC-MS/MS was used as analytical method. The limit of quantification (LOQ) was 0.5035 mg/L. The range of linearity was 0.05 µg/L to 11 µg/L.

3. Observation and measurements

Immobilisation of midge larvae, intoxication symptoms and physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

The EC_x values were determined with Probit analysis using linear maximum likelihood regression (Chi^2-Test). NOEC and LOEC were calculated with Fisher's Exact Binomial Test with Bonferroni Correction.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	20.6°C ± 0.2°C
pH:	7.9 to 8.0
Dissolved oxygen (mg/L):	8.2 to 8.4
Dissolved oxygen (% saturation):	90 to 94
Light/dark transition period:	16 h light / 8 h dark
Light intensity:	500-1000 lux

B. Analytical Findings

Analytical verification of test solutions revealed that concentrations in all freshly prepared test levels on day 0 ranged between 97.0 and 103 % of nominal concentrations (average 101 %). In aged test levels on day 2 concentrations ranged between 101 and 107 % (average 104 %) of nominal. Biological results are reported as nominal. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960

Nominal concentrations µg a.i./L	Day 0		Day 2	
	µg a.i./L	% of nominal	µg a.i./L	% of nominal
Control	<0.5035	-	<0.5035	-
3.125	3.03	97	3.15	101
6.25	6.04	97	6.41	103
12.5	12.9	103	13.2	106
25.0	25.2	101	26.7	107
50.0	51.6	103	52.1	104
100	103	103	104	104
	Mean: 101% of nominal		Mean: 104% of nominal	

C. Biological Findings

Observations on immobilisation and sublethal intoxication symptoms are listed as follows:

Table: Effects of BYI 02960 on *Chironomus riparius* at day 2

nominal concentrations (µg a.i./L)	introduced	immobile	% mortality	symptoms observed
Control	40	0	0.0	no
3.125	40	0	0.0	no
6.25	40	0	0.0	no
12.5	40	1	2.5	no
25.0	40	1	2.5	no
50.0	40	12	30*	no
100	40	34	85*	yes**

* significantly different from the control ($\alpha < 0.05$)
** reduced mobility

The sensitivity of the test organisms was tested with 3,5-dichlorophenol as a toxic reference on a regular basis.

D. Validity Criteria

All validity criteria were met. The validity criterion of control mortality less than 10% is fulfilled. The validity criterion of oxygen saturation above 60% is fulfilled.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

Biological endpoints reported as:	Nominal concentration
48-hour-figures:	
EC_{50}:	61.7 µg a.i./L (95% confidence limits 41.4 to 109 µg a.i./L)
NOEC:	25 µg a.i./L[#]
EC_{10}:	30.0 µg a.i./L (95% confidence limits 7.9 to 44.0 µg a.i./L)
EC_{20}:	38.4 µg a.i./L (95% confidence limits 15.3 to 54.5 µg a.i./L)

[#] based on not statistically significant immobilization at the 50 and 100 µg/L test concentrations and absence of statistically significant effects at 12.5 and 25 µg/L..

CONCLUSION

The acute effect of BYI 02960 on *Chironomus riparius* can be quantified as a 48-hour-EC_{50} of 61.7 µg a.i./L (95% confidence limits 41.4 to 109 µg a.i./L). The highest concentration with no observed immobilisation and no sublethal behavioural effects can be set to 25 µg a.i./L.

Report:	**KIIA 8.3.1.2/02; Bruns, E. (2011)**
Title:	Acute toxicity of BYI 02960-succinamide to larvae of *Chironomus riparius* in a 48 h static laboratory test system
Report No:	EBRVP202
Document No:	M-417386-01-2
Guidelines:	**No specified guideline; study is performed according to general aspects as quoted under OECD Guideline No. 202**
Deviations:	**According to test system**
GLP:	**Yes (certified laboratory)** **Screening of water for contaminants was not performed under GLP as described in the study report**

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of BYI 02960 – succinamide (Sample description: TOX 09343-00 (Batch ID: BCOO 6329-2-10); purity 97.8% w/w) to larvae of *Chironomus riparius*.

Chironomus riparius (first instars, less than 2 to 3 days old, 40 per treatment level) were exposed in a static system over a period of 48 hours to nominal concentrations of 26, 36, 51, 71 and 100 mg pure metabolite (p.m.)/L, respectively. In addition, a water control was tested. Four replicates, containing 10 animals each, were tested for the test item concentrations and the control.

The analytical findings in all freshly prepared test levels on day 0 in reference to nominal concentrations ranged between 103 and 106 % (average 104%). In aged test levels on day 2 there were analytical findings between 102 and 107% (average 104%) of nominal. Due to the high recoveries at the beginning of the exposure and the analytical findings after 2 days, all results are based on nominal concentrations.

As the primary endpoint, a concentration causing 50% immobility to larvae of *Chironomus riparius* (48 h -EC_{50}) was determined. Additionally, possible occurrence of symptoms was recorded and evaluated after 48 hours of exposure.

Following 48 h of exposure 0, 0, 2.5, 2.5, and 15% immobilisation was observed among daphnids exposed to the 26, 36, 51, 71 and 100 mg p.m./L nominal treatment levels, respectively. No

immobilisation occurred in the water control groups. There were no other abnormal signs indicative for toxicity observed during the test period of 48 hours in the control or the treatments.

The 48-hour-EC_{50} was > 100 mg p.m./L and the 48-hour-NOEC was determined to be 71 mg p.m./L based on the statistically significant findings at 100 mg/L.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960 – succinamide (BCS-CR74729)
Type of test material:	Substance, technical (pure metabolite)
Chemical state and description:	White powder
Batch No.:	BCOO 6329-2-10
Sample description:	TOX 09343-00
Purity:	97.8% w/w
Storage conditions:	Room temperature from +10°C to +30°C

2. Test solutions

Vehicle:	None
Controls:	Water control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Chironomus riparius*
Common name:	1st instar midge larvae
Strain:	From University of Frankfurt
Source:	Inhouse culture since 2006
Age at study initiation:	Less than 2 to 3 days old
Feeding during test:	0.01 mL of an aqueous fish food suspension (50 g Tetra Phyll/L) added at test start
Maintenance of culture:	
Temperature:	20 ± 2°C.
Photoperiod:	16-hour light, 8-hour dark
Food:	The hatched larvae are fed with green algae and an aqueous suspension of a plant material based fish food (Tetra Phyll®).

B. Study design and methods

1. In life dates: May 27 to August 25, 2011

2. Design of biological test

Chironomus riparius (less than 2 to 3 days old) were exposed to BYI 02960 – succinamide (purity 97.8% w/w) in a static system over a period of 48 hours. Nominal concentrations were 26, 36, 51, 71 and 100 mg pure metabolite (p.m.)/L, respectively. In addition a water control was tested. Each vessel (glass beakers) served as one replicate filled with 25 mL Elendt-medium (M7). 10 larvae were used per replicate. The test was conducted with 4 replicates per treatment level.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0 and day 2 from all concentrations. HPLC-UV was used as analytical method. The limit of quantification (LOQ) was <0.273 mg/L. The range of linearity was 0.011 to 2.180 mg/L.

4. Observation and measurements

Immobilisation of midge larvae, intoxication symptoms and physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

The ECx values were determined with Probit analysis using linear maximum likelihood regression (Chi2-Test). NOEC and LOEC were calculated with William's test. Statistical Software "ToxRat Professional", version 2.10.05 was used.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	20.2°C to 20.7 °C
pH:	7.5 to 7.8
Dissolved oxygen (mg/L):	7.6 to 7.8
Dissolved oxygen (% saturation):	86 to 88
Light/dark transition period:	16 h light / 8 h dark
Light intensity:	mean: 657 Lux
Hardness:	195.8 mg/L $CaCO_3$
Alkalinity:	53.4 mg/L $CaCO_3$

B. Analytical Findings

Analytical verification of test solutions on day 0 revealed measured concentrations between 103 and 106 % (average 104 %) of nominal. In aged test levels on day 2 there were analytical findings between 102 and 107% (average 104 %) of nominal. Biological results are reported as nominal. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960 – succinamide

Nominal concentrations	Day 0		Day 2	
mg p.m./L	mg p.m./L	% of nominal	mg p.m./L	% of nominal
control	<0.273		<0.273	
26	26.8	103	27.7	107
36	37.4	104	37	103
51	52.7	103	52	102
71	73.8	104	75	106
100	105	106	104	104
	Mean: 104% of nominal		Mean: 104% of nominal	

C. Biological Findings

Observations on immobilisation and sublethal intoxication symptoms are listed as follows:

Table: Effects of BYI 02960 – succinamide on *Chironomus riparius* at day 2

nominal concentrations (mg p.m./L)	introduced	immobile	% mortality	symptoms observed
Control	40	0	0.0	no
26	40	0	0.0	no
36	40	0	0.0	no
51	40	1	2.5	no
71	40	1	2.5	no
100	40	6	15*	no

* significantly different from the control (α< 0.05), p.m. = pure metabolite

D. Validity Criteria

The validity criterion of control mortality less than 10% is fulfilled. The validity criterion of oxygen saturation above 60% is fulfilled.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

Biological endpoints reported as:	nominal
48-hour-figures:	
EC_{50}:	**> 100 mg p.m./L**
NOEC:	71 mg p.m./L
EC_{10}:	89.2 mg p.m./L (95% confidence limits 91.5 to 321.9 mg p.m./L)
EC_{20}:	> 100 mg p.m./L

CONCLUSION

The acute effect of BYI 02960 – succinamide on Chironomus riparius can be quantified as a 48-hour-EC50 of > 100 mg p.m./L. The highest concentration with no observed immobilisation and no sublethal behavioural effects (NOEC) can be set to 71 mg p.m./L.

Report:	**KIIA 8.3.1.2/03; Bruns, E. (2012)**
Title:	Acute toxicity of BYI 02960-azabicyclosuccinamide (BCS-CS64875) to larvae of *Chironomus riparius* in a 48 h static laboratory test system
Report No:	EBRVP207
Document No:	M-424404-01-1
Guidelines:	**OECD Guideline 235 (2011)**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** **Screening of water for contaminants was not performed under GLP as described in the study report**

EXECUTIVE SUMMARY

The objective of this 48 hour (h) toxicity test was to evaluate the acute immobilisation to larvae of Chironomus riparius (1st instar) caused by BYI 02960-azabicyclosuccinamide (BCS-CS64875; Sample description: TOX 09342-00 (Batch ID: SES 11732-3-1); purity 48.0 % w/w).

Midge larvae (less than 2 to 3 days old, 30 per test concentration) were exposed in a static system over a period of 48 hours to concentrations of 26, 36, 51, 71 and 100 mg pure metabolite (p.m.)/L. In addition a water control was tested. Six replicates, containing 5 animals each, were tested for the test item concentrations and the control.

The analytical findings in all freshly prepared test levels on day 0 in reference to nominal concentrations ranged between 107 and 112% (average 110%). In aged test levels on day 2 analytical findings ranged between 117 and 120% (average 119%) of nominal. Due to the high recoveries at the beginning of the exposure and the analytical findings after 2 days, all results are based on nominal concentrations.

24 and 48 hours after test initiation, the number of immobilised larvae (animals showing no swimming movements within 15 seconds after slight agitation of the vessel) were recorded for each test vessel separately with a binocular. Additional observations for sub-lethal effects were performed and recorded for each test vessel separately. Significant features of the test medium (e.g. presence of undissolved material) were also noted.

The concentration causing 50 % immobility to larvae of Chironomus riparius (48 h -EC50) was determined. Additionally, possible occurrence of symptoms was recorded and evaluated after 48 hours of exposure.

After 48 hours of exposure 0, 0, 3.3, 3.3, and 20% immobilisation was observed in the 26, 36, 51, 71, and 100 mg p.m./L treatment level, respectively. No immobilisation occurred at control level.

The 48-hour-EC50 was >100 mg p.m./L, the 48-hour-NOEC was determined to be 71 mg p.m./L based on the statistically analysis of the findings.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960-azabicyclosuccinamide Na-Salt
Type of test material:	Substance, technical (metabolite)
Chemical state and description:	Light brown liquid
Batch number:	SES 11732-3-1
Sample description:	TOX 09342-00
Content:	48.0% w/w
Storage conditions:	Approved until 2012-03-22 , storage at 5 ± 5°C

2. Test solutions

Vehicle:	None
Controls:	Water control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Chironomus riparius*
Common name:	1st instar midge larvae
Strain:	From University of Frankfurt
Source:	Inhouse culture since 2006
Age at study initiation:	Less than 2 to 3 days old

Feeding during test:	0.01 mL of an aqueous fish food suspension (50 g Tetra Phyll/L) added at test start
Maintenance of culture:	
Temperature:	20 ± 2°C.
Photoperiod:	16-hour light, 8-hour dark
Food:	Aqueous suspension of a plant material based fish food (Tetra Phyll®).

B. Study design and methods

1. In life dates: 25 November – 12 December 2011

2. Design of biological test

Chironomus riparius (less than 2 to 3 days old) were exposed to BYI 02960-azabicyclosuccinamide (purity 48.0% w/w) in a static system over a period of 48 hours. Nominal concentrations were 0 (control), 26, 36, 51, 71 and 100 mg pure metabolite (p.m.)/L, respectively. Each vessel (glass beaker) served as one replicate filled with 10 mL Elendt-medium (M7). Five larvae were used per replicate.

The test was conducted with 6 replicates per treatment level.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0 and day 2. HPLC-MS/MS was used as analytical method. The limit of quantification (LOQ) was 0.5 mg/L.

4. Observation and measurements

Immobilisation of midge larvae, intoxication symptoms and physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis:

The ECx values were determined with Probit analysis using linear maximum likelihood regression (Chi2-Test). NOEC and LOEC values were calculated with the U-test after Bonferroni-Holm and the William's t-test. Statistical Software "ToxRat Professional", version 2.10.05 was used.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	20.7°C – 21.2°C
pH:	7.8 to 7.9
Dissolved oxygen (mg/L):	7.7 to 7.8
Light/dark transition period:	16 h light/ 8 h dark
Light intensity:	500-1000 lux

B. Analytical Findings

The analytical findings in all freshly prepared test levels on day 0 in reference to nominal concentrations ranged between 107 and 112% (average 110%). In aged test levels on day 2 analytical findings ranged between 117 and 120% (average 119%) of nominal. Biological results are reported as nominal. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960-azabicyclosuccinamide

Nominal concentrations [mg p.m/L]	Day 0		Day 2	
	Analysed conc. mean of two analyses each [mg p.m./L]	% of nominal	Analysed conc. mean of two analyses each [mg p.m./L]	% of nominal
control	< 0.631	-	< 0.631	-
26	27.8	107	30.3	117
36	40.2	112	43.1	120
51	57.0	112	60.1	118
71	79.0	111	85.0	120
100	109	109	120	120
Average		**110**		**119**

C. Biological Findings

Observations on immobilisation and sublethal intoxication symptoms are listed as follows:

Table: Effects of BYI 02960-azabicyclosuccinamide on *Chironomus riparius* at day 2

	Number of larvae			
Treatment [mg p.m./L]	Introduced	Mobile	Immobile	% Immobility
control	30	30	0	0.0
26.0	30	30	0	0.0
36.0	30	30	0	0.0
51.0	30	29	1	3.33
71.0	30	29	1	3.33
100	30	24	6	20.0

The control mortality of 0.0% at 48 h will be compensated using Abbott`s formula.

The sensitivity of the test organisms was tested with 3,5-dichlorophenol as a toxic reference on a regular basis.

D. Validity Criteria

All validity criteria were met: Control mortality was below 15% within 48 hours. Dissolved oxygen was > 3 mg oxygen/L in the control and in all test concentrations.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

Biological endpoints reported as:	nominal
48-hour-figures:	
EC_{50}:	> 100 mg p.m./L
NOEC:	71 mg p.m./L
EC_{10}:	81.27 mg p.m./L (95% CI 60.69 to 111.06 µg p.m./L)
EC_{20}:	102.77 mg p.m./L (95% CI 83.5 to 221.27 µg p.m./L)

CONCLUSION

The acute effect of BYI 02960-azabicyclosuccinamide on *Chironomus riparius* can be quantified as a 48-hour-EC_{50} of >100 mg p.m./L. The highest concentration with no observed immobilisation and no sublethal behavioural effects (NOEC) can be set to 71 mg p.m./L based on statistical analysis.

The following study on the acute toxicity of 6-CNA to *Chironomus tentans* was reviewed during the Annex I inclusion of imidacloprid and in the DAR was concluded to be valid, a short summary of the study findings is provided here.

Report:	**KIIA 8.3.1.2/04; Bowers, L.M. & Lam, C.V. (1998)**
Title:	Acute Toxicity of 6-chloronicotinic acid (a metabolite of Imidacloprid) to *Chironomus tentans* Under Static Renewal Conditions
Report No:	108127
Document No:	M-048448-01-1
Guidelines:	**American Society for Testing and Materials (ASTM, 1987)** **U.S. Environmental Protection Agency (USEPA; 1975, 1982, 1985)**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

A static limit test was conducted to determine the acute toxicity of 6-chloronicotinic acid (Batch number: 9931, lot R: 10017517, 97% a.i.) to *Chironomus tentans.*

Three replicates of ten organisms each were prepared at 1 mg/L test level, and one replicate of ten organisms was prepared for the dilution water control. The test solutions were renewed on Day 2. Solutions of 6-chloronicotinic acid were prepared and analyzed to determine stability after two days at room temperature. Analysis revealed recoveries of 99.2 and 99.2% of Day 0 injections and demonstrated stability of the test item in water over two days.

The primary measure for acute toxicity was mortality. Observations for Chironomus survival, sublethal and behavioral effects were recorded daily.

One larva was found dead in the control level on Day 3. No mortalities or sublethal effects were noted at the 1 mg p.m./L test concentration during the exposure period.

Therefore, the lowest-observed-effect-concentration (LOEC) was greater than 1 mg p.m./L and the no-observed-effect-concentration (NOEC) was 1 mg p.m./L. The 96-hour Chironomus tentans LC50 was greater than 1 mg p.m./L.

IIA 8.3.1.3 Acute toxicity for representative species of aquatic crustaceans

Additional data on crustacean species other than Daphnia magna representing the freshwater biocoenosis are not available. However, the saltwater shrimp Americamysis bahia is also a data requirement under US EPA guidelines and was examined using an acute flow-through test. The data are summarized under point 8.11 “marine species”.

IIA 8.3.1.4 Acute toxicity for repr. species of aquatic gastropod molluscs

In general, gastropod molluscs are considered to be significantly less sensitive than Daphnia and only in cases where direct use on surface water is intended, data for molluscs are required. BYI 02960 will not be applied to surface water; nevertheless, some evidence for the potential risk to additional invertebrates can be obtained from the available shell deposition test on the marine oyster Crassostrea virginica. This test is summarized under point 8.11. “marine species”.

IIA 8.3.2 Chronic toxicity to aquatic invertebrates

As discussed above chronic tests on aquatic invertebrates were performed for BYI 02960 and for the metabolites, BYI 20960-succinamide, DFA and 6-CNA.

IIA 8.3.2.1 Chronic toxicity in Daphnia magna (21-day)

Report:	**KIIA 8.3.2.1/01; Riebschlaeger, T. (2011)**
Title:	Effects of BYI 02960 (tech.) on development and reproductive output of the waterflea *Daphnia magna* in a static-renewal laboratory test system
Report No:	EBRVP209
Document No:	M-414066-01-2
Guidelines:	**OECD-Guideline No. 211** **EC Council Regulation No 440/2008, Method C.20** **U.S. FIFRA72-4 (1982)** **U.S. EPA- OPPTS Guideline 850.1300**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** **Screening of contaminants in water was not performed according to GLP**

EXECUTIVE SUMMARY

The aim of the study was to determine the long-term effects of BYI 02960 (Sample description: TOX 08508-01 (Batch ID: 2009-000239); purity 96.2 % w/w) on development, reproductive capacity and behaviour of *Daphnia magna*.

Daphnia magna (<24 hour old neonates, 10 animals per study group) were exposed in a static-renewal system over a period of 21 days to nominal concentrations of 0.8, 1.6, 3.2, 6.4, 12.8 and 25.6 mg a.i./L. In addition, a water and solvent control were tested. Test solutions were renewed in 48 hours (working days) respectively 72 hours (weekend) intervals.

The accompanying chemical analysis of BYI 02960 in the freshly prepared test solutions at start of the chosen exposure intervals revealed recoveries between 102% and 109% (mean: 105%) of the corresponding nominal concentrations. The corresponding concentrations of the aged test solutions at the end of the exposure intervals ranged between 102% and 117% (mean: 107%) of nominal. All results submitted by this report are related to nominal test concentrations of the active ingredient.

Endpoints measured and recorded were the total living offspring per surviving parental animal, the parental age at first offspring emergence as well as the rate of parental survivors and the survivors' body-length and dry body mass at the end of the study.

No mortalities were observed for any treatment level. However, for some of the other parameters detrimental effects could be observed.

The overall-NOEC was determined to be 3.2 mg a.i./L, based on a reduced parental body length at test termination. The corresponding LOEC is 6.4 mg a.i./L. The "Maximum Acceptable Toxicant Concentration" (MATC), calculated as geometric mean between NOEC and LOEC, is 4.5 mg a.i./L (nominal).

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-01
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Storage conditions:	+10 to +30 °C

2. Test solutions

Vehicle:	Dimethylformamide (DMF)
Concentration of vehicle:	100 µL/L
Controls:	Water and solvent control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Daphnia magna*
Common name:	Water flea
Strain:	Genotype No. 2 ; (Bradley, 1988) equal to type B (Baird 1991)
Source:	Inhouse-culture
Age at study initiation:	< 24 hour old neonates
Feeding during test:	0.2 mg TOC per test vessel with 100 mL (corresponding to 1x108 cells/L)
Maintenance of culture:	
Temperature:	20 ± 2°C
Photoperiod:	16 hours light, 8 hours dark
Food:	Green alga *Desmodesmus subspicatus*

B. Study design and methods

1. In life dates May 4 to July 5, 2011

2. Design of biological test

Daphnia magna (<24 hour old neonates at test start) were exposed to BYI 02960; (purity 96.2% w/w) in a semi-static system over a period of 21 days. Nominal concentrations were 0.8, 1.6, 3.2, 6.4, 12.8 and 25.6 mg a.i./L. In addition a water and solvent control were tested. The vessels were glass beakers (250 mL filled with 100 mL test medium). The test was conducted with 10 replicates of individually exposed animals per treatment.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0 (fresh), day 2 (aged), day 9 (fresh), day 12 (aged), day 19 (fresh) and day 21 (aged) from all concentrations. The LOD and LOQ were 0.02 µg/L and 0.05 µg/L, respectively. The range of linearity was 0.05 to 11 µg/L.

4. Observation and measurements

Immobilisation of daphnids, symptoms, data on reproduction and growth as well as physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

Quantitative data like offspring counts and parental growth measurements were analysed on variance homogeneity (Levene's Test) and normal distribution (Shapiro Wilk's Test) on a 5% level of significance.

Parametric procedures for homogeneous data involved subjecting reproduction data to standard ANOVA. If significant differences among the means were indicated, multiple comparison procedures (e.g. Dunnett's multiple t-test procedure) or, in case of monotonous decrease of responses, adequate step down trend-tests (e.g. Williams multiple sequential t-test procedure) were performed on a 5% level of significance ($p \leq 0.05$), to indicate which treatment groups differed significantly from the control. For non-parametric procedures the Mann-Whitney-Wilcoxon U-test for independent samples was applicable, and alternatively multiple comparison procedure with Bonferroni-Correction was used.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	19.6 to 21.2 °C
pH:	7.7 to 8.1
Dissolved oxygen (mg/L):	8.6 to 9.5
Aeration used:	no
Photoperiod:	16 hrs light; 8 hrs dark
Hardness:	13 to 15 German degrees (dH)
Alkalinity:	3 dH as carbonate hardness
Conductivity:	488 to 563 µmhos/cm

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of 102% to 109% (mean: 105%) in freshly prepared and 102% to 117% (mean: 107%) in aged test solutions of nominal. Thus, biological results are reported as nominal. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960

nominal concentrations (mg a.i./L)	Medium	Measured concentrations (mg a.i./L)						% of nominal
		day 0	day 2	day 9	day 12	day 19	day21	
0.8	(fresh)	0.836		0.847		0.828		104 to 117
	(aged)		0.858		0.939		0.837	
1.6	(fresh)	1.66		1.69		1.64		102 to 111
	(aged)		1.78		1.74		1.63	
3.2	(fresh)	3.31		3.45		3.47		104 to 111
	(aged)		3.4		3.55		3.32	
6.4	(fresh)	6.7		6.74		6.53		102 to 108
	(aged)		6.76		6.74		6.91	
12.8	(fresh)	13.2		13.9		13.6		103 to 110
	(aged)		14		13.6		13.4	
25.6	(fresh)	27.5		27.4		26.9		105 to 108
	(aged)		27.6		27.3		27.1	

C. Biological Findings

Observations on immobilization, reproduction and growth are listed as follows:

Table: Effect of BYI 02960 on survival and reproduction of *Daphnia magna*

treatment level [mg a.i./L]	mean size of adult females [mm ± SD]	mean weight of adult females [mg ± SD]	% adult survival	total offspring per female [n ± SD]	parent age at 1st offspring emergence [days]	% offspring dead or affected
water control	4.5 ± 0.2	0.97 ± 0.1	100	126.1 ± 22.3	9.1	0
solvent control	4.6 ± 0.1	0.98 ± 0.1	100	126.2 ± 17.6	9.4	0
0.8	4.5 ± 0.3	0.97 ± 0.2	100	132.8 ± 31.1	9	0
1.6	4.4 ± 0.1	0.89 ± 0.2	100	122.3 ± 19.7	9.2	0
3.2	4.4 ± 0.1	0.83 ± 0.1	100	121.2 ± 21.9	9.4	0
6.4	4.3 * ± 0.2	0.98 ± 0.2	100	107.7 ± 31.4	9.3	0
12.8	4.2 * ± 0.2	0.83 ± 0.1	100	102.8 *± 24.3	9.6	0
25.6	3.9 * ± 0.2	0.63 *± 0.1	100	85.4 *± 23.3	9.6	0

* significant difference from solvent control by 5%; Williams t-test, $p \leq 0.5$

No sublethal behavioural changes were observed.

D. Validity Criteria

Control mortality was less than 20%. Mean number of live offspring per parent was greater than 60 in the control.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

NOEC (overall):	**3.2 mg a.i./L**
NOEC (immobilisation):	≥ 25.6 mg a.i./L
NOEC (time to first brood):	≥ 25.6 mg a.i./L
NOEC (living neonates/adult):	6.4 mg a.i./L
NOEC (adult body length):	3.2 mg a.i./L
NOEC (adult dry weight):	12.8 mg a.i./L

CONCLUSION

The chronic effect of BYI 02960 on growth and reproduction of *Daphnia magna* can be quantified as an overall-NOEC of 3.2 mg a.i./L. The corresponding LOEC is 6.4 mg a.i./L. Therefore the "Maximum Acceptable Toxicant Concentration" (MATC), calculated as geometric mean between NOEC and LOEC, is 4.5 mg a.i./L (nominally).

Report:	**KIIA 8.3.2.1/02; Riebschlaeger, T. (2012)**
Title:	Influence of BYI02960-succinamide (tech.) on development and reproductive output of the waterflea *Daphnia magna* in a static-renewal laboratory test system
Report No:	EBRVP185
Document No:	M-424700-01-2
Guidelines:	**OECD-Guideline No. 211** **EC Council Regulation No 440/2008, Method C.20** **U.S. FIFRA72-4 (1982)** **U.S. EPA- OPPTS Guideline 850.1300**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** Screening of contaminants in water was not performed according to GLP.

EXECUTIVE SUMMARY

The aim of the study was to determine the long-term effects of BYI 02960-succinamide (Sample description: TOX 09343-01 (Batch ID: BCS-CR74729-01-01); purity 97.8 % w/w) on development, reproductive capacity and behaviour of *Daphnia magna* during 21 days of exposure.

Daphnia magna (<24 hour old neonates, 10 replicates per treatment) were exposed in a static-renewal system over a period of 21 days to nominal concentrations 3.5, 8.1, 18.7, 43.3 and 100 mg pure metabolite (p.m.)/L. In addition, a water control was tested. Test medium renewal took place on Mondays, Wednesdays and Fridays, immediately after new test solutions had been prepared. The test was conducted with 10 replicates of individually exposed animals.

The chemical analysis of BYI02960-succinamide in the freshly prepared test solutions at start of the chosen exposure intervals revealed recoveries between 103% and 111% (mean: 106%) of the corresponding nominal concentrations. The corresponding concentrations of the aged test solutions at the end of the exposure intervals ranged between 104% and 109% (mean: 106%) of nominal. Based on analytical findings the biological endpoints are reported as nominal figures.

The endpoints measured and recorded were the total living offspring per surviving parental animal, the parental age at first offspring emergence as well as the rate of parental survivors and the survivors' body-length and dry body mass at the end of the study. Although no mortalities occurred at any test level, effects on some of the other endpoints were recorded.

The overall-NOEC was determined to be 43.3 mg p.m./L based on an increased age at first reproduction at the highest test concentration of 100 mg p.m./L. The corresponding LOEC is 100 mg p.m./L. The "Maximum Acceptable Toxicant Concentration" (MATC), calculated as geometric mean between NOEC and LOEC, is 65.8 mg p.m./L (nominal).

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960-succinamide (BCS-CR74729)
Type of test material:	Substance, technical (pure metabolite)
Chemical state and description:	White powder
Batch number:	BCOO 6329-2-10
Batch ID:	BCS-CR74729-01-01
Sample description:	TOX 09343-01
IUPAC name:	4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}-4-oxobutyric acid
Purity:	97.8% w/w
Expiration date:	2012-03-13
Storage conditions:	+10 to +30 °C

2. Test solutions

Vehicle:	None
Controls:	Deionised water
Evidence of undissolved material:	None

3. Test organisms

Species:	*Daphnia magna*
Common name:	Water flea
Strain:	Genotype No. 2 ; (Bradley, 1988) equal to type B (Baird 1991)
Source:	Inhouse-culture
Age at study initiation:	< 24 hour old neonates
Feeding during test:	0.2 mg TOC per test vessel with 100 mL (corresponding to 1x108 cells/L)
Maintenance of culture:	
Temperature:	20 ± 2°C
Photoperiod:	16 hours light, 8 hours dark
Food:	Green alga *Desmodesmus subspicatus*

B. Study design and methods

1. In life dates November 8 to December 5, 2011

2. Design of biological test

Daphnia magna (<24 hour old neonates at test start) were exposed to BYI 02960-succinamide; (purity 97.8 % w/w) in a static renewal system over a period of 21 days. Renewals took place on days 2, 5, 7, 9, 12, 14, 16 and 19. Nominal concentrations were 3.5, 8.1, 18.7, 43.3 and 100 mg pure metabolite/L. In addition a water control was tested. The vessels were glass beakers (250 mL filled with 100 mL test medium). The test was conducted with 10 replicates with one daphnid each.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0 (fresh), day 2 (aged), day 9 (fresh), day 12 (aged), day 19 (fresh) and day 21 (aged) from all concentrations. The LOQ was 10.9 µg/L. The range of linearity was 10.9 to 2180 µg/L.

4. Observation and measurements

Immobilisation of daphnids, symptoms of intoxication, data on reproduction and growth as well as physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

Quantitative data like offspring counts and parental growth measurements were analysed on variance homogeneity (e.g. Bartlett's Test) and normal distribution (e.g. Kolmogorroff-Smirnov Test) on a 5% level of significance using the treatment levels and untreated control as co-variates.

Parametric procedures for homogeneous data involved subjecting reproduction data to standard ANOVA. If significant differences among the means were indicated, multiple comparison procedures (e.g. Dunnett's multiple t-test procedure or, in case of monotonous decrease of responses, adequate step down trend-tests (e.g. Williams multiple sequential t-test procedure) were performed on a 5% level of significance ($p \leq 0.05$), to indicate which treatment groups differed significantly from the control. For non-parametric procedures the Mann-Whitney-Wilcoxon U-test for independent samples was applicable, and alternatively multiple comparison procedure with Bonferroni-Correction was used.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	20.4 to 21.8 °C
pH:	6.9 to 8.0
Dissolved oxygen (mg/L):	8.5 to 9.1
Aeration used:	no
Photoperiod:	16 hrs light; 8 hrs dark
Hardness:	14 German degrees (dH)
Alkalinity:	3 dH as carbonate hardness
Conductivity:	627 to 633 µmhos/cm

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of 103% to 111% (mean: 106%) of nominal in freshly prepared and 104% to 109% (mean: 106%) of nominal in aged test solutions. Biological results are reported as nominal. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960-succinamide

Nominal concentrations (mg p.m./L)	medium	Measured concentration (mg p.m./L)						% of nominal
		day 0	day 2	day 9	day 12	day 19	day21	
3.5	(fresh)	3.75		3.76		3.72		104 to 108
	(aged)		3.77		3.66		3.71	
8.1	(fresh)	8.67		8.63		8.59		106 to 107
	(aged)		8.69		8.63		8.56	
18.7	(fresh)	19.6		19.4		19.8		103 to 106
	(aged)		20.0		19.6		19.8	
43.3	(fresh)	47.3		48.1		44.7		103 to 111
	(aged)		47.0		45.3		45.2	
100	(fresh)	106		105		103		103 to 108
	(aged)		107		107		108	

C. Biological Findings

Observations on immobilization, reproduction and growth are listed as follows:

Table: Effect of BYI 02960-succinamide on survival and reproduction of *Daphnia magna*

Treatment mg pure metabolite/L (nominally)	parental endpoints			reproductive endpoints			
	body length (mm ± SD)	dry body mass (mg ± SD)	survival (%)	total offspring per parent animal (n ± SD)	parent age at first offspring emergence (days ± SD)	neonates (% of total offspring)	
						affected	dead
control	4.3 ± 0.2	0.9 7± 0.2	100	104.9 ± 16.0	9.0 ± 0.0	0	0
3.5	4.2 ± 0.1	0.86 ± 0.2	100	101.9 ± 12.5	9.1 ± 0.3	0	0
8.1	4.2 ± 0.2	0.90 ± 0.2	100	92.7 ± 18.8	9.2 ± 0.4	0	0
18.7	4.2 ± 0.1	0.91 ± 0.2	100	90.0 ± 15.4	9.0 ± 0.0	0	0
43.3	4.3 ± 0.0	0.83 ± 0.2	100	98.5 ± 8.8	9.0 ± 0.0	0	0
100	4.2 ± 0.2	0.90 ± 0.2	100	90.6 ± 31.7	9.7 *± 1.9	0	0

* statistically significant difference from untreated control (William's t-test, p <0.05, one sided)

No sublethal behavioural changes were observed.

D. Validity Criteria

All validity criteria were met. No mortality occurred in the control. Mean number of live offspring per parent was greater than 60 in the control.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

NOEC (immobilisation):	100 mg pure metabolite/L
NOEC (time to first brood):	43.3 mg pure metabolite/L
NOEC (living neonates/adult):	100 mg pure metabolite/L
NOEC (adult body length):	100 mg pure metabolite/L
NOEC (adult dry weight):	100 mg pure metabolite/L
NOEC (overall):	**43.3 mg pure metabolite/L**

CONCLUSION

The chronic effect of BYI 02960-succinamide on growth and reproduction of *Daphnia magna* can be quantified as an overall-NOEC of 43.3 mg pure metabolite/L. This NOEC is based on an increased age at first reproduction at the highest test concentration of 100 mg pure metabolite/L. The corresponding LOEC is 100 mg pure metabolite/L. The "Maximum Acceptable Toxicant Concentration" (MATC), calculated as geometric mean between NOEC and LOEC, is 65.8 mg pure metabolite/L (nominal).

IIA 8.3.2.2 Chronic toxicity for representative species of aquatic insects

From the insecticidal activity of BYI 02960 a specific activity against aquatic insects may be anticipated. To address the chronic toxicity of the insecticide, the growth and development test for *Chironomus riparius* (OECD 219) was undertaken.. Chronic tests on the midge were also performed with the soil metabolites 6-CNA and DFA following the rationale described earlier considering the major structural differences to the parent compound.

As previously noted DFA is a very strong acid that cannot be handled as the free acid, it was therefore tested as the sodium salt with the knowledge that dissociation will occur in the aquatic system.

Report:	**KIIA 8.3.2.2/01 Bruns, E. (2011)**
Title:	*Chironomus riparius* 28-day chronic toxicity test with BYI 02960 (tech.) in a water-sediment system using spiked water
Report No:	EBRVP025
Document No:	M-401792-01-2
Guidelines:	**OECD Guideline 219**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** **Screening of contaminants in water was not performed according to GLP**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2% w/w) on emergence and development of *Chironomus riparius*.

Midge larvae of *Chironomus riparius* (1st instar larvae, 2-3 days old, 4 replicates of 20 per treatment and control) were exposed in a static water sediment system (spiked-water exposure) over a period of 28 days to nominal concentrations of 1.25, 2.50, 5.00, 10.0, 20.0 and 40.0 µg a.i. /L. In addition a water control and solvent control were tested. Four replicates per concentration and control with 20 animals each were tested.

Recoveries of active substance were measured three times during the study: 1 hour, 7 days and 28 days after application in one additional test container of each nominal initial test concentration.

Chemical analysis of overlying water and pore water over time reflect expected aquatic fate data with high recoveries of 85 % to 110 % (mean 99 %) at the beginning of the exposure period in the overlying water of all test concentrations.

On day 7 recoveries from 37 % to 83 % (mean 60 %) were found. Recoveries from 30 % to 52 % (mean 41 %) of nominal test concentrations were found on day 28.

Chemical analysis of the pore water over time yield 0.6 % of nominal concentration on day 0, 2.0 % on day 7 and 2.2 %, on day 28.

Initial nominal concentrations were used for reporting and evaluation of the results. Additionally, the results were calculated to initial measured concentrations.

Emergence, sex and development rates were determined.

The start of emergence was reduced for one day at test concentration of 20.0 µg a.i./L. No emergence was observed at the highest test concentration of 40.0 µg a.i. /L. The overall NOEC was determined to be 10.0 µg a.i./L (initial measured: 10.5 µg a.i./L based on emergence rate and development rate.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Specification No.:	102000022313
Batch number:	2009-000239 (Batch code: BYI 02960-01-03)
Sample description:	TOX 08508-00
CAS#:	951659-40-8
IUPAC Name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Stability:	Expiry date: 16 Jan 2011, when stored at +25 ± 5°C (storage conditions from +2°C to +30°C are also acceptable)

2. Test solutions

Vehicle:	Dimethylformamide (DMF)
Controls:	Water control and solvent control

3. Test organisms

Species:	*Chironomus riparius*
Common name:	Midge
Strain:	University of Frankfurt am Main (Germany)
Source:	Inhouse-culture since 2006
Age at study initiation:	1st instar larvae (2-3 days old)
Maintenance of culture:	
Temperature:	20 ± 2°C
Photoperiod:	16 hours light, 8 hours dark
Food:	Green algae and an aqueous suspension of a plant material based fish food (Tetra Phyll®).

B. Study design and methods

1. In-life dates: January 21 to February 25, 2010

2. Design of biological test

Chironomus riparius first instar larvae were exposed to BYI 02960 (purity 96.2 % w/w) in an artificial water-sediment system over a period of 28 days. Nominal concentrations were 1.25, 2.50, 5.00, 10.0, 20.0 and 40.0 µg a.i./L. In addition a water control and solvent control were tested. The vessels were glass beakers (0.6 L) filled with a 1.5 cm layer of artificial sediment and a 6.0 cm layer of water (M7-medium). The test was conducted with 4 replicates per treatment, 4 replicates for the water control and 4 for the solvent control

sediment layer:	1.5 cm
quartz sand	75.80%
sphagnum moss peat	4%
kaolinite	20%
calcium carbonate	0.20%

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0 (1 hour), day 7 and day 28 from all concentrations. BYI 02960 (purity 96.2 % w/w) served as analytical standard. The LOQ was 0.115 µg a.i./L.

4. Observation and measurements

Emergence of midges and development rates as well as physical-chemical water parameters were assessed as indicated below in the result section. The test vessels were observed at least three times per week to make a visual assessment of any behavioural differences compared to the control. The sex, time point of emergence and number of emerged midges was recorded daily during the period of emergence.

5. Statistical analysis

The statistically different distribution between sexes compared to the assumption of 50% females and 50% males are judged by a χ^2-r x 2 table test. EC_x values (e.g. x = 15, 50) and confidence intervals after 28 days were calculated by probit (or logit, weibit, etc.) analysis or in case of failure by non parametric-methods from the appropriate parameters (endpoints). The LOEC determinations from the appropriate parameters (endpoints) were done, using the ANOVA procedure ($\alpha = 0.05$, one sided) and properly selected multiple t-tests. In case of a limit test (comparison of control and one treatment group only) the Student t-test can be used. Statistical evaluations were done using the commercial program ToxRat Professional.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	20.2°C to 20.7°C
pH:	8.3 to 8.7
Dissolved oxygen (mg/L):	7.5 to 8.5
Dissolved oxygen (% saturation):	84.2 % (for 7.5 mg/L)
Aeration used:	yes
Photoperiod:	16 hours light; 8 hours dark
Light intensity:	500 to 1000 Lux
Hardness:	249.2 to 267.0 mg/L as $CaCO_3$)
Alkalinity:	124.6 to 231.4 mg/L as $CaCO_3$)
Sediment pH:	6.9
Sediment water content:	31.50%
Sediment organic carbon content	2%

B. Analytical Findings

Chemical analysis of overlying water and pore water over time reflect expected aquatic fate data with high recoveries of 85 % to 110 (mean 99 %) at the beginning of the exposure period in the overlying water of all test concentrations. On day 7, recoveries from 37 % to 83 % (mean 60 %) were found. Recoveries from 30 % to 52 % (mean 41 %) of nominal test concentrations were found on day 28. Chemical analysis of the pore water over time yield 0.6 % of nominal concentration on day 0, 2.0% on day 7 and 2.2%, on day 28. Biological results are reported as mean measured. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960

initial nominal concentration [µg a.i./L])	analytical results of BYI 02960, means of two analyses each [µg a.i./L]					
	day 0 (1 hour)		day 7		day 28	
	analysed	% of nominal	analysed	% of nominal	analysed	% of nominal
	overlying water					
control	< 0.1115		< 0.1115		< 0.1115	
solvent control	< 0.1115		< 0.1115		< 0.1115	
1.25	1.22	98	0.671	54	0.382	31
2.5	2.13	85	1.23	49	0.738	30
5.0	4.41	88	1.83	37	1.82	36
10.0	10.5	105	6.31	63	4.76	48
20.0	21.3	107	16.5	83	10.3	52
40.0	43.8	110	29.7	74	20.3	51
average %		**99**		**60**		**41**
	pore water [1)]					
control	< 0.1115		< 0.1115		< 0.1115	
solvent control	< 0.1115		< 0.1115		< 0.1115	
1.25	0.214	1	0.283	1.5	0.3	1.5
2.5	0.184	0.5	0.543	1.3	0.524	1.4
5.0	0.314	0.4	0.838	1	1.330	1.9
10.0	0.688	0.5	3.33	2.3	4.10	2.9
20.0	2.32	0.7	9.62	2.9	8.95	2.6
40.0	4.62	0.7	19.2	3.0	16.6	2.8
average %		**0.6**		**2.0**		**2.2**

1) = calculated to the real volume of pore water and the applied amount of a.i.

C. Biological Findings

Start of emergence was on day 13 and 14 for the controls and test concentrations from 1.25 to 10.0 µg a.i. /L. The start of emergence was reduced for one day at test concentration of 20.0 µg a.i./L. No emergence was observed at the highest test concentration of 40.0 µg a.i. /L. Emergence and development rates were observed as follows:

Table: Effect of BYI 02960 on survival and reproduction of *Chironomus riparius*

Initial nominal test concentration (µg a.i./L)	number of emerged midges (introduced)	emergence of introduced larvae (pooled sex)			development rate (1/d)		
		total %	male %	female %	pooled sex	male	female
Controls (pooled)	144 (160)	90	41.3	48.8	0.058	0.064	0.052
1.25	70 (80)	87.5	42.5	45	0.058	0.064	0.053
2.5	74 (80)	92.5	42.5	50	0.057	0.064	0.051
5	67 (80)	83.8	41.3	42.5	0.058	0.065	0.052
10	71 (80)	88.8	41.3	47.5	0.059	0.065	0.054
20	42* (80)	52.5	18.8	33.8	0.050*	0.058*	0.044*
40	0 (80)	-	-	-	-	-	-

* significant difference ($\alpha = 0.05$)

The Exact r-x-2-Table-Test indicates no statistically different distribution between sexes compared to the assumption of 50% females and 50% males. Therefore males and female were pooled for all further endpoint calculations to increase the statistical power.

A statistical significant ($\alpha = 0.05$) effect on emergence rate was evaluated for 20.0 µg a.i. /L, resulting in an NOEC of 10.0 µg a.i./L.

For the development rate (pooled sex) a statistical significance was evaluated at the highest test concentration with emergence of 20.0 µg a.i./L, resulting in an NOEC of 10.0 µg a.i./L

D. Validity Criteria

Mean number of live offspring per parent was greater than 60 in the control. The emergence in the control(s) was >70% of introduced larvae at the end of the test. The emergence was between 12 and 23 days after their introduction into the control vessels. The oxygen content in the water body was > 60 % of saturation at the end of the test in all test vessels. The pH of the overlying water was between 6 and 9 in all test vessels. The water temperature did not to differ by more than ± 1°C over the whole exposure period.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

Endpoints based on nominal concentrations:

Endpoints	NOEC [µg a.i./L]	LOEC [µg a.i./L]	EC_{15} [µg a.i./L]	EC_{50} [µg a.i./L]
emergence rate (pooled sex) (95 % confidence limits)	10.0	20.0	13.4 (11.9 – 15.2)	20.3 (18.5 – 22.2)
development rate (pooled sex) (95 % confidence limits)	10.0	20.0	20.6 (19.9 – 21.7)	32.6 (27.9 – 48.7)

Endpoints based on initial measured concentrations:

Endpoints	NOEC [µg a.i./L]	LOEC [µg a.i./L]	EC_{15} [µg a.i./L]	EC_{50} [µg a.i./L]
emergence rate (pooled sex) (95 % confidence limits)	10.5	21.3	14.3 (12.6 – 16.1)	21.8 (19.8 – 23.8)
development rate (pooled sex) (95 % confidence limits)	10.5	21.3	21.9 (21.2 – 23.1)	35.3 (30.2 – 51.8)

CONCLUSION

The chronic effect of (BYI 02960) on development and emergence of *Chironomus riparius* can be quantified as an overall-NOEC of 10.0 µg a.i./L (initial measured: 10.5 µg a.i./L).

Report:	**KIIA 8.3.2.2/02; Bruns, E. (2011)**
Title:	*Chironomus riparius* 28-day chronic toxicity test with Sodium difluoroacetate in a water-sediment system using spiked water – limit test
Report No:	EBRVP181
Document No:	M-415913-01-2
Guidelines:	**OECD Guideline No. 219**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** **Screening of contaminants in water was not performed according to GLP.**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of sodium difluoroacetate (Na-salt of BYI 02960-DFA) (Sample description: TOX 08988-01 (Batch ID: 2009-000239), purity > 99.0% w/w) on emergence and development of *Chironomus riparius*.

Midge larvae of *Chironomus riparius* (1st instar larvae; 2-3 days old, 20 per replicate with 6 replicates) were exposed in a static water-sediment system (spiked-water exposure) over a period of 28 days to a nominal concentration of 100 mg pure metabolite/L. In addition a water control was tested.

Recoveries of the metabolite were measured three times during the study: 1 hour, 7 days and 28 days after application in one additional test container of the limit test concentration of 100 mg p.m./L and controls of the overlying water and the pore water of the sediment.

Chemical analysis of sodium difluoroacetate in the overlying water over time showed high recoveries of 105% at the beginning of the exposure period of the limit test concentration. On day 7 and 28 the determined amount of sodium difluoroacetate was 95.1% and 98.3%, respectively.

Chemical analysis of the pore water over time yield 1.55% of nominal on day 0, 6.61% on day 7 and 7.95%, on day 28.

The initial nominal limit concentration was used for reporting and evaluation of the results.

Emergence, sex and development rates were determined. The overall-NOEC was determined to be the limit dose tested, ≥ 100 mg pure metabolite/L.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	Sodium difluoroacetate (Na-salt of BYI 02960-DFA)
Type of test material:	Substance, technical (pure metabolite)
Chemical state and description:	White powder
Batch ID:	2009-000239
Batch No.	BCOO 6092-3-1
Code:	BCS-AB60481
Sample description:	TOX 08988-01
Purity:	> 99.0% w/w
Storage conditions:	Expiry date: 25 Apr 2011, to be stored at +10 to +30°C

2. Test solutions

Vehicle:	None
Controls:	Water control

3. Test organisms

Species:	*Chironomus riparius*
Common name:	Midge
Strain:	University of Frankfurt am Main (Germany)
Source:	Inhouse-culture since 2006
Age at study initiation:	1st instar larvae (2-3 days old)
Feeding during test:	0.5 to 1 mg Tetraphyll per test vessel at least three times per week
Maintenance of culture:	
Temperature:	20 ± 2°C
Photoperiod:	16 hours light, 8 hours dark
Food:	Green algae and an aqueous suspension of a plant material based fish food (Tetra Phyll®).

B. Study design and methods

1. In life dates 18 Jan to 22 Feb, 2011

2. Design of biological test

Chironomus riparius first instar larvae were exposed to sodium difluoroacetate (Na-salt of BYI 02960-DFA) (code: BCS-AB60481; purity > 99.0 % w/w) in an artificial static water-sediment system (spiked water) over a period of 28 days. The nominal concentration was 100 mg pure metabolite/L. In addition a water control was tested. The vessels were glass beakers (0.6 L; diameter 9.5 cm) filled with 1.5 cm layer of sediment and a 6.0 cm layer of M7 test medium. The test was conducted with 20 larvae per replicate (6 replicates). During the study the larvae were fed at least about three times per week with a commercial ornamental fish food extract (trade name Tetra Phyll®) as used for the breeding. An appropriate amount of this suspension (about 0.5 - 1 mg Tetraphyll® /Larvae/day) was added to each test container.

Sediment:	
sediment layer:	1.5 cm
quartz sand	75.0%
sphagnum moss peat	4%
kaolinite	20%
calcium carbonate	0.1%

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0 (1 hour), day 7 and day 28 from all concentrations. The test substance served as analytical standard. HPLC-MS/MS was used as analytical method. The limit of quantification (LOQ) was 1.048 µg/L.

4. Observation and measurements

The test vessels were also observed at least three times per week to make a visual assessment of any behavioural differences compared to the control. The sex, time point of emergence and number of emerged midges was recorded daily during the period of emergence. As only fully emerged adults are relevant for the endpoints of this study, larvae which did not yet mature were not taken into account for emergence rates and development time. To determine number and sex of emerged adults, the covering plates of each test container were carefully moved and the midges, which mostly stayed at the sides of the vessels, were enumerated; after identification of the sex (male midges have feathered antennae) midges were removed. Emergence of midges and development rates as well as physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

The statistically different distribution between sexes compared to the assumption of 50% females and 50% males are judged by a χ^2-r x 2 table test. EC_x values (e.g. x = 15, 50) and confidence intervals after 28 days were calculated by probit (or logit, weibit, etc.) analysis or in case of failure by non-parametric-methods from the appropriate parameters (endpoints). The LOEC determinations from the appropriate parameters (endpoints) were done, using the ANOVA procedure ($\alpha = 0.05$, one sided) and selected multiple t-tests. In case of a limit test (comparison of control and one treatment group only) the Student t-test can be used. Statistical evaluations were performed using the commercial program ToxRat Professional.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	20.3°C to 20.5°C
pH:	8.2 to 8.5
Dissolved oxygen (mg/L):	7.7 to 8.8 mg/L
Dissolved oxygen (% saturation):	85.8 % (at 7.7 mg/L)
Aeration used:	yes
Photoperiod:	16 hours light; 8 hours dark
Light intensity:	852 Lux
Hardness:	302.6 to 320.4 mg/L as CaCO3)
Alkalinity:	106.8 to 213.6 mg/L as CaCO3)
Sediment pH:	6.7
Sediment water content:	32.6 %
Sediment organic carbon content	2.1 %

B. Analytical Findings

Chemical analysis of Sodium difluoroacetate in the overlying water over time showed high recoveries of 105 % at the beginning of the exposure period of the limit test concentration. On day 7 and 28 the determined amount of Sodium difluoroacetate was 95.1 % and 98.3 %, respectively.

Chemical analysis of the pore water over time yield 1.55 % of nominal on day 0, 6.61 % on day 7 and 7.95 %, on day 28. Biological results are reported as initial nominal concentrations. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BCS-AB60481

Nominal concentration (mg p.m./L)	Analytical results of sodium difluoroacetate [mg p.m./L]					
	day 0 (1 hour)		day 7		day 28	
	analytical	% of nominal	analytical	% of nominal	analytical	% of nominal
	overlying water					
control	< LOQ		< LOQ		< LOQ	
100	105	105	95.1	95.1	98.3	98.3
	pore water [1)]					
control	< LOQ		< LOQ		< LOQ	
100	26.6	1.55	101	6.61	104	7.95

[1)] Calculated to the real volume of pore water per test vessel

C. Biological Findings

Start of emergence was on day 13 and 14 for the controls and the limit test concentration of 100 mg p.m./L. 95.8% of the inserted (n= 120) larvae maturated to adults in the controls after 28 days. Observations on emergence and development rate are listed as follows:

Table: Effect of BCS-AB60481 on survival and reproduction of *Chironomus riparius*

Nominal concentration (mg p.m./L)	Number of emerged midges (introduced)	Emergence of introduced larvae			Development rate (1/d) ; pooled sex
		total %	male %	female %	
Control	115 (120)	95.8	50.8	45.0	0.062
100	108 (120)	90.0	48.3	41.7	0.063

The Chi^2-Test indicates no statistically different distribution between sexes compared to the assumption of 50% females and 50% males. Therefore male and female results were pooled for further statistical analyses to increase the statistical power. There was no statistically significant difference in emergence between the control and solvent control and at the limit test concentrations of 100 mg p.m./L as compared to the control findings. For the development rate (pooled sex) there was no statistical significant difference as compared to the control for the limit test concentration.

D. Validity Criteria

Mean number of live offspring per parent was greater than 60 in the control. The emergence in the control(s) was >70% of introduced larvae at the end of the test. The emergence was between 12 and 23 days after their introduction into the control vessels. The oxygen content in the water body was > 60% of saturation at the end of the test in all test vessels. The pH of the overlying water was between 6 and 9 in all test vessels. The water temperature did not to differ by more than ± 1°C over the whole exposure period.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

	Sodium difluoroacetate [mg p.m./L]	
Endpoints *	NOEC	LOEC
emergence rate (pooled sex)	≥ 100	> 100
development rate (pooled sex)	≥ 100	> 100

* Based on nominal initial concentrations

CONCLUSION

The chronic effect of sodium difluoroacetate (Na-salt of BYI 02960-DFA; BCS-AB60481) on emergence and development of *Chironomus riparius* can be quantified as an overall-NOEC, based on emergence and development of ≥ 100 mg pure metabolite/L the concentration tested.

Report:	**KIIA 8.3.2.2/03; Bruns, E. (2011)**
Title:	*Chironomus riparius* 28-day chronic toxicity test with 6-Chloronicotinic acid in a water-sediment system using spiked water – limit test
Report No.:	EBRVP183
Document No.:	M-416604-02-2
Guidelines:	**OECD Guideline 219**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** **Screening of contaminants in water was not performed according to GLP**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of 6-Chloronicotinic acid (Sample description: AZ 16813 (code: AE F161089; purity 98.8%) on emergence and development *Chironomus riparius*.

Midge larvae of *Chironomus riparius* (1st instar larvae; 2-3 days old, 20 per replicate with 6 replicates) were exposed in a static water-sediment system (spiked water exposure) over a period of 28 days to a nominal concentration of 100 mg pure metabolite/L (limit test). In addition, a water and a solvent control were tested. The study was conducted with six replicates per treatment level with 20 animals each.

Recoveries of the metabolite were measured three times during the study: 1 hour, 7 days and 28 days after application in one additional test container of the limit test concentration of 100 mg p.m./L and controls of the overlying water and the pore water of the sediment.

Chemical analysis of overlying water and pore water over time showed high recoveries of 102% at the beginning of the exposure period in the overlying water of the limit test concentration. On day 7 and 28 the determined amount of 6-Chloronicotinic acid was 89.6% and 90.6%, respectively.

Chemical analysis of the pore water over time yielded 1.29% of nominal on day 0, 6.66% on day 7 and 7.29%, on day 28, respectively.

The initial nominal limit concentration was used for reporting and evaluation of the results.

Emergence, sex ratio and development rates were determined.

There was no statistical significant difference for emergence and development rate between the controls and the limit test concentration of 100 mg p.m./L

Therefore, the overall-NOEC was determined to be ≥ 100 mg pure metabolite/L based on the limit test concentration.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	6-Chloronicotinic acid (6-CNA)
Type of test material:	Substance, technical (pure metabolite)
Chemical state and description:	Beige powder
Origin Batch No.:	M12653
Batch Code:	AE F161089 00 1B99 0001
Sample description:	AZ 16813
Purity:	98.8%
Stability:	Expiry date: 2012-07-09, when stored at +5 ± 5°C

2. Test solutions

Vehicle:	None
Controls:	Water control

3. Test organisms

Species:	*Chironomus riparius*
Common name:	Midge
Strain:	University of Frankfurt am Main (Germany)
Source:	Inhouse-culture since 2006
Age at study initiation:	1st instar larvae (2-3 days old)
Feeding during test:	0.5 to 1 mg Tetraphyll per test vessel at least three times per week
Maintenance of culture:	
Temperature:	20 ± 2°C
Photoperiod:	16 hours light, 8 hours dark
Food:	Green algae and an aqueous suspension of a plant material based fish food (Tetra Phyll®).

B. Study design and methods

1. In-life dates January 18 to February 22, 2011

2. Design of biological test

Chironomus riparius first instar larvae were exposed to 6-Chloronicotinic acid (code: AE F161089 00 1B99 0001; purity 98.8 %) in a static water-sediment system (spiked water) over a period of 28 days. The nominal concentration was 100 mg pure metabolite/L.

The test was conducted with 6 replicates. In addition a water and a solvent control were tested. The vessels were glass beakers (0.6 L; diameter 9.5 cm) filled with 1.5 cm layer of sediment and a 6.0 cm layer of M7 test medium. The test was conducted with 20 larvae per replicate (6 replicates). During the study the larvae were fed at least about three times per week with a commercial ornamental fish food extract (trade name Tetra Phyll®) as used for the breeding. An appropriate amount of this suspension (about 0.5 - 1 mg Tetraphyll® /Larvae/day) was added to each test container.

Sediment:

sediment layer:	1.5 cm (140 g)
quartz sand	75.0%
sphagnum moss peat	4%
kaolinite	20%
calcium carbonate	1.0%

3. Method of analytical verification

For the analytical verification of test item concentrations samples were taken at day 0 (1 hour), day 7 and day 28 from all treatment levels. The test substance served as analytical standard. HPLC-MS/MS was used as analytical method. The limit of quantification (LOQ) was 1.145 µg/L.

4. Observation and measurements

The test vessels were observed at least three times per week to make a visual assessment of any behavioural differences compared to the control. The sex, time point of emergence and number of emerged midges was recorded daily during the period of emergence. As only fully emerged adults are relevant for the endpoints of this study, larvae which did not yet mature were not taken into account for emergence rates and development time. To determine number and sex of emerged adults, the covering plates of each test container were carefully moved and the midges, which mostly stayed at the sides of the vessels, were enumerated; after identification of the sex (male midges have feathered antennae) midges were removed.

Physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

Sex ratio: The statistically different distribution between sexes compared to the assumption of 50% females and 50% males are judged by a χ^2-r x 2 table test. In case of emergence rate (no indications of statistically difference) male and female results were pooled for analyses to increase the statistical power. In case of developmental rate statistical analyses were done separately for each sex.

The LOEC and NOEC determinations from the appropriate parameters (endpoints) were done using the ANOVA procedure ($\alpha = 0.05$, one sided) and properly selected multiple t-tests using a commercial program. In case of a limit test (comparison of control and one treatment group only) the STUDENT t-test was used.

Calculations were carried out using Microsoft Excel® spreadsheets. All further statistical evaluations were done using the commercial program ToxRat Professional.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	20.5°C to 20.7°C
pH:	8.4 to 8.6
Dissolved oxygen (mg/L):	7.6 to 8.9 mg/L
Dissolved oxygen (% saturation):	85.3 % (at 7.6 mg/L)
Aeration used:	yes
Photoperiod:	16 hours light; 8 hours dark
Light intensity:	852 Lux
Hardness:	284.8 to 338.2 mg/L as $CaCO_3$)
Alkalinity:	106.8 to 213.6 mg/L as $CaCO_3$)
Sediment pH:	6.7
Sediment water content:	32.6%
Sediment organic carbon content	2.1%

B. Analytical Findings

Chemical analysis of overlying water and pore water over time showed high recoveries of 102% at the beginning of the exposure period in the overlying water of the limit test concentration. On day 7 and 28 the determined amount of 6- chloronicotinic acid was 89.6 % and 90.6 %, respectively.

Chemical analysis of the pore water over time yielded 1.29% of nominal on day 0, 6.66% on day 7 and 7.29% on day 28. Biological results are reported as initial nominal concentrations. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of 6-chloronicotinic acid (AE F161089)

	6-chloronicotinic acid [mg p.m./L]					
Nominal concentration [mg p.m./L]	Day 0 (1 hour)		Day 7		Day 28	
	analytical	% of nominal	analytical	% of nominal	analytical	% of nominal
	overlying water					
control	< LOQ		< LOQ		< LOQ	
100	102	102	89.6	89.6	90.6	90.6
	pore water [1])					
control	< LOQ		< LOQ		< LOQ	
100	22.9	1.29	103	6.66	91.9	7.29

[1)] Calculated to the real volume of pore water per test vessel

C. Biological Findings

Start of emergence was at day 13 for the controls and the limit test concentration of 100 mg p.m./L. 87.5% of the inserted (n= 120) larvae maturated to adults in the controls after 28 days. Observations on emergence, sex ratio and development rate are listed as follows:

Table: Effect of 6-chloronicotinic acid on survival and development of *Chironomus riparius*

Nominal concentration (mg p.m./L)	Number of emerged midges (introduced)	Emergence of introduced larvae			Development rate (1/d); pooled sex
		total %	male %	female %	
Control	105 (120)	87.5	41.7	45.8	0.064
100	106 (120)	88.3	50.0	38.3	0.066

The Chi^2-Test indicates no statistically different distribution between sexes compared to the assumption of 50% females and 50% males. Therefore male and female results were pooled for further statistical analyses to increase the statistical power.

There was no statistical significant difference in emergence between the control and solvent control and at the limit test concentrations of 100 mg p.m./L as compared to the control findings. For the development rate (pooled sex) there was no statistical significant difference as compared to the control for the limit test concentration.

D. Validity Criteria

Mean number of live offspring per parent was greater than 60 in the control. The emergence in the control(s) was > 70% of introduced larvae at the end of the test. The emergence was between 12 and 23 days after their introduction into the control vessels. The oxygen content in the water body was > 60% of saturation at the end of the test in all test vessels. The pH of the overlying water was between 6 and 9 in all test vessels. The water temperature did not to differ by more than ± 1°C over the whole exposure period.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints, based on nominal initial concentration, can be derived:

Endpoints (mg p.m./L)	NOEC	LOEC
emergence rate (pooled sex)	≥ 100	> 100
development rate (pooled sex)	≥ 100	> 100

CONCLUSION

The chronic effect of 6-Chloronicotinic acid (AE F161089) on growth and reproduction of *Chironomus riparius* can be quantified as an overall-NOEC of ≥ 100 mg pure metabolite/L, the limit concentration tested.

IIA 8.3.2.3 Chronic toxicity for repr. species of aquatic gastropod molluscs

This study is not triggered as BYI 02960 is not intended to be used directly on surface water (overspray). Acute toxicity to the saltwater mollusc *Crassostrea virginica*, indicates no particular concern to this taxonomic group (Annex II, 8.11.) and therefore, chronic tests were not deemed necessary.

IIA 8.3.3 Aquatic field testing

The available studies on laboratory indicator species give a detailed picture of BYI 02960's toxicity to aquatic non-target organisms with a special focus on invertebrates. The derived endpoints provide a good overview on potentially sensitive species and fields of concern. A risk assessment for the intended

uses of BYI 02960 used as a plant protection product can be undertaken without the requirement for testing at (model) field scale. Therefore no additional semifield or field tests have been undertaken.

IIA 8.4 Effects on algal growth and growth rate (2 species)

Testing on algae was performed for BYI 02960 and several major metabolites, testing on the BYI 02960-azabicyclosuccinamide was not performed as the indication from testing of the other metabolites did not indicate a concern. This is in agreement with the recommendation of the Guidance Document on Aquatic ecotoxicology which proposed a factor of 100 to the most sensitive (parent) species to indicate a necessity for further testing. The difference for BYI 02960 between the toxicity to algae and chironomids is > 1000 allowing a significant margin of safety if the metabolite should be more toxic than the parent to algae.

Growth tests with algae are considered a chronic test as several generations are develop during the test period of 72h.

Report:	**KIIA 8.4/01; [redacted], C.S. & [redacted] C.V. (2010)**
Title:	Toxicity of BYI 02960 Technical to the Green Alga *Pseudokirchneriella subcapitata*
Report No:	EBRVP030
Document No:	M-397552-01-1
Guidelines:	**EPA OPPTS 850.5400** **OECD Guideline 201** **FIFRA 123-2**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: BCS-CR74729-01-01); purity 96.2%) on the growth of green alga (*Pseudokirchneriella subcapitata*).

Cultures of *Pseudokirchneriella subcapitata* with an initial cell density of 10000 cells/mL were exposed in a static system over a period of 96 hours to nominal concentrations of 5.0, 10, 20, 40 and 80 mg a.i./L (corresponding to analytically verified concentrations of 5.9,11, 23, 47 and 95 mg a.i./L). In addition, a water control and solvent control were tested.

72 and 96 hour growth rates based on cell density and visual assessment of potential cell deformations were used as endpoints. No physical abnormalities were observed in the control and in the treatment groups during the study. Based on analytical findings the biological endpoints are reported as nominal figures.

The 96-hour-E_rC_{50} was >80 mg a.i./L, the 96-hour-NOEC was determined to be 80 mg a.i./L the highest concentration tested..

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difluoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Storage conditions:	Room temperature
Water solubility:	Approximately 80 mg a.i./L under test conditions

2. Test solutions

Vehicle:	Dimethylformamide (DMF)
Concentration of vehicle:	0.1 mL/L
Method of preparation:	Sonicated for one our
Controls:	Water control and solvent control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Pseudokirchneriella subcapitata*
Common name:	Green alga
Source:	University of Texas (UTEX), Austin, Texas, USA, received 6/14/05
Maintenance of pre-culture:	
Temperature:	24 ± 2°C.
Photoperiod:	24 hours light

B. Study design and methods

1. In life dates March 1 to 5, 2010

2. Design of biological test

Green alga (*Pseudokirchneriella subcapitata*) were exposed to BYI 02960 (purity 96.2%) in a static system over a period of 96 hours. This study was conducted up to the functional limit of solubility in the test system, which was determined to be approximately 80 mg a.i./L. Nominal concentrations were 5.0, 10, 20, 40 and 80 mg a.i./L. In addition, a water control and solvent control was tested. Each vessel (Sterile Erlenmeyer flasks covered by inverted glass beakers; 250 mL) filled with 100 mL filter sterilized 1xAAP media (0.20 mm filter) with an initial pH of 7.5 ± 0.1, served as one replicate. At test initiation the cell density was 10000 cells/mL. The test was conducted with 3 replicates per treatment level.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at 0 and 96 hours from all concentrations. High-performance liquid chromatography (HPLC) was used as analytical method. The limit of quantification (LOQ) was 0.62 mg/L. The range of linearity was 0.001 to 0.5 mg/L.

4. Observation and measurements

Growth rates, observation on cell abnormalities and physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

Raw or transformed data from treatment groups were compared to controls for normality and homogeneity of variance using the Shapiro-Wilks test and Levene's test of equal variance, respectively. If normality and homogeneity of variance were demonstrated for the raw or transformed values, then parametric analyses were conducted using analysis of variance (ANOVA) followed by Dunnett's test. If normality and/or homogeneity of variance were not demonstrated on raw or transformed values, nonparametric procedures were used.

The ranks of the raw values were determined, and then an analysis of variance and a one-tailed Dunnett's test were performed on these ranks. The EC_{50}s, and the respective 95% confidence intervals, were calculated with help of regression analysis for cell density, cumulative biomass, and growth rate.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	24.4 to 24.6 °C
pH:	7.3 to 7.4 at test start and 10.0 to 10.2 at test termination
Photoperiod:	24 hours light
Light source	Cool white fluorescent
Light intensity:	Mean: 4036 lux (range: 3740 – 4540 lux)
Conductivity:	Mean 104 µmhos/cm (range: 95 to 116 µmhos/cm)

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of 5.9,11, 23, 47 and 95 mg a.i./L calculated as arithmetic mean. Biological results are reported as nominal. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960

Nominal Conc. (mg a.i./L)	measured day 0		measured day 4		Mean Measured	Mean % Nominal
	mg a.i./L	% nominal	mg a.i./L	% nominal		
Control	< LOQ	---	< LOQ	---	< LOQ	---
Solvent Control	< LOQ	---	< LOQ	---	< LOQ	---
5	5.8	117	5.9	118	5.9	117
10	11	111	12	117	11	114
20	23	113	23	116	23	115
40	48	120	46	115	47	117
80	94	118	96	120	95	119

C. Biological Findings

Observations on growth rates are listed as follows:

Table: Effect of BYI 02960 on growth-inhibition of *Pseudokirchneriella subcapitata*

Nominal Concentration (mg a.i./L)	72-h-Mean growth rate (cell density)	72-h-% inhibition compared to pooled control	96-h-Mean growth rate (cell density)	96-h-% inhibition compared to pooled control
Control	0.069946	NA	0.059591	NA
Solvent Control	0.067933	NA	0.059691	NA
Pooled Controls	0.06894	NA	0.059641	NA
5	0.070266	-1.9	0.059024	1.0
10	0.070646	-2.5	0.058943	1.2
20	0.071208	-3.3	0.06018	-0.9
40	0.070429	-2.2	0.0592	0.7
80	0.069627	-1.0	0.059782	-0.2

No cell abnormalities were observed.

D. Validity Criteria

The validity criteria were fulfilled.

0 to 72 Hour Control Growth Validity Criteria:	
Control Biomass Increase (minimum recommended multiplication factor is 16):	A factor of approximately 144 (mean of control and solvent control groups)
Mean coefficient of variation for section-by-section specific growth rates (days 0-1, 1-2, 2-3) in the controls (criterion is ≤ 35%)	13%
Coefficient of variation for average specific growth rates during the 0 to 72 hour test period in replicate control cultures (criterion is ≤ 7%)	1.0%

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

72 h and 96-h-figures (growth rate):

E_rC_{50}	**>80 mg a.i./L**
highest concentration with no effect (NOEC):	80 mg a.i./L
lowest concentration with effect (LOEC):	>80 mg a.i./L

CONCLUSION

The effect of BYI 02960 on *Pseudokirchneriella subcapitata* can be quantified as a 96-hour-E_rC_{50} of >80 mg a.i./L. The highest concentration with no observed growth inhibition and no cell deformations can be set to 80 mg a.i./L, the highest tested concentration.

Report:	**KIIA 8.4/02; Bruns, E. (2011)**
Title:	*Pseudokirchneriella subcapitata* growth inhibition test with BCS-AB60481 – limit test
Report No:	EBRVP077
Document No:	M-409118-01-2
Guidelines:	**OECD Guideline 201**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of sodium difluoroacetate (Na-salt of DFA; Sample description: TOX 08988-01; code: BCS-AB60481; purity > 99.0% w/w) on growth rate of *Pseudokirchneriella subcapitata*.

Cultures of *Pseudokirchneriella subcapitata* with an initial cell density of 10,000 cells/mL and 6 replicates were exposed in a static system over a period of 72 hours to a nominal concentration of 10 mg p.m./L. In addition a water control was tested.

Quantitative amounts of BCS-AB60481 were measured in all treatment groups and in the control on day 0 and day 3 of the exposure period. Analytical findings on day 0 and on day 3 were 103% of nominal and 100% of nominal, respectively. Based on analytical findings the biological endpoints are reported as nominal figures.

72 hour growth rate based on cell density was used to determine the endpoints. The 72-hour-E_rC_{50} was > 10 mg/L, the 72-hour-NOEC was determined to be 10 mg/L.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	Sodium difluoroacetate (Na-salt of BYI 02960-DFA)
Type of test material:	Substance, technical (pure metabolite)
Chemical state and description:	White powder
Batch number:	BCS-AB60841-01-01
Code:	BCS-AB60481
Sample description:	TOX 08988-01
Purity:	> 99.0% w/w
Storage conditions:	+10 to +30°C

2. Test solutions

Vehicle:	None
Controls:	Water control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Pseudokirchneriella subcapitata*
Common name:	Green alga
Source:	Collection of Algal Cultures, Inst. for Plant Physiology, University of Goettingen, Nikolausberger Weg 18, 37077 Goettingen, Germany
Maintenance of pre-culture:	
Temperature:	22 ± 2°C
Photoperiod:	Permanent light

B. Study design and methods

1. In life dates December 3, 2010 to February 27, 2011

2. Design of biological test

Green alga (*Pseudokirchneriella subcapitata*) were exposed to sodium difluoroacetate (Na-salt of DFA; code: BCS-AB60481; purity > 99.0% w/w) in a static system over a period of 72 hours. Nominal concentrations were 10 mg/L. In addition a water control was tested. Each vessel (Erlenmeyer flasks;

300 mL) served as one replicate filled with 150 mL test solution. At test initiation the cell density was 10,000 cells/mL. The test was conducted with 6 replicates per treatment level.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at 0 and 72 hours from all concentrations. HPLC-MS/MS was used as analytical method. The limit of quantification (LOQ) was 0.998 µg/L. The range of linearity was 0.11 µg/L to 11 µg/L.

4. Observation and measurements

Growth rates, observation on cell abnormalities and physical-chemical water parameters were assessed as indicated below in the result section. Growth inhibition was calculated using algae biomass per volume. The surrogate for biomass was cell density (used as response parameter), measurable by direct counting of algae cells per volume or indirectly by calculation of cell numbers after measurement of optical cell density.

5. Statistical analysis

Not applicable.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	Mean 21.4°C (range 21.3°C to 22.1°C)
pH:	Ranged from 7.7 to 7.9 in the controls; ranged from 7.7 to 7.8 at 10 mg/L
Photoperiod:	Permanent light
Light source	Cool white fluorescent lamps (Sylvania Standard F W / 133-T8)
Light intensity:	Mean 7280 lux (range 7030 to 7620 lux)

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations calculated as arithmetic mean. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BCS-AB60481

Nominal concentration [mg/L]	Measured concentration [mg/L] average day 0	% recovery	Measured concentration [mg/L] average day 3	% recovery
control	< 0.000998	-	< 0.000998	-
10 mg/L	10.3	103	10.0	100

C. Biological Findings

Observations on growth rates are listed as follows:

Table: Effect of BCS-AB60481 on 72-hour-growth-inhibition of *Pseudokirchneriella subcapitata*

Nominal Concentration [mg/L]	Cell Number/mL after 72 h (means)	(0-72h)-Average Specific Growth Rates [$days^{-1}$]	Inhibition of Average Specific Growth Rate [%]
Control	679 000	1.406	-
10	573 000	1.349	4

test initiation with 10,000 cells/mL

No cell abnormalities were observed.

D. Validity Criteria

The validity criteria were fulfilled. Biomass increased in the control by more than 16-fold within the evaluation period. Mean percent coefficient of variation of sectional growth rates from day 0-1, day 1-2, and day 2-3 in the control did not exceed 35%. Percent coefficient of variation of the average growth rate in each control replicate did not exceed 7%.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

72-hour-figures (growth rate):

E_rC_{50}	**> 10 mg p.m./L**
highest concentration with no effect (NOE_rC):	10 mg p.m./L

CONCLUSION

The effect of sodium difluoroacetate (Na-salt of BYI 02960-DFA; BCS-AB60481) on *Pseudokirchneriella subcapitata* can be quantified as a 72-hour-E_rC_{50} of > 10 mg p.m./L. The highest concentration with no observed growth inhibition and no cell deformations can be set to 10 mg p.m./L the limit concentration tested.

Report:	**KIIA 8.4/03; Sobczyk, H. (2011)**
Title:	*Pseudokirchneriella subcapitata* growth inhibition test with BYI 02960 – succinamide – limit test
Report No:	EBRVP184
Document No:	M-414090-01-2
Guidelines:	**OECD Guideline 201**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The objective of this 72 hour growth inhibition test was, to verify the assumption that the BYI 02960 – succinamide (Sample description: TOX 09343-00 (Batch ID: BCS-CR74729-01-01); purity 97.8% w/w) will cause no adverse effects on the growth of the green algae *Pseudokirchneriella subcapitata.*

Cultures of *Pseudokirchneriella subcapitata* with an initial cell density of 10,000 cells/mL and 6 replicates were exposed in a static system over a period of 72 hours to a nominal concentration of 10 mg p.m./L (limit test). In addition a water control and a solvent control were tested.

Quantitative amounts of BYI 02960 – succinamide were measured in all treatment groups and in the control on day 0 and day 3 of the exposure period. The analytical findings of BYI 02960 – succinamide found on day 0 were 114 % of nominal. On day 3 analytical findings of 113 % of nominal were found. All results are based on nominal test concentrations of the metabolite.

72 hour growth rate based on cell density were used to determine the endpoints. The 72-hour-E_rC_{50} was > 10.0 mg pure metabolite/L, the 72-hour-NOE_rC was determined to be 10.0 mg pure metabolite/L.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960 – succinamide
Type of test material:	Substance, technical (pure metabolite)
Chemical state and description:	White powder
Batch number:	BCOO 6329-2-10
Code:	BCS-CR74729
Sample description:	TOX 09343-00
CAS name:	4-{[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino}-4-oxobutyric acid
Purity:	97.8% w/w
Storage conditions:	+10 – +30°C

2. Test solutions

Vehicle:	DMF
Concentration of vehicle:	0.1 mL/L
Controls:	Water control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Pseudokirchneriella subcapitata*
Common name:	Green alga
Source:	Collection of Algal Cultures, Inst. For Plant Physiology, University of Göttingen, Nikolausberger Weg 18, 37077 Göttingen, Germany
Maintenance of pre-culture:	
Temperature:	22 ± 2°C
Photoperiod:	Permanent light

B Study design and methods

1. In life dates June 24 to September 2, 2011

2. Design of biological test

Green algae (*Pseudokirchneriella subcapitata*) were exposed to BYI 02960 – succinamide (purity 97.8% w/w) in a static system over a period of 72 hours. Nominal concentrations were 10 mg p.m./L.

In addition a water and a solvent control were tested. Each replicate consisted of a vessel (Erlenmeyer flasks; 300 mL) filled with 150 mL of test solution. At test initiation the cell density was 10,000 cells/mL. The test was conducted with 6 replicates per treatment level.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0 and day 3. HPLC-UV was used as analytical method. The limit of quantification (LOQ) was 136 µg/L. The range of linearity was 11 µg/L to 2180 µg/L.

4. Observation and measurements

Growth rates, observation on cell abnormalities and physical-chemical water parameters were assessed as indicated below in the result section.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	mean 21.5°C (range 21.3°C to 22.2°C)
pH:	ranged from 7.8 to 8.2 in the controls
Photoperiod:	permanent light
Light source	cool white fluorescent lamps
Light intensity:	mean 8417 lux (range 8220 to 8790 lux)

B. Analytical Findings

Analytical verification of test solutions determined in the concentration of BYI 02960 – succinamide on day 0 to be 114% of nominal and on day 3 to be 113% of nominal was found, respectively. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960 – succinamide

Nominal concentration [mg p.m /L]	1. determination [mg p.m /L]	2. determination [mg p.m /L]	average [mg p.m /L]	% recovery
control	< 0.136	< 0.136	< 0.136	-
solvent control	< 0.136	< 0.136	< 0.136	-
10 (day 0)	11.4	11.4	11.4	114
10 (day 3)	11.4	11.3	11.3	113

C. Biological Findings

Observations on growth rates are listed as follows:

Table: Effect of BYI 02960 – succinamide on 72-hour-growth-inhibition of *Pseudokirchneriella subcapitata*

Nominal Concen-tration [mg p.m./L]	0 - 24 h		0 - 48 h		0 - 72 h		
	Average Specific Growth Rates [days^{-1}]	Inhibition of Average Specific Growth Rate [%]	Average Specific Growth Rates [days^{-1}]	Inhibition of Average Specific Growth Rate [%]	Cell Number/ mL after 72 h (means)	Average Specific Growth Rates [days^{-1}]	Inhibition of Average Specific Growth Rate [%]
control	1.742	-	1.560	-	753 000	1.440	-
solvent control	1.713	-	1.536	-	738 000	1.434	-
pooled control	1.727	-	1.548	-	746 000	1.437	-
10.0	1.706	1.2	1.525	1.5	743 000	1.435	0.1

No cell abnormalities were observed.

D. Validity Criteria

The validity criteria were fulfilled.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

E_rC_{50}	**> 10.0 mg p.m./L**
NOE_rC	≥ 10.0 mg p.m./L

CONCLUSION

The effect of BYI 02960 – succinamide on *Pseudokirchneriella subcapitata* can be quantified as a 72 hour-E_rC_{50} of > 10.0 mg pure metabolite/L. The highest concentration with no observed growth inhibition and no cell deformations can be set to ≥ 10.0 mg pure metabolite/L the limit concentration tested.

Report:	**KIIA 8.4/04; Bruns, E. (2012)**
Title:	*Pseudokirchneriella subcapitata* growth inhibition test with 6-chloronicotinic acid
Report No:	EBRVP242
Document No:	M-424145-01-2
Guidelines:	**OECD Guideline 201**
Deviations:	**In the highest test concentration of 100 mg p.m./L 6 test vessels were used in the study. In 3 of these vessels the pH was adjusted to 8.2 (pH of control) before introducing the algae. The temperature ranged between 19.4 and 23.0°C. These deviations had no impact on the outcome of the study.**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the influence of 6-chloronicotinic acid (specified by origin batch no.: M12653, certificate no.: AZ 16813 and LIMS no.: 1022273; analysed purity: 98.8% w/w) on the growth rate of the green alga *Pseudokirchneriella subcapitata*.

Cultures of *Pseudokirchneriella subcapitata* with an initial cell density of 10,000 cells/mL were exposed in a static system over a period of 72 hours to nominal concentrations of 6.25, 12.5, 25.0, 50.0, 100 (pH not adjusted) and 100 (pH adjusted) mg pure metabolite/L in comparison to a control.

In the highest test concentration of 100 mg p.m./L 6 test vessels were used in the study. In 3 of these vessels the pH was adjusted to 7.5 - 8.2 (pH of control) before introducing the algae, whereas in the remaining (not pH adjusted) vessels the pH was 5.1 – 6.6.

Quantitative amounts of 6 - chloronicotinic acid were measured in all treatment groups and in the control on day 0 and day 3 of the exposure period. The analytical findings of 6 - chloronicotinic acid in the treatment levels found on day 0 were 100 % to 112 % of nominal (average 105 %). On day 3 analytical findings of 98.7 % to 110 % of nominal (average 105 %) were found. All results are based on nominal test concentrations of the metabolite.

The 72 hour growth rate based on cell density was used to determine the endpoints.

Test concentration of 100 mg p.m./L not pH adjusted (pH 5.1 – 6.6):

The E_rC_{50} was 114 mg p.m./L, the E_rC_{10} was 80.5 mg p.m./L and the NOE_rC was 50.0 mg p.m./L.

Test concentration of 100 mg p.m./L pH adjusted (pH 7.5 – 8.2):

The E_rC_{50} was > 100 mg p.m./L, the E_rC_{10} was 130 mg p.m./L and the NOE_rC was ≥ 100 mg p.m./L.

The observed results demonstrate that the effects detected at 100 mg pure metabolite/L are caused by the highly acidic nature of the solutions and are not of relevance in the natural environment, the proposed end-points for risk assessment are therefore the value derived from the pH adjusted test .

The (0 - 72h)-E_rC_{50} for 6 - chloronicotinic acid is > 100 mg p.m./L,

the (0 - 72h) - NOE_rC is ≥ 100 mg p.m./L.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	6-chloronicotinic acid (6-CNA)
Type of formulation:	Substance, technical (pure metabolite)
Chemical state and description:	Beige powder
Batch No.:	M12653
Code:	AE F161089 00 1B99 0001
Sample description:	AZ 16813
Purity:	> 98.8% w/w
Storage conditions:	+5 ± 5°C

2. Test solutions

Vehicle:	None
Controls:	Water control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Pseudokirchneriella subcapitata*
Common name:	Green alga
Source:	Collection of Algal Cultures, Inst. for Plant Physiology, University of Goettingen, Nikolausberger Weg 18, 37077 Goettingen, Germany
Maintenance of pre-culture:	
Temperature:	19.4 to 23.0°C
Photoperiod:	permanent light

B. Study design and methods

1. In life dates November 11 to December 20, 2011

2. Design of biological test

Green alga (*Pseudokirchneriella subcapitata*) were exposed to the metabolite 6 - chloronicotinic acid in a chronic multigeneration test for 3 days under static exposure conditions to nominal concentrations of 6.25, 12.5, 25.0, 50.0, 100 (pH not adjusted) and 100 (pH adjusted) mg pure metabolite/L. In addition, a water control was tested.

Each vessel (Erlenmeyer flasks; 300 mL) served as one replicate filled with 150 mL test solution. At test initiation the cell density was 10,000 cells/mL.

The test was conducted with 3 replicates per test level and 6 replicates per control.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at 0 and 72 hours from all concentrations. HPLC-MS/MS was used as analytical method. The limit of quantification (LOQ) was 0.11 µg/L. The range of linearity was 0.11 µg/L to 11 µg/L, with an injection volume of 2.5 µL. The correlation coefficient was 0.9995.

4. Observation and measurements

Growth rates, observation on cell abnormalities and physical-chemical water parameters were assessed as indicated below in the result section. Growth inhibition was calculated using algae biomass per volume. The surrogate for biomass was cell density (used as response parameter); measurable by direct

counting of algae cells per volume or indirectly by calculation of cell numbers after measurement of optical cell density.

5. Statistical analysis

Statistical Software "ToxRat Professional", version 2.10 was used.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	19.4 to 23°C (mean 21.0°C)
pH:	Ranged from 7.8 to 8.2 in the controls; from 7.8 to 8.0 in 6.25 mg/L; from 7.5 to 8.1 in 12.5 mg/L; from 7.6 to 8.1 in 25 mg/L; from 7.4 to 7.9 in 50 mg/L; from 5.1 to 6.2 in 100 mg/L (pH not adjusted) and from 7.5 to 8.2 in 100 mg/L (pH adjusted)
Photoperiod:	Permanent light
Light source	Cool white fluorescent lamps
Light intensity:	Mean 7939 lux (range 7430 to 8540 lux)

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations calculated as arithmetic mean. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of 6-chloronicotinic acid

Nominal concentration [mg/L]	Measured concentration [mg/L] average day 0	% recovery	Measured concentration [mg/L] average day 3	% recovery
control	< 0.0009831	--	< 0.0009831	--
6.25	6.99	112	6.70	107
12.5	13.1	105	13.5	108
25.0	25.6	102	26.3	105
50.0	50.4	101	49.8	99.6
100	100	100	98.7	98.7
100 (pH adjusted)	111	111	110	110
	mean	**105**	**mean**	**105**

C. Biological Findings

No cell abnormalities were observed.

Observations on growth rates are listed as follows:

Table: Effect of 6 - chloronicotinic acid on 72-hour-growth-inhibition of *Pseudokirchneriella subcapitata*

nominal concentration [mg p.m./L]	cell number after 72 h (means) per mL	(0-72h)-average specific growth rates [days^{-1}]	inhibition of average specific growth rate [%]
control	1 022 000	1.540	--
6.25	1 075 000	1.555	-1.0
12.5	1 277 000	1.612	-4.7
25.0	1 543 000	1.679	-9.0
50.0	1 206 000	1.595	-3.6
100	238 000	1.055	31.5
100 (pH adjusted)	860 000	1.483	3.7

test initiation with 10000 cells/mL
-% inhibition: increase in growth relative to the control

D. Validity Criteria

Biomass increased in the control by more than 16-fold within the evaluation period. Mean percent coefficient of variation of sectional growth rates from day 0-1, day 1-2, and day 2-3 in the control did not exceed 35%. Percent coefficient of variation of the average growth rate in each control replicate did not exceed 7%. The validity criteria are therefore fulfilled.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

Test concentration of 100 mg p.m./L not pH adjusted (pH 5.1 – 6.6):

Average growth rate (0 - 72 h)	E_rC_{50}	114 mg p.m./L
	E_rC_{10}	80.5 mg p.m./L
	LOE_rC	100 mg p.m./L
	NOE_rC	50.0 mg p.m./L

Test concentration of 100 mg p.m./L pH adjusted (pH 7.5 – 8.2):

Average growth rate (0 - 72 h)	**E_rC_{50}**	**> 100 mg p.m./L**
	E_rC_{10}	130 mg p.m./L
	LOE_rC	> 100 mg p.m./L
	NOE_rC	≥ 100 mg p.m./L

CONCLUSION

Test concentration of 100 mg p.m./L not pH adjusted (pH 5.1 – 6.6):

The (0 - 72h)-E_rC_{50} for 6 - chloronicotinic acid is 114 mg p.m./L,

the (0 - 72h)-E_rC_{10} is 80.5 mg p.m./L,

the (0 - 72h)-NOE_rC is 50.0 mg p.m./L.

Test concentration of 100 mg p.m./L pH adjusted (pH 7.5 – 8.2):

The (0 - 72h)-E_rC_{50} for 6 - chloronicotinic acid is > 100 mg p.m./L,

the (0 - 72h)-E_rC_{10} is 130 mg p.m./L,

the (0 - 72h) - NOE_rC is ≥ 100 mg p.m./L.

The observed results demonstrate that the effects detected at 100 mg pure metabolite/L are caused by the highly acidic nature of the solutions and are not of relevance in the natural environment, the proposed end-points for risk assessment are therefore the value derived from the pH adjusted test .

The (0 - 72h)-E_rC_{50} for 6 - chloronicotinic acid is > 100 mg p.m./L,

the (0 - 72h) - NOE_rC is ≥ 100 mg p.m./L.

IIA 8.5 Effects on sediment dwelling organisms

Based on the European triggers and the EU-Aquatic Guidance Document, studies with sediment dwelling organisms are required if, in the water-sediment study, > 10% of applied radioactivity represented by the parent compound is present in the sediment at or after day 14, and the chronic NOEC for *Daphnia* is < 0.1 mg/L. A further trigger may be a high acute toxicity to *Chironomus* in combination with an occurrence in the sediment at greater than 10 %.

In laboratory studies (see Section 5, point 9.7) BYI 02960 was found in sediment in amounts exceeding 10%, however under more natural conditions, but still confined conditions of an outdoor micro-cosm study the amount of BYI 02960 was much lower (< 10% for the test at 10 µg/L).

The test species recommended for testing sediment dwelling organisms is *Chironomus riparius*, this species has been tested as a second sensitive indicator species for invertebrates. For BYI 02960, the acute toxicity to *Chironomus* was considerably higher than that observed Daphnia. Considering the physico-chemical properties of BYI 02960 (aquatic solubility and log Koc) a spiked water test is considered more relevant for the use of BYI 02960 to determine the risk to benthic organisms. This test was summarized under 8.2.3.3 above but is considered to address also the risk to sediment dwelling organisms.

For the metabolites of BYI 02960 there is little concern for the benthic compartment as the K_{OC} values are very low, testing on chironomids indicates that the metabolites are much less toxic than the parent and further testing is not required.

According to US and Canadian directives toxicity studies with sediment organisms are not required due to the low K_{OC}-value of BYI 02960 and the metabolites suggesting no relevant exposure of the sediment compartment

IIA 8.5.1 Acute test

An acute test to the test species *Chironomus riparius* was summarized under 8.3.1.2. This test allows conclusions on the intrinsic toxicity to the midge. However, a sediment substrate is not included into the test. For reasons of completeness, the summary of the conclusions of the test is summarized here again.

Report:	**KIIA 8.5.1/01; Bruns, E. (2011)**
Title:	Acute toxicity of BYI 02960 (tech.) to larvae of *Chironomus riparius* in a 48 h static laboratory test system
Report No:	EBRVP026
Document No:	M-414739-01-2
Guidelines:	**No specified guideline; study is performed according to general aspects as quoted under OECD Guideline No. 202**
Deviations:	**According to test system**
GLP:	**Yes (certified laboratory)** **Screening of water for contaminants was not performed under GLP as described in the study report**

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2%) on larvae of *Chironomus riparius*.

Following 48 h of exposure 0, 0, 2.5, 2.5, 30, and 85% immobilisation was observed among daphnids exposed to the 3.125, 6.25, 125.5, 25.0, 50.0, and 100 mg a.i./L treatment levels, respectively. No immobilisation occurred in the water control. Therefore, the 48-hour-EC_{50} was 61.7 µg a.i./L (95% confidence limits 41.4 to 109 µg a.i./L), the 48-hour-NOEC was determined to be 25 µg a.i./L.

IIA 8.5.2 Chronic test

The testing rationale was described under section 8.5., chronic spiked water test with the recommended species, *Chironomus riparius* ,are available for BYI 02960, DFA and 6 CNA and summarized under 8.3.2.2. For reasons of completeness, the conclusions of test for the parent compound are summarized here again.

Report:	**KIIA 8.5.2/01 Bruns, E. (2011)**
Title:	*Chironomus riparius* 28-day chronic toxicity test with BYI 02960 (tech.) in a water-sediment system using spiked water
Report No:	EBRVP025
Document No:	M-401792-01-2
Guidelines:	**OECD Guideline 219**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** **Screening of contaminants in water was not performed according to GLP**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2% w/w) on emergence and development of *Chironomus riparius*.

The start of emergence was reduced for one day at test concentration of 20.0 µg a.i./L. No emergence was observed at the highest test concentration of 40.0 µg a.i. /L. The NOEC based on emergence rate and development rate was determined to be 10.0 µg a.i./L (initial measured: 10.5 µg a.i./L..

IIA 8.6 Effects on aquatic plants

In Europe tests on aquatic plants are not required for non-herbicidal substances. Hence, as BYI 02960 is a selective insecticide, tests on higher plants are not required.

For The US and Canada data must be provided for the macrophyte *Lemna*, therefore a 7 day growth inhibition test is available. As there is no indication that the metabolites have a relevant activity no testing for the metabolites has been performed.

A general comment is considered appropriate with respect to the evaluation of effects in *Lemna* studies based on two parameters, i.e. frond number and dry weight or total frond area. “Growth rate” is the only and relevant response variable, which can then be used in risk assessments. This issue is addressed within the OECD guideline 221 in paragraph 51 (finalized in 2006). It is clearly stated that the response variable “average specific growth rate” is independent of the absolute level of the specific growth rate of the control, slope of the concentration-response curve or on test duration, and is therefore the

preferred measure for reliable endpoint calculations. The response variable "yield" is a USEPA national requirement and, although calculated and reported, should not be used for comparing the toxicity of toxicants.

This argumentation in OECD 221 reflects the actual status of scientific discussions, and is considered more relevant than the corresponding chapter 2.4.1 in the Aquatic Guidance Document (issued in 2002).

Report:	**KIIA 8.6/01; Banman, C.S., Alexander, T.M & Lam, C.V. (2010)**
Title:	Toxicity of BYI 02960 Technical to Duckweed (*Lemna gibba* G3) Under Static-Renewal Conditions
Report No:	EBRVP043
Document No:	M-398376-01-1
Guidelines:	**OECD Test Guideline 221:** **FIFRA Guideline 123-2** **OPPTS 850.4400**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239; purity 96.2%) on growth of duck weed (*Lemna gibba*).

Cultures of *Lemna gibba* with an initial density of 12 fronds per vessel were exposed in a static renewal (one renewal at day 3) system over a period of 7 days to nominal concentrations of 5.0, 10, 20, 40 and 80 mg a.i./L (corresponding to analytically verified concentrations of 4.02, 8.17, 16.0, 34.2 and 67.7 mg a.i./L (80 to 86% of nominal)). In addition a water control and solvent control (< 0.5 mg a.i./L) were tested. Growth was determined by frond counts on Days 0, 3, 5 and 7 and frond dry weights from Day 0 and Day 7. Frond numbers and total frond area at each occasion were used to determine the endpoints. Based on analytical findings the biological endpoints are reported as mean measured figures.

The EC_{50} regarding growth inhibition was > 67.7 mg a.i./L for both, frond number and dry weight.

The NOEC was determined to be 34.2 mg a.i./L and 67.7 mg a.i./L for frond number and dry weight, respectively.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difluoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2%
Storage conditions:	Room temperature
Water solubility:	Approximately 80 mg a.i./L under test conditions

2. Test solutions

Vehicle:	Dimethylformamide (DMF)
Concentration of vehicle:	0.1 mL/L
Method of preparation:	Highest test level: sonicated approximately 4 hours (Day 0) and approximately 5 hours (Day 3), inverted several times; Remaining test levels: inverted several times
Controls:	Water control and solvent control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Lemna gibba*
Common name:	Duck weed
Strain:	G3
Source:	United States Department of Agriculture Fruit Laboratory, Beltsville Maryland (received on June 3, 2004).
Maintenance of pre-culture:	
Temperature:	25.0 ± 2.0 °C
Photoperiod:	24 hours light

B. Study design and methods

1. In life dates April 30 to May 7, 2010

2. Design of biological test

Duck weed (*Lemna gibba*) were exposed to BYI 02960 (purity 96.2%) in a static renewal (one renewal at day 3) system over a period of 7 days. Nominal concentrations were 5.0, 10, 20, 40, and 80 mg a.i./L. In addition a water control and a solvent control were tested. Each vessel (borosilicate glass crystallisation dishes; 250 mL) filled with 100 mL 20xAAP with an initial pH of 7.5 ± 0.1, served as one replicate. At test initiation the number of fronds was 12 fronds per vessel. The test was conducted with 3 replicates per treatment level.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0 (new), day 3 (new and old) and day 7 (old) from all concentrations. BYI 02960 (99.4% w/w) served as analytical standard. The limit of quantification (LOQ) was 0.5 mg a.i./L.

4. Observation and measurements

Growth rates, observation on cell abnormalities and physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

Raw or transformed data from treatment groups were compared to controls for normality and homogeneity of variance using the Shapiro-Wilks test and Levene's test of equal variance, respectively. If normality and homogeneity of variance were demonstrated for the raw or transformed values, then parametric analyses were conducted using analysis of variance (ANOVA) followed by Dunnett's test.

If normality and/or homogeneity of variance were not demonstrated on raw or transformed values, nonparametric procedures were used. The ranks of the raw values were determined, and then an analysis of variance and a one-tailed Dunnett's test were performed on these ranks. Statistical analyses were conducted using SAS.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarised as follows:

Test temperature:	24.62 to 25.01 °C (mean: 24.78 °C)
pH:	7.9 to 9.2
Photoperiod:	24 hours light
Light intensity:	5070 to 6370 lux (mean: 5764 lux)
Conductivity:	1509 to 1578 µmhos/cm (mean: 1553 µmhos/cm)

B. Analytical Findings

Analytical verification of test solutions resulted in measured concentrations of <0.5 (control and solvent control), 4.02, 8.17, 16.0, 34.2 and 67.7 mg a.i./L (80 to 86% of nominal) calculated as arithmetic mean. Based on these analytical findings the biological endpoints are reported as mean measured figures. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960

	Day 0		Day 3 (old)		Day 3 (new)		Day 7		Mean	
Nominal Conc. (mg a.i./L)	Measured Conc. (mg a.i./L)	% nominal	Measured Conc. (mg a.i./L)	% nominal	measured Conc. (mg a.i./L)	Day 3 (new) % nominal	Measured Conc. (mg a.i./L)	Day 7 % nominal	Mean measured Conc. (mg a.i/L)	Mean measured % nominal
Control	< 0.5	---	< 0.5	---	< 0.5	---	< 0.5	---	< 0.5	---
Solvent Control	< 0.5	---	< 0.5	---	< 0.5	---	< 0.5	---	< 0.5	---
5	3.39	68	3.55	71	4.46	89	4.67	93	4.02	80
10	6.7	67	7.2	72	9.14	91	9.63	96	8.17	82
20	13.1	66	14.1	71	17.2	86	19.6	98	16	80
40	26	65	29	72	41.7	104	40.3	101	34.2	86
80	52.4	65	61.7	77	77	96	79.9	100	67.7	85

C. Biological Findings

Growth inhibition was observed as listed below.

Table: Effect of BYI 02960 on growth-inhibition (frond number) of *Lemna gibba*

Mean measured test levels [mg a.i./L]	Final frond no. (replicate means, day 7)	Growth Rate for Frond Numbers [day^{-1}]	% inhibition (growth rate for frond no.)
Control	238	0.017771	---
Solvent Control	217	0.01722	---
Pooled Controls	228	0.017496	---
4.02	208	0.016966	3.0
8.17	215	0.017145	2.0
16.0	191	0.016469	5.9
34.2	219	0.017233	1.5
67.7	169*	0.015701*	10.3

* Statistically different from controls ($p<0.05$)

Table: Effect of BYI 02960 on growth-inhibition (dry weight) of *Lemna gibba*

Mean measured test levels [mg a.i./L]	Dry weight (means, day 7) [g]	Growth Rate for Frond Dry Weight [day^{-1}]	% inhibition (growth rate for dry weight)
Control	0.0272	0.016273	---
Solvent Control	0.0252	0.015811	---
Pooled Controls	0.0262	0.016042	---
4.02	0.0238	0.015467	3.6
8.17	0.0249	0.015717	2.0
16.0	0.0230	0.015261	4.9
34.2	0.0280	0.016315	-1.7
67.7	0.0251	0.014814	7.7

No cell abnormalities were observed.

D. Validity Criteria

The validity criterion of a doubling time less than 60 hours (2.5 days) in the control is fulfilled.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

Test Substance	BYI 02960 Technical
Test Object	*Lemna gibba* G3
Exposure	7-Day, static-renewal
7-day E_rC_{50} – growth rate for frond numbers	**> 67.7 mg a.i./L**
7-day E_bC_{50} – cumulative biomass for frond numbers	**> 67.7 mg a.i./L**
7-day EC_{50} – frond count	> 67.7 mg a.i./L
7-day EC_{50} – frond dry weight	> 67.7 mg a.i./L
7-day E_rC_{50} – growth rate for frond dry weight	> 67.7 mg a.i./L
Lowest Concentration With an Effect (LOEC)	67.7 mg a.i./L (frond counts)
Highest Concentration Without Toxic Effect (NOEC)	34.2 mg a.i./L (frond counts)

CONCLUSION

The NOEC and LOEC in the 7-day exposure study of *Lemna gibba* G3 to BYI 02960 technical were 34.2 and 67.7 mg a.i./L, respectively for the endpoints of 7 day frond counts, cumulative biomass for

fronds and growth rate for frond counts. EC_{50} values could not be calculated for any end-point and were determined to be greater than the highest test concentration (> 67.7 mg a.i./L).

IIA 8.7 Effects on bees

The acute oral and contact toxicity had been determined for BYI 02960 and plant metabolites to which bees could potentially be exposed, these include the metabolites BYI 02960-DFEAF, BYI 02960-OH, BYI 02960-CHMP, DFA, 6-CNA.

Additionally 10 day feeding studies have been performed for BYI 02960 the metabolites BYI 02960-DFEAF, BYI 02960-OH, BYI 02960-CHMP, DFA, 6-CNA, these studies are summarised under point 8.16.1.

A study on the effects of exposure af larvae to BYI 02960 has also been performed and is summarised under point 8.16.

IIA 8.7.1 Acute oral toxicity

Report:	**KIIA 8.7.1/01; Schmitzer, S. & Sekine, T. (2008)**
Title:	Effects of BYI 02960 (Acute Contact and Oral) on Honey Bees (*Apis mellifera* L.) in the Laboratory
Report No:	41121035
Document No:	M-308904-02-1
Guidelines:	**OECD Guideline 213** **OECD Guideline 214**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of BYI 02960 (Sample description: TOX 08080-00 (Batch ID: NLL 7780-44-6); purity: 99.5% w/w) on mortality of the honey bee (*Apis mellifera*) after oral or contact exposure.

In the oral dose response test 30 adult worker honey bees were exposed for 48 hours to doses of 2.8, 2.1, 1.3, 0.68, 0.34 and 0.17 µg a.i. per bee by feeding (values based on the actual intake of the test item). For the contact dose response test 30 honey bees were exposed for 96 hours to doses of 200.0, 100.0, 50.0, 25.0 and 12.5 µg a.i. per bee by topical application. The contact toxicity test was prolonged for 48 hours due to increasing mortality between 24 and 72 hours, up to a maximum of 96 hours.

In addition, negative controls [oral: a) 50% aqueous sugar solution and b)acetone/sugar solution; contact test: a) tap water + 0.5% Adhäsit and b) acetone] and a toxic reference (Dimethoate; 400 g/L nominal) at nominal dose rates of 0.30, 0.15, 0.08 and 0.05 µg dimethoate/bee were tested.

In the oral toxicity test, no mortality occurred in both the negative control and the solvent control group. The oral LD_{50} values (24 + 48 h) of the test item BYI 02960 were calculated to be 1.3 and 1.2 µg a.i./bee, respectively. Behavioural abnormalities (e.g. movement coordination problems and apathy) were observed in the four highest treatment groups during the first 4 hours in the oral toxicity test. After 24 hours and 48 hours no behavioural abnormalities were observed among the surviving bees.

In the contact toxicity test, 10% and 3.3% mortality occurred in the negative control (water + 0.5% Adhäsit) and the solvent control (acetone) group, respectively. The contact LD_{50} values (24, 48, 72 and 96 h) of the test item BYI 02960 were determined to be >200, >200, 158.4 and 122.8 µg a.i./bee,

respectively. No significant test item related behavioural abnormalities occurred in the contact toxicity test except for a few bees among the two highest dose groups (200.0 and 100.0 µg a.i./bee) showing movement coordination problems and/or apathy from 48h onwards.

MATERIAL AND METHODS

A. Materials

1. Test Material

Test item:	BYI 02960
Type:	Substance, technical
Chemical state and description:	Solid, pink
Batch No.:	NLL 7780-44-6
Sample description:	TOX 08080-00
Purity:	99.5% w/w according to certificate of analysis
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Solubility:	In water: 3.24 g/L In acetone: not available at start of study
Stability of test compound:	Expiry date: 16.07.2008, when stored at +25 ± 5°C in original container in the dark

2. Vehicle and/or positive control

Oral Test:	a) 50% w/w sugar (syrup) solution (in tap water); b) 50% sugar solution with solvent (45% water, 5% acetone, 50% sugar)
Contact Test:	a) Tap water with 0.5% Adhäsit* (applied after anesthetization with CO_2); b) Acetone (applied after anesthetization with CO_2) * (Adhäsit improves spreading of the test droplet on the water-repellent hairs on the thorax of bees)

Wetting Agent:

Name:	Adhäsit
Batch No.:	0150207
Nominal content of active substance:	100 g/L Marlopon (nominal)
Manufacturer:	Spiess-Urania Chemicals GmbH, Heidenkampsweg 77, 20097 Hamburg, Germany
Stability:	Expiry date: 12/2009, when stored in original container, at room temperature (10 °C to 30 °C), in the dark
Target Amount in this Study:	0.5%

Reference Item:

The information concerning the reference item according to the substance container label and data sheet:

Name:	Perfekthion EC (BAS 152 11 I)
Manufacturer:	BASF AG, Agricultural Center Limburgerhof, D-67114 Limburgerhof
Batch No.:	1814
Nominal content of active ingredient:	Dimethoate: 400 g/L
Analytical content of active ingredient:	Dimethoate: 395.9 g/L according to certificate of analysis
Certificate of Analysis Study Code:	330040_1
Type:	Insecticide
Chemical state and description:	Liquid, blue
Density:	1.066 g/cm^3
Solubility:	In water: emulsifiable

Stability:	Expiry date: 01.11.2008, when stored in refrigerator (4 ± 4°C), in original container in the dark. In water: reference item is considered stable under test conditions

3. Test organisms

Species:	*Apis mellifera carnica* L.
Common name:	Honey bee
Age or developmental stage at test start:	Female adult worker bees
Source:	Disease-free and queen-right honeybee colonies, bred by IBACON

B. Study design and methods

1. In life dates April 23 to July 13, 2008

2. Experimental treatments

Test units were stainless steel chambers of 10 cm x 8.5 cm x 5.5 cm (length x width x height), the front side was a removable glass sheet, the bottom was perforated with 98 ventilation holes (Ø 1 mm), the inner walls were lined with filter paper.

10 bees were used per replicate unit, 3 replicates per treatment group (i.e. 30 individuals per treatment group).

Exposure time for both tests was 48 hours. The contact test was prolonged for a further 48 hours due to increasing mortality between 24 and 72 hours up to a maximum of 96 hours.

Food was commercial ready-to-use syrup (Apiinvert; 30% Saccharose, 31% Glucose, 39% Fructose) *ad libitum* was supplied via syringes directly after treatment.

Bees in the oral test were starved for 20 minutes prior to test start.

Bees in the contact test were anesthetized for *ca.* 20 seconds with CO_2 until they were completely immobilized immediately before application (only in the contact test).

Control:

Contact test: a) CO_2/tap water + Adhäsit[1]; b) CO_2/acetone

Oral test: a) Aqueous sugar solution; b) Aqueous sugar solution + acetone

Test item:

Contact Test:

Nominal dosage 200.0, 100.0, 50.0, 25.0 and 12.5 µg a.i./bee

Oral Test:

Nominal dosage 5.0, 2.5 1.3, 0.63, 0.31 and 0.16 µg a.i./bee

Measured dosage 2.8, 2.1, 1.3, 0.68, 0.34 and 0.17 µg a.i./bee

Toxic reference item:

Contact test:

Nominal dosage 0.30, 0.20, 0.15 and 0.10 µg Dimethoate per bee

Oral Test:

Nominal dosage 0.30, 0.15, 0.08 and 0.05 µg Dimethoate per bee

Measured dosage 0.26, 0.16, 0.08 and 0.06 µg Dimethoate per bee

Application of the test item in the contact test:

Bees were anaesthetized with CO_2 in the contact test. A single 5 µL droplet of BYI 02960 in an appropriate carrier (acetone) was placed on the dorsal bee thorax using a Burkard – Applicator. For the control one 5 µL droplet of a) tap water containing 0.5% Adhäsit* and b) pure acetone was used. The reference item was also applied in 5 µL (dimethoate made up in acetone). A 5 µL droplet was chosen in deviation to the guideline recommendation of a 1 µL droplet, since a higher volume ensured a more reliable dispersion of the test item; Ibacon experience has proven that higher volumes are suitable and no adverse effects on the outcome of the study are to be expected.

Application of the test item in the oral test:

Appropriate amounts of BYI 02960 or reference item dilutions in acetone were mixed with syrup (ready-to-use syrup; Apiinvert, Suedzucker, D-97195 Ochsenfurt; composition of the sugar component: 30% Saccharose, 31% Glucose, 39% Fructose) in order to achieve the required test concentrations in a final dilution of 50% syrup solution (45% water, 50% syrup and 5% acetone (w/w)). For the controls, the same proportion of syrup, water and acetone was used (solvent control) and similarly, 50 % aqueous syrup solution was used for the negative control. The treated food was offered in syringes, which were weighed before and after introduction into the cages (duration of uptake was 1 – 6 hours for the test item treatments). After a maximum of 6 hours, the syringes containing the treated food were removed, weighed and replaced by ones containing fresh, untreated food.

The target dose levels (*e.g.* 5.0 µg a.i./bee nominal) would have been obtained if 20 mg/bee of the treated food was ingested. In practice, higher (or lower) dose levels were obtained as the bees had a higher or lower uptake of the test solutions than the nominal 20 mg/bee.

3. Observation and measurements:

The number of dead bees was determined after 4 hours (first day); 24 and 48 hours (contact and oral test); 72 and 96 hours (contact test). Behavioural abnormalities (vomiting, apathy, intensive cleaning) were assessed after 4 hours (first day); 24 and 48 hours (contact and oral test); 72 and 96 hours (contact test).

4. Statistical analysis

Results obtained with the bees treated with the test item and the reference item were compared to those obtained with the control in both the contact and oral tests.

The contact and oral LD_{50} of the test item were estimated with Probit Analysis (according to Finney 1971). The contact and oral LD_{50} of the reference item were estimated according to moving average computations (Thompson and Weil, 1952). The LD_{50} calculation was carried out taking into account the mortality data corrected by control mortality using Abbott's formula (1925).

The NOED was estimated using Fisher Exact Test (pairwise comparison, one-sided greater, $\alpha = 0.05$), which is a distribution-free test and does not require testing for normality or homogeneity prior to analysis.

The software used to perform the statistical analysis was ToxRat Professional, Version 2.09 and 2.10, ® ToxRat Solutions GmbH, © 2005.

[1] Adhäsit was used to improve the spreading of the test droplet on the bee body. Adhäsit is non-toxic to honey bees.

RESULTS AND DISCUSSION

A. Environmental Parameters

Measurements of climatic parameters during the test are summarized as follows:

Test temperature:	25°C
Relative air humidity:	43 to 71%
Light intensity:	Darkness (except during observation)
Ventilation:	Ventilation to avoid possible accumulation of pesticide vapour
Recording:	Test conditions were continuously recorded with an electronic data logger and documented in the raw data

B. Biological Findings

Contact Test:

Mortality occurred in all treatment groups in a dose related pattern. The contact test was prolonged for a further 48 hours up to 96 hours due to increasing mortality between 24 and 72 hours. The contact dose levels of 200.0 to 12.5 µg a.i./bee resulted in mortality ranging from 73.3% to 6.7% at the end of the test (96 hours after application).

The mortality in the water control and the solvent control were 10.0% and 3.3%, respectively.

A prolongation was necessary because mortality was still increasing between 24 and 72 hours.

Behavioural abnormalities (e.g. movement coordination problems) were found in a single bee at 50.0 µg a.i./bee dose level during the 4-hour assessment. These behavioural impairments were found at two highest dose levels from 48 to 96 hours following the application.
There were behavioural abnormalities consistent with the observed toxicity in the reference item test.

Table: Mortality and behavioural abnormalities of the bees in the contact toxicity test

	after 4 hours		after 24 hours		after 48 hours		after 72 hours		after 96 hours	
	mortality	behav. abnorm.	mortality	behav. abnorm.	mortality	behav. abnorm.	mortality	behav. abnorm.	mortality	behav. abnorm.
dosage [µg a.i./bee]	mean %	mean %	mean %	mean %	mean %	mean %	mean %	mean %	mean %	mean %
test item										
200.0	0.0	0.0	0.0	0.0	36.7	6.7	60.0	33.3	73.3	26.7
100.0	3.3	0.0	3.3	0.0	23.3	6.7	33.3	6.7	36.7	10.0
50.0	0.0	3.3	6.7	0.0	6.7	0.0	20.0	0.0	23.3	0.0
25.0	0.0	0.0	0.0	0.0	6.7	0.0	13.3	0.0	13.3	0.0
12.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.7	0.0
water	0.0	0.0	0.0	0.0	0.0	0.0	6.7	0.0	10.0	0.0
solvent	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.3	0.0
reference item		0.0								
0.30	33.3	26.7	96.7	0.0	96.7	0.0	96.7	3.3	96.7	3.3
0.20	3.3	33.3	80.0	0.0	90.0	0.0	96.7	3.3	96.7	3.3
0.15	0.0	0.0	30.0	0.0	36.7	0.0	43.3	36.7	43.3	16.7
0.10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.0	6.7	6.7

results are averages from three replicates (ten bees each) per dosage/control

behav. abnorm. = behavioural abnormalities; water = CO_2/water-treated control
solvent = CO_2/solvent control

Oral Test:

Mortality occurred in all treatment groups (except at 0.34 µg a.i./bee): oral doses of 2.8, 2.1, 1.3 and 0.68 µg a.i./bee, respectively, resulted in mortality ranging between 100.0 and 6.7% (48 hours after application). 3.3% mortality occurred at 0.17 µg/bee.

There was no mortality in the water and the solvent control.

During the 4 hours check, dis-coordinated movements and apathy occurred in the dose level of 2.8, 2.1, 1.3 and 0.68 µg a.i./bee groups, respectively. After 24 and 48 hours no further behavioural impairments were observed in any of test item treatment groups.

There were behavioural abnormalities consistent with the observed toxicity in the reference item test.

Table: Mortality and behavioural abnormalities of the bees in the oral toxicity test

	after 4 hours		after 24 hours		after 48 hours	
	mortality	behav. abnorm.	mortality	behav. abnorm.	mortality	behav. abnorm.
uptaken dosage [µg a.i./bee]	mean %	mean %	mean %	mean %	mean %	mean %
test item						
2.8	73.3	26.7	100.0	0.0	100.0	0.0
2.1	46.7	53.3	90.0	0.0	90.0	0.0
1.3	46.7	40.0	53.3	0.0	53.3	0.0
0.68	0.0	6.7	6.7	0.0	6.7	0.0
0.34	0.0	0.0	0.0	0.0	0.0	0.0
0.17	0.0	0.0	0.0	0.0	3.3	0.0
water	0.0	0.0	0.0	0.0	0.0	0.0
solvent	0.0	0.0	0.0	0.0	0.0	0.0
reference item						
0.26	0.0	50.0	100.0	0.0	100.0	0.0
0.16	0.0	0.0	80.0	3.3	83.3	0.0
0.08	0.0	0.0	10.0	0.0	10.0	0.0
0.06	0.0	0.0	3.3	0.0	3.3	0.0

results are averages from three replicates (ten bees each) per dosage/control

behav. abnorm. = behavioural abnormalities; solvent = solvent control

C. Validity Criteria

The contact and oral test are considered valid as the control mortality in each case was ≤ 10% and the LD_{50} values obtained with the reference item (dimethoate) were within the required ranges.

D. Biological Endpoints Derived

An overview of the endpoints derived for both acute contact and acute oral toxicity test is given below:

Acute contact toxicity test	Contact $LD_{50\ (24h)}$ of BYI 02960: > 200.0 µg a.i./bee Contact $LD_{50\ (48h)}$ of BYI 02960: > 200.0 µg a.i./bee Contact $LD_{50\ (72h)}$ of BYI 02960: 158.4 µg a.i./bee Contact $LD_{50\ (96h)}$ of BYI 02960: 122.8 µg a.i./bee Contact $NOED_{(72h)}$ of BYI 02960: 25 µg a.i./bee
Acute oral toxicity test	Oral $LD_{50\ (24\ h)}$ of BYI 02960: 1.3 µg a.i./bee Oral $LD_{50\ (48\ h)}$ of BYI 02960: 1.2 µg a.i./bee Oral $NOED_{(48h)}$ of BYI 02960: 0.68 µg a.i./bee
Behavioural Abnormalities:	Discoordinated movements and apathy occurred in both toxicity tests.

CONCLUSION

The contact LD_{50} values (24, 48, 72 and 96 h) of the test item BYI 02960 were determined to be >200, >200, 158.4 and 122.8 µg a.i./bee, respectively. The oral LD_{50} values (24 + 48 h) of the test item BYI 02960 were calculated to be 1.3 and 1.2 µg a.i./bee, respectively.

Behavioural abnormalities such as dis-coordinated movements and apathy occurred in both toxicity tests.

Report:	**KIIA 8.7.1/02; Schmitzer, S. (2010)**
Title:	Effects of BYI 02960-difluoroethyl-amino-furanone (Acute Contact and Oral) on Honey Bees (*Apis mellifera* L.) in the Laboratory
Report No:	60291035
Document No:	M-398557-01-2
Guidelines:	**OECD Guideline 213** **OECD Guideline 214**
Deviations:	**For the contact test, a 5 µL droplet was chosen (for any of the treatments) in deviation to the guideline recommendation of 1 µL**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of BYI 02960-difluoroethyl-amino-furanone on the honey bee (*Apis mellifera*) after oral or contact exposure.

In the oral dose response test 30 adult female worker honey bees per dose level were exposed for 48 hours to doses of 81.5, 54.3, 26.8, 13.7 and 6.7 µg a.i./bee by feeding (values based on the actual intake of the test item). For the contact toxicity test 30 worker bees per dose level were exposed for 48 hours to doses of 100.0, 50.0, 25.0, 12.5 and 6.3 µg a.i. per bee by topical application .

In addition, negative controls [oral: a) water/sugar and b) acetone/sugar; contact: a) tap water and b) acetone] and a toxic reference (Dimethoate; 400 g/L nominal) at nominal rates of 0.30, 0.15, 0.08 and 0.05 µg dimethoate/bee. Mortality and abnormal behaviour were recorded 4, 24 and 48 hours after test start, respectively. The LD_{50} (48h) of the test item was determined to be > 100.0 µg a.i./bee (highest dose level tested) in the contact toxicity test and > 81.5 µg a.i./bee in the oral toxicity test, respectively. No test item related behavioural abnormalities occurred neither in the contact nor in the oral toxicity test.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI02960-difluoroethyl-amino-furanone (BCS-CC98193)
Type:	Pure metabolite
Chemical state and description:	Solid, brown
Batch No.:	LUI 3009-8-1
Purity:	99.2% w/w according to certificate of analysis
CAS#:	1134834-71-1
Solubility:	In acetone: not indicated
Stability of test compound:	Expiry date: 04.05.2013, when stored at +5 ± 5°C in original container in the dark

Control

Oral Test:	a) 50 % (w/w) aqueous sugar solution (in tap water); b) 50% (w/w) sugar solution (45% water, 5% acetone, 50% sugar)
Contact Test:	a) Tap water with 0.5% Adhäsit* (applied after anesthetization with CO_2) b) Acetone (applied after anesthetization with CO_2) * (Adhäsit improves spreading of the test droplet on the water-repellent hairs on the thorax of bees)

Wetting Agent

Name:	Adhäsit
Batch No.:	0150207
Analytical content of active ingredient:	100 g/L Marlopon (nominal)
Manufacturer:	Spiess-Urania Chemicals GmbH, Heidenkampsweg 77, 20097 Hamburg, Germany
Storage:	Expiry Date: 12/2011, when stored in original container, at room temperature (20 ± 5°C), in the dark
Target Amount in this Study:	0.5%

Reference Item

The information concerning the reference item according to the substance container label and data sheet:

Name:	Perfekthion EC (BAS 152 11 I)
Manufacturer:	BASF AG, Agricultural Center Limburgerhof, D-67114 Limburgerhof
Batch No.:	90924-06
Analytical content of active ingredient:	nominal: Dimethoate: 400 g/L analyzed: Dimethoate: 414.8 g/L according to certificate of analysis
Certificate of Analysis Study Code:	346282_32
Type of formulation:	EC
Aggregate State at Room Temperature:	Liquid
Colour:	Blue
Density:	1.074 g/cm^3
Solubility:	In water: emulsifiable
Stability:	Expiry date: October 07, 2011 Storage: in original container, in refrigerator (≤10 °C), in the dark

2. Test organisms

Species:	*Apis mellifera carnica* L.
Common name:	Honey bee
Age or developmental stage at test start:	Adult female worker bees
Source:	Honey bee colonies, disease-free and queen-right, bred by IBACON

B. Study design and methods

1. In life dates: August 16 to 19, 2010

2. Experimental treatments:

Test units were stainless steel chambers of 10 cm x 8.5 cm x 5.5 cm (length x width x height), the front side was a removable glass sheet, the bottom was perforated with 98 ventilation holes (Ø 1 mm), the inner walls were lined with filter paper.

10 bees were used per replicate unit, 3 replicates per treatment group (i.e. 30 individuals per treatment group).

Exposure time for both tests was 48 hours.

Food was commercial ready-to-use syrup (Apiinvert; 30% Saccharose, 31% Glucose, 39% Fructose).

Bees in the oral test were starved for 20 minutes prior to test start.

Bees in the contact test were anesthetized for *ca.* 20 seconds with CO_2 until they were completely immobilized immediately before application (only in the contact test).

Control:

Contact test:	a) CO_2/tap water + Adhäsit[2];	b) CO_2/acetone
Oral test:	a) Aqueous sugar solution;	b) Aqueous sugar solution + acetone

Test item:

Contact Test:

Nominal dosage	100.0, 50.0, 25.0, 12.5 and 6.3 µg a.i./bee

Oral Test:

Nominal dosage	100.0, 50.0, 25.0, 12.5 and 6.3 µg a.i./bee
Measured dosage	81.5, 54.3, 26.8, 13.7 and 6.7 µg a.i./bee

Toxic reference item:

Contact test:

Nominal dosage	0.30, 0.20, 0.15 and 0.10 µg Dimethoate per bee

Oral Test:

Nominal dosage	0.30, 0.15, 0.08 and 0.05 µg Dimethoate per bee
Measured dosage	0.30, 0.14, 0.08 and 0.05 µg Dimethoate per bee

Application of the test item in the contact test:

Bees were anaesthetized with CO_2 in the contact test. A single 5 µL droplet of the test item BYI 02960-difluoroethyl-amino-furanone in an appropriate carrier (acetone) was placed on the dorsal bee thorax using a Burkard - Applicator. For the controls, one 5 µL droplet of a) tap water with 0.5% Adhäsit and b) pure acetone was used, respectively. The reference item was also applied in a 5 µL droplet (dimethoate made up in acetone).

A 5 µL droplet was chosen in deviation to the guideline recommendation of 1 µL, since a higher volume ensured a more reliable dispersion of the test item; IBACON experience has proven that higher volumes are suitable and no adverse effects on the outcome of the study are to be expected.

Application of the test item in the oral test:

Appropriate amounts of BYI02960-difluoroethyl-amino-furanone or reference item dilutions in acetone were mixed with syrup (ready-to-use syrup; Apiinvert, Südzucker, D-97195 Ochsenfurt; composition of the sugar component: 30% Sucrose, 31% Glucose, 39% Fructose) in order to achieve the required test concentrations in a final dilution of 50% syrup solution (45% water, 50% syrup and 5% acetone (w/w)). For the controls, the same proportion of syrup, water and acetone was used (solvent control) and similarly, 50 % aqueous syrup solution was used for the negative control. The treated food was offered in syringes, which were weighed before and after introduction into the cages (duration of uptake was 3

[2] Adhäsit was used to improve the spreading of the test droplet on the bee body. Adhäsit is non-toxic to honey bees.

hours 20 minutes to 6 hours for the test item treatments). After a maximum of 6 hours, the syringes were removed, weighed and replaced by ones containing fresh, untreated food.

The mean target dose levels (*e.g.* 100 µg a.i./bee nominal) would have been obtained if exactly 20 mg/bee of the treated food were ingested. In practice, uptake of the treated sugar solutions differed slightly from the nominal 20 mg/bee and results are given based on the measured consumption.

3. Observation and measurements:

The number of dead bees was determined after 4 hours (first day); 24 and 48 hours (contact and oral test). Behavioural abnormalities (vomiting, apathy, intensive cleaning) were assessed after 4 hours (first day); 24 and 48 hours (contact and oral test).

Result evaluation:

Results obtained from the bees treated with test item were compared to those obtained from the toxic standard and the controls.

The contact and oral LD_{50} of the reference item were estimated according to moving average computations (Thompson and Weil, 1952).

If necessary, the LD_{50} calculation was carried out taking into account the mortality data corrected by control mortality using Abbott's formula (1925).

The software used to perform the statistical analysis was ToxRat Professional, Version 2.10.05, ® ToxRat Solutions GmbH.

RESULTS AND DISCUSSION

A. Environmental Parameters

Measurements of climatic parameters during the test are summarized as follows:

Test environment:	Incubator
Test temperature:	24 - 25°C
Relative air humidity:	61 to 86 %
Light intensity:	Darkness (except during observation)
Ventilation:	Ventilation to avoid possible accumulation of pesticide vapour
Recording:	Test conditions were continuously recorded with electronic data logger and documented in the raw data

B. Biological Findings

Observations:

Oral Test:

In the oral toxicity test, the maximum nominal dose level of BYI02960 - difluoroethyl - amino - furanone (i.e. 100 µg a.i./bee) was not achieved, because the bees did not ingest the full volume of treated sugar solution, even when offered over a period of 6 hours. The actual intake at the 100 µg a.i./bee - treatment level resulted on average in 81.5 µg a.i./bee. Oral mean doses of 81.5, 54.3, 26.8, 13.7 and 6.7 µg a.i./bee resulted in no mortality in any of the dose levels. No mortality occurred in the solvent control group or in the water control group (50% sugar solution),

Table: Mortality and behavioural abnormalities of the bees in the oral toxicity test

	after 4 hours		after 24 hours		after 48 hours	
	mortality	behav. abnorm.	mortality	behav. abnorm.	mortality	behav. abnorm.
consumed	mean	mean	mean	mean	mean	mean
µg a.i./bee	%	%	%	%	%	%
test item						
81.5	0.0	0.0	0.0	0.0	0.0	0.0
54.3	0.0	0.0	0.0	0.0	0.0	0.0
26.8	0.0	0.0	0.0	0.0	0.0	0.0
13.7	0.0	0.0	0.0	0.0	0.0	0.0
6.7	0.0	0.0	0.0	0.0	0.0	0.0
water control	0.0	0.0	0.0	0.0	0.0	0.0
solvent control	0.0	0.0	0.0	0.0	0.0	0.0
reference item						
0.30	16.7	43.3	96.7	0.0	100.0	0.0
0.14	3.3	20.0	76.7	0.0	83.3	0.0
0.08	0.0	0.0	10.0	3.3	20.0	0.0
0.05	0.0	0.0	0.0	0.0	3.3	0.0

results are averages from three replicates (ten bees each) per dosage/control

behav. abnorm. = behavioural abnormalities

Contact Test:

At the end of the contact toxicity test (48 hours after application), one single bee died in the 25.0 µg a.i./bee dose group (i.e. 3.3% mortality). At the other dose levels (100.0, 50.0, 12.5 and 6.3 µg a.i./bee) no mortality occurred. No mortality occurred in the water control group (water + 0.5% Adhäsit) and there was 3.3% mortality in the solvent control group (acetone).

No test item induced behavioural effects were observed at any time in the contact toxicity test.

Table: Mortality and behavioural abnormalities of the bees in the contact toxicity test

	after 4 hours		after 24 hours		after 48 hours	
	mortality	behav. abnorm.	mortality	behav. abnorm.	mortality	behav. abnorm.
dosage	mean	mean	mean	mean	mean	mean
µg a.i./bee	%	%	%	%	%	%
test item						
100.0	0.0	0.0	0.0	0.0	0.0	0.0
50.0	0.0	0.0	0.0	0.0	0.0	0.0
25.0	0.0	0.0	0.0	0.0	3.3	0.0
12.5	0.0	0.0	0.0	0.0	0.0	0.0
6.3	0.0	0.0	0.0	0.0	0.0	0.0
water control	0.0	0.0	0.0	0.0	0.0	0.0
solvent control	0.0	0.0	0.0	0.0	3.3	0.0
reference item						
0.30	0.0	23.3	86.7	0.0	93.3	0.0
0.20	0.0	3.3	83.3	6.7	83.3	6.7
0.15	0.0	0.0	3.3	0.0	16.7	3.3
0.10	0.0	0.0	0.0	0.0	3.3	0.0

results are averages from three replicates (ten bees each) per dosage/control

behav. abnorm. = behavioural abnormalities

No test item induced behavioural effects were observed at any time in the oral toxicity test.

C. Validity Criteria

The validity criterion of control mortality <10% is fulfilled; the validity criterion regarding the performance of the toxic reference is fulfilled for both contact and oral toxicity test.

CONCLUSION

The toxicity of BYI 02960-difluoroethyl-amino-furanone was tested in both an acute contact and oral (dose response) toxicity test on honey bees. The LD_{50} (48h) of the test item was determined to be > 100.0 µg a.i./bee (highest dose level tested) in the contact toxicity test and > 81.5 µg a.i./bee in the oral toxicity test, respectively. No test item related behavioural abnormalities occurred either in the contact or in the oral toxicity test.

Report:	**KIIA 8.7.1/03; Schmitzer, S. (2011)**
Title:	Effects of BYI 02960-hydroxy (Acute Contact and Oral) on Honey Bees (*Apis mellifera* L.) in the Laboratory
Report No:	63901035
Document No:	M-409606-01-2
Guidelines:	**OECD Guideline 213** **OECD Guideline 214**
Deviations:	**For the contact test, a 5 µL droplet was chosen (for any of the treatments) in deviation to the guideline recommendation of 1 µL**
GLP	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of BYI 02960-hydroxy on the honey bee (*Apis mellifera*) after oral or contact exposure.

In the oral limit test 50 honey bees (adult female worker bees) were exposed for 48 hours to a single dose of 105.3 µg a.i./bee by feeding (value based on the actual intake of the test item). For the contact limit test 50 worker bees were exposed for 48 hours to a single dose of 100.0 µg a.i./bee by topical application.

In addition, negative controls [oral: a) water/sugar and b) acetone/sugar; contact: a) tap water and b) acetone] and a toxic reference (Dimethoate; 400 g/L nominal) at nominal rates of 0.30, 0.15, 0.08 and 0.05 µg dimethoate/bee were tested. Mortality and abnormal behaviour were recorded 4, 24 and 48 hours after test start, respectively.

The LD_{50} (48h) of the test item was determined to be > 100.0 µg a.i./bee in the contact toxicity test and > 105.3 µg a.i./bee in the oral toxicity test.

No test item related behavioural abnormalities occurred in the contact test. One bee showed dis-coordinated movements during the 4 hour assessment (before dying) in the oral toxicity test.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960-hydroxy (BCS-CQ74364)
Type:	Pure metabolite
Chemical state and description:	Solid, brown
Batch No.:	SES 11215-7-10
Purity:	95.5% w/w according to certificate of analysis
Solubility:	In acetone: soluble
Stability of test compound:	Expiry date: 15.12.2012, when stored at +5 ± 5°C in original container in the dark

Control

Oral Test:	a) 50 % (w/w) aqueous sugar solution (in tap water); b) 50% (w/w) sugar solution (45% water, 5% acetone, 50% sugar)
Contact Test:	a) Tap water with 0.5% Adhäsit* (applied after anesthetization with CO_2) b) Acetone (applied after anesthetization with CO_2) * (Adhäsit improves spreading of the test droplet on the water-repellent hairs on the thorax of bees)

Wetting Agent

Name:	Adhäsit
Batch No.:	0180201
Analytical content of active ingredient:	100 g/L Marlopon (nominal)
Manufacturer:	Spiess-Urania Chemicals GmbH, Heidenkampsweg 77, 20097 Hamburg, Germany
Storage:	Expiry Date: 02/2013, when stored in original container, at room temperature (20 ± 5°C), in the dark
Target Amount in this Study:	0.5%

Reference Item

The information concerning the reference item according to the substance container label and data sheet:

Name:	Perfekthion EC (BAS 152 11 I)
Manufacturer:	BASF AG, Agricultural Center Limburgerhof, D-67114 Limburgerhof
Batch No.:	90924-06
Analytical content of active ingredient:	nominal: Dimethoate: 400 g/L analyzed: Dimethoate: 414.8 g/L according to certificate of analysis
Certificate of Analysis Study Code:	346282_32
Type of formulation:	EC
Aggregate State at Room Temperature:	Liquid
Colour:	Blue
Density:	1.074 g/cm^3
Solubility:	In water: emulsifiable
Stability:	Expiry date: October 07, 2011 Storage: in original container, in refrigerator (≤10 °C), in the dark

2. Test organisms

Species:	*Apis mellifera carnica* L.
Common name:	Honey bee
Age or developmental stage at test start:	Adult female worker bees
Source:	Honey bee colonies, disease-free and queen-right, bred by IBACON

B. Study design and methods

1. In life dates: 10 to 13 May, 2011

2. Experimental treatments:

Test units were stainless steel chambers of 10 cm x 8.5 cm x 5.5 cm (length x width x height), the front side was a removable glass sheet, the bottom was perforated with 98 ventilation holes (Ø 1 mm), the inner walls were lined with filter paper.

10 bees were used per replicate unit, 5 replicates per treatment group (i.e. 50 individuals per treatment group).

Exposure time for both tests was 48 hours.

Food was commercial ready-to-use syrup (Apiinvert; 30% Saccharose, 31% Glucose, 39% Fructose).

Bees in the oral test were starved for 20 minutes prior to test start.

Bees in the contact test were anesthetized for *ca.* 20 seconds with CO_2 until they were completely immobilized immediately before application (only in the contact test).

Control:

Contact test: a) CO_2/tap water + Adhäsit[3]; b) CO_2/acetone

Oral test: a) Aqueous sugar solution; b) Aqueous sugar solution + acetone

Test item:

Contact Test:

Nominal dosage 100.0 µg a.i./bee

Oral Test:

[3] Adhäsit was used to improve the spreading of the test droplet on the bee body. Adhäsit is non-toxic to honey bees.

Nominal dosage	100.0 µg a.i./bee
Measured dosage	105.3 µg a.i./bee

Toxic reference item:

Contact test:	
Nominal dosage	0.30, 0.20, 0.15 and 0.10 µg Dimethoate per bee
Oral Test:	
Nominal dosage	0.30, 0.15, 0.08 and 0.05 µg Dimethoate per bee
Measured dosage	0.26, 0.16, 0.08 and 0.05 µg Dimethoate per bee

Application of the test item in the contact test:

Bees were anaesthetized with CO_2 in the contact test. A single 5 µL droplet of the test item BYI 02960-hydroxy in an appropriate carrier (acetone) was placed on the dorsal bee thorax using a Burkard - Applicator. For the controls, one 5 µL droplet of a) tap water with 0.5% Adhäsit and b) pure acetone was used, respectively. The reference item was also applied in a 5 µL droplet (dimethoate made up in acetone).

A 5 µL droplet was chosen in deviation to the guideline recommendation of 1 µL, since a higher volume ensured a more reliable dispersion of the test item; IBACON experience has proven that higher volumes are suitable and no adverse effects on the outcome of the study are to be expected.

Application of the test item in the oral test:

Appropriate amounts of BYI 02960-hydroxy or reference item dilutions in acetone were mixed with syrup (ready-to-use syrup; Apiinvert, Südzucker, D-97195 Ochsenfurt; composition of the sugar component: 30% Saccharose, 31% Glucose, 39% Fructose) in order to achieve the required test concentrations in a final dilution of 50% syrup solution (45% water, 50% syrup and 5% acetone (w/w)). For the controls, the same proportion of syrup, water and acetone was used (solvent control) and similarly, 50% aqueous syrup solution was used for the negative control. The treated food was offered in syringes, which were weighed before and after introduction into the cages (duration of uptake was one hour 45 minutes for the test item treatments). After a maximum of one hour 45 minutes, the uptake was complete and the syringes were removed, weighed and replaced by ones containing fresh, untreated food.

The mean target dose levels (*e.g.* 100 µg a.i./bee nominal) would have been obtained if exactly 20 mg/bee of the treated food were ingested. In practice, uptake of the treated sugar solutions differed slightly from the nominal 20 mg/bee and results are given based on the measured consumption.

3. Observation and measurements:

The number of dead bees was determined after 4 hours (first day); 24 and 48 hours (contact and oral test). Behavioural abnormalities (vomiting, apathy, intensive cleaning) were assessed after 4 hours (first day); 24 and 48 hours (contact and oral test).

Result evaluation:

Results obtained with the bees treated with the test item and the reference item were compared to those obtained with the control in both the contact and oral tests.

The contact and oral LD_{50} values of the reference item were estimated according to moving average computations (Thompson and Weil, 1952).

The software used to perform the statistical analysis was ToxRat Professional, Version 2.10.05, ® ToxRat Solutions GmbH.

RESULTS AND DISCUSSION

A. Environmental Parameters

Measurements of climatic parameters during the test are summarized as follows:

Test environment:	Incubator
Test temperature:	24 - 25°C
Relative air humidity:	50 to 68 %
Light intensity:	Darkness (except during observation)
Ventilation:	Ventilation to avoid possible accumulation of pesticide vapour
Recording:	Test conditions were continuously recorded with electronic data logger and documented in the raw data

B. Biological Findings

Observations:

Oral Test:

In the oral toxicity test, the maximum nominal test level of BYI 02960-hydroxy (100 µg a.i./bee) corresponded to an actual intake of 105.3 µg a.i./bee. This dose level led to 4.0% mortality after 48 hours.

No mortality occurred in the solvent and in the water control group (50% sugar solution), respectively.

One bee in the test item treatment showed a dis-coordinated movement (before dying) during the 4-hours assessment.

Table: Mortality and behavioural abnormalities of the bees in the oral toxicity test

	after 4 hours		after 24 hours		after 48 hours	
	mortality	behav. abnorm.	mortality	behav. abnorm.	mortality	behav. abnorm.
consumed dosage µg a.i./bee	mean %	mean %	mean %	mean %	mean %	mean %
test item						
105.3	2.0	2.0	4.0	0.0	4.0	0.0
water	0.0	0.0	0.0	0.0	0.0	0.0
solvent	0.0	0.0	0.0	0.0	0.0	0.0
reference item						
0.26	40.0	54.0	100.0	0.0	100.0	0.0
0.16	8.0	10.0	92.0	0.0	98.0	0.0
0.08	0.0	0.0	0.0	0.0	0.0	0.0
0.05	0.0	0.0	0.0	0.0	0.0	0.0

results are averages from five replicates (ten bees each) per dosage/control
behav. abnorm. = behavioural abnormalities
water = water control; solvent = solvent control

Contact Test:

At the end of the contact toxicity test (48 hours after application), there was 0.0% mortality at 100.0 µg a.i./bee. No mortality occurred in the solvent control group (acetone) and there was 2.0% mortality in the water control group (water + 0.5% Adhäsit).

No test item induced behavioural effects were observed at any time in the contact toxicity test.

Table: Mortality and behavioural abnormalities of the bees in the contact toxicity test

	after 4 hours		after 24 hours		after 48 hours	
	mortality	behav. abnorm.	mortality	behav. abnorm.	mortality	behav. abnorm.
dosage	mean	mean	mean	mean	mean	mean
µg a.i./bee	%	%	%	%	%	%
test item						
100.0	0.0	0.0	0.0	0.0	0.0	0.0
water	0.0	0.0	0.0	0.0	2.0	0.0
solvent	0.0	0.0	0.0	0.0	0.0	0.0
reference item						
0.30	18.0	44.0	92.0	6.0	96.0	4.0
0.20	0.0	0.0	80.0	4.0	84.0	6.0
0.15	0.0	0.0	22.0	2.0	32.0	14.0
0.10	2.0	0.0	2.0	0.0	2.0	0.0

results are averages from five replicates (ten bees each) per dosage/control
behav. abnorm. = behavioural abnormalities;
water = CO_2/water-treated control; solvent = CO_2/solvent control

C. Validity Criteria

The validity criterion of control mortality <10% is fulfilled; the validity criterion regarding the performance of the toxic reference is fulfilled for both contact and oral toxicity test, respectively.

D. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

Contact toxicity test:

Since no mortality occurred in the 100.0 µg a.i./bee treatment group, the contact LD_{50} can be considered to be > 100.0 µg a.i./bee.

Oral toxicity test:

There was 4.0% mortality in the 105.3 µg a.i./bee treatment group, therefore, the oral LD_{50} can be considered as > 105.3 µg a.i./bee.

The contact and oral LD_{50} (24 h) values of the reference item (dimethoate) were calculated to be 0.17 and 0.12 µg a.i./bee, respectively.

CONCLUSION

The toxicity of BYI 02960-hydroxy was tested in both an acute contact and oral toxicity test on honey bees. The LD_{50} (48h) of the test item was determined to be > 100.0 µg a.i./bee in the contact toxicity test and > 105.3 µg a.i./bee in the oral toxicity test, respectively.

No test item related behavioural abnormalities occurred in the contact test. One bee showed dis-coordinated movements during the 4 hour assessment (before dying) in the oral toxicity test.

Report:	**KIIA 8.7.1/04; Schmitzer, S. (2010)**
Title:	Effects of Difluoroacetic acid (Acute Contact and Oral) on Honey Bees (*Apis mellifera* L.) in the Laboratory
Report No:	56331035
Document No:	M-367915-01-2
Guidelines:	**OECD Guideline 213** **OECD Guideline 214**
Deviations:	**For the contact test, a 5 µL droplet was chosen (for all of the treatments) in deviation to the guideline recommendation of 1 µL**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of difluoroacetic acid on the honey bee (*Apis mellifera*) after oral or contact exposure.

In the oral limit test, 50 honey bees (adult female worker bees) were exposed for 48 hours to a single dose of 107.9 µg a.i./bee by feeding (value based on the actual intake of the test item). For the contact limit test 50 worker were exposed for 48 hours to a single dose of 100.0 µg a.i. per bee by topical application.

In addition, negative controls [contact test: a) tap water and b) acetone; oral: a) water/sugar and b) acetone/sugar] and a toxic reference (Dimethoate; 400 g/L nominal) at nominal rates of 0.30, 0.15, 0.08 and 0.05 µg dimethoate/bee were tested.

Mortality and abnormal behaviour were recorded 4, 24 and 48 hours after test start, respectively.

The LD_{50} (48h) of the test item was determined to be > 100.0 µg a.i./bee in the contact toxicity test and > 107.9 µg a.i./bee in the oral toxicity test, respectively. No test item related behavioural abnormalities occurred neither in the contact nor in the oral toxicity test.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	Difluoroacetic acid (BCS-AA56716)
Type:	Pure metabolite
Chemical state and description:	Liquid, colourless
Batch No.:	BCOO 5984-1-1
Purity:	95.8% w/w according to certificate of analysis
CAS#:	381-73-7
Solubility:	In water: miscible (according to test facility) In acetone: miscible (according to test facility)
Stability of test compound:	Expiry date: 29.07.2010, when stored at +25 ± 5°C in original container in the dark

Control

Oral Test:	a) 50 % (w/w) aqueous sugar solution (in tap water); b) 50% (w/w) sugar solution (45% water, 5% acetone, 50% sugar)
Contact Test:	a) Tap water with 0.5% Adhäsit* (applied after anesthetization with CO_2) b) Acetone (applied after anesthetization with CO_2) * (Adhäsit improves spreading of the test droplet on the water-repellent hairs on the thorax of bees)

Wetting Agent

Name:	Adhäsit
Batch No.:	0150207
Analytical content of active ingredient:	100 g/L Marlopon (nominal)
Manufacturer:	Spiess-Urania Chemicals GmbH, Heidenkampsweg 77, 20097 Hamburg, Germany
Storage:	Expiry Date: 12/2011, when stored in original container, at room temperature (20 ± 5°C), in the dark
Target Amount in this Study:	0.5%

Reference Item

The information concerning the reference item according to the substance container label and data sheet:

Name:	Perfekthion EC (BAS 152 11 I)
Manufacturer:	BASF AG, Agricultural Center Limburgerhof, D-67114 Limburgerhof
Batch No.:	90924-06
Analytical content of active ingredient:	nominal: Dimethoate: 400 g/L analyzed: Dimethoate: 414.8 g/L according to certificate of analysis
Certificate of Analysis Study Code:	346282_32
Type of formulation:	EC
Aggregate State at Room Temperature:	Liquid
Colour:	Blue
Density:	1.074 g/cm^3
Solubility:	In water: emulsifiable
Stability:	Expiry date: October 07, 2011 Storage: in original container, in refrigerator (≤10 °C), in the dark

2. Test organisms

Species:	*Apis mellifera carnica* L.
Common name:	Honey bee
Age or developmental stage at test start:	Adult female worker bees
Source:	Honey bee colonies, disease-free and queen-right, bred by IBACON

B. Study design and methods

1. In life dates: April 12 to 15, 2010

2. Experimental treatments:

Test units were stainless steel chambers of 10 cm x 8.5 cm x 5.5 cm (length x width x height), the front side was a removable glass sheet, the bottom was perforated with 98 ventilation holes (Ø 1 mm), the inner walls were lined with filter paper.

10 bees were used per replicate unit, 5 replicates per treatment group (i.e. 50 individuals per treatment group).
Exposure time for both tests was 48 hours.

Food was commercial ready-to-use syrup (Apiinvert; 30% Saccharose, 31% Glucose, 39% Fructose).

Bees in the oral test were starved for 20 minutes prior to test start.

Bees in the contact test were anesthetized for *ca.* 20 seconds with CO_2 until they were completely immobilized immediately before application (only in the contact test).

Control:

Contact test:	a) CO_2/tap water + Adhäsit[4];	b) CO_2/acetone
Oral test:	a) Aqueous sugar solution;	b) Aqueous sugar solution + acetone

Test item:

Contact Test:	
Nominal dosage	100.0 µg a.i./bee
Oral Test:	
Nominal dosage	100.0 µg a.i./bee
Measured dosage	107.9 µg a.i./bee

Toxic reference item:

Contact test:	
Nominal dosage	0.30, 0.20, 0.15 and 0.10 µg Dimethoate per bee
Oral Test:	
Nominal dosage	0.30, 0.15, 0.08 and 0.05 µg Dimethoate per bee
Measured dosage	0.23, 0.15, 0.08 and 0.05 µg Dimethoate per bee

Application of the test item in the contact test:

Bees were anaesthetized with CO2 in the contact test. A single 5 µL droplet of the test item difluoroacetic acid in an appropriate carrier (acetone) was placed on the dorsal bee thorax using a Burkard - Applicator. For the controls, one 5 µL droplet of a) tap water with 0.5% Adhäsit and b) pure acetone was used, respectively. The reference item was also applied in a 5 µL droplet (dimethoate made up in acetone).

A 5 µL droplet was chosen in deviation to the guideline recommendation of 1 µL, since a higher volume ensured a more reliable dispersion of the test item; IBACON experience has proven that higher volumes are suitable and no adverse effects on the outcome of the study are to be expected.

Application of the test item in the oral test:

Appropriate amounts of difluoroacetic acid or reference item dilutions in acetone were mixed with syrup (ready-to-use syrup; Apiinvert, Südzucker, D-97195 Ochsenfurt; composition of the sugar component: 30% Saccharose, 31% Glucose, 39% Fructose) in order to achieve the required test concentrations in a final dilution of 50 % syrup solution (45% water, 50% syrup and 5% acetone

[4] Adhäsit was used to improve the spreading of the test droplet on the bee body. Adhäsit is non-toxic to honey bees.

(w/w)). For the controls, the same proportion of syrup, water and acetone was used (solvent control) and similarly, 50% aqueous syrup solution was used for the negative control. The treated food was offered in syringes, which were weighed before and after introduction into the cages (duration of uptake was 4.45 hours for the test item treatments). After a maximum of 4.45 hours, the syringes containing the treated food were removed, weighed and replaced by ones containing fresh, untreated food.

The target dose levels (*e.g.* 100 µg a.i./bee nominal) would have been obtained if 20 mg/bee of the treated food was ingested. In practice, higher (or lower) dose levels were obtained as the bees had a higher or lower uptake of the test solutions than the nominal 20 mg/bee.

3. Observation and measurements:

The number of dead bees was determined after 4 hours (first day); 24 and 48 hours (contact and oral test). Behavioural abnormalities (vomiting, apathy, intensive cleaning) were assessed after 4 hours (first day); 24 and 48 hours (contact and oral test).

Result evaluation:

Results obtained from the bees treated with test item were compared to those obtained from the toxic standard and the controls.

Results obtained with the bees treated with test item and the reference item was compared to those obtained with the control in both the contact and oral tests.

The contact and oral LD_{50} values of the reference item were estimated with Probit Analysis (according to Finney 1971).

The software used to perform the statistical analysis was ToxRat Professional, Version 2.10, ® ToxRat Solutions GmbH, © 2009.

RESULTS AND DISCUSSION

A. Environmental Parameters

Measurements of climatic parameters during the test are summarized as follows:

Test environment:	Incubator
Test temperature:	25°C
Relative air humidity:	35 to 61 %
Light intensity:	Darkness (except during observation)
Ventilation:	Ventilation to avoid possible accumulation of pesticide vapour
Recording:	Test conditions were continuously recorded with electronic data logger and documented in the raw data

B. Biological Findings

Observations:

Oral Test:

In the oral toxicity test, no test item related mortality occurred throughout the entire testing period.

No test item induced behavioural effects were observed at any time in the oral toxicity test.

Table: Mortality and behavioural abnormalities of the bees in the oral toxicity test

consumed dosage	after 4 hours mortality mean %	after 4 hours behav. abnorm. mean %	after 24 hours mortality mean %	after 24 hours behav. abnorm. mean %	after 48 hours mortality mean %	after 48 hours behav. abnorm. mean %
test item µg a.i./bee						
107.9	0.0	0.0	0.0	0.0	0.0	0.0
water	0.0	0.0	0.0	0.0	2.0	0.0
solvent	0.0	0.0	0.0	0.0	0.0	0.0
reference item µg a.i./bee						
0.23	34.0	34.0	100.0	0.0	100.0	0.0
0.15	2.0	8.0	70.0	10.0	72.0	2.0
0.08	0.0	0.0	2.0	0.0	2.0	0.0
0.05	0.0	0.0	0.0	0.0	0.0	0.0

results are averages from five replicates (ten bees each) per dosage/control
behav. abnorm. = behavioural abnormalities
water = water control; solvent = solvent control

Contact Test:

At the end of the contact toxicity test (48 hours after application), 4% test item related mortality occurred. In the water control (water + 0.5% Adhäsit) and in the solvent control (acetone), 2% and 0% mortality occurred, respectively.

No test item induced behavioural effects were observed at any time in the contact toxicity test.

Table: Mortality and behavioural abnormalities of the bees in the contact toxicity test

dosage	after 4 hours mortality mean %	after 4 hours behav. abnorm. mean %	after 24 hours mortality mean %	after 24 hours behav. abnorm. mean %	after 48 hours mortality mean %	after 48 hours behav. abnorm. mean %
test item µg a.i./bee						
100.0	0.0	0.0	4.0	0.0	4.0	0.0
water	0.0	0.0	0.0	0.0	0.0	0.0
solvent	0.0	0.0	0.0	0.0	0.0	0.0
reference item µg a.i./bee						
0.30	42.0	50.0	98.0	2.0	100.0	0.0
0.20	18.0	8.0	86.0	10.0	96.0	0.0
0.15	4.0	0.0	60.0	0.0	68.0	0.0
0.10	0.0	0.0	2.0	0.0	12.0	0.0

results are averages from five replicates (ten bees each) per dosage/control
behav. abnorm. = behavioural abnormalities;
water = CO_2/water-treated control; solvent = CO_2/solvent control

C. Validity Criteria

The validity criterion of control mortality <10% is fulfilled; the validity criterion regarding the performance of the toxic reference is fulfilled for both contact and oral toxicity test, respectively.

D. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

Contact toxicity test:

Since 4% mortality occurred at the tested dose level of 100.0 µg a.i./bee, the contact LD_{50} can be considered to be > 100.0 µg a.i./bee.

Oral toxicity test:

There was no mortality during the entire test period, therefore, the oral LD_{50} can be considered as > 107.9 µg a.i./bee.

CONCLUSION

The toxicity of Difluoroacetic acid was tested in both an acute contact and oral toxicity test on honey bees. The LD_{50} (48h) of the test item was determined to be > 100.0 µg a.i./bee in the contact toxicity test and > 107.9 µg a.i./bee in the oral toxicity test, respectively. No test item related behavioural abnormalities occurred either in the contact or in the oral toxicity test.

Report:	**KIIA 8.7.1/05; Schmitzer, S. (2010)**
Title:	Effects of 6-chloronicotinic acid (Acute Contact and Oral) on Honey Bees (*Apis mellifera* L.) in the Laboratory
Report No:	60281035
Document No:	M-395279-01-2
Guidelines:	**OECD Guideline 213** **OECD Guideline 214**
Deviations:	**For the contact test, a 5 µL droplet was chosen (for any of the treatments) in deviation to the guideline recommendation of 1 µL**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of 6-chloronicotinic acid on the honey bee (*Apis mellifera*) after oral or contact exposure.

In the oral limit test 50 honey bees (adult female worker bees) were exposed for 48 hours to a single dose of 107.1 µg a.i. per bee by feeding (value based on the actual intake of the test item). For the contact limit test 50 worker bees per treatment were exposed for 48 hours to a single dose of 100.0 µg a.i. per bee by topical application.

In addition, negative controls [oral: a) water/sugar and b) acetone/sugar; contact: a) tap water and b) acetone] and a toxic reference (Dimethoate; 400 g/L nominal) at nominal rates of 0.30, 0.15, 0.08 and 0.05 µg dimethoate/bee were tested.

Mortality and abnormal behaviour were recorded 4, 24 and 48 hours after test start.

The LD_{50} (48h) of the test item was determined to be > 100.0 µg a.i./bee in the contact toxicity test and > 107.1 µg a.i./bee in the oral toxicity test, respectively. No test item related behavioural abnormalities occurred neither in the contact nor in the oral toxicity test.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	6-chloronicotinic acid (AE F161089; BYI 02960-6-CNA)
Type:	Pure metabolite
Chemical state and description:	Solid, beige
Batch No.:	M12653
Purity:	98.8% w/w according to certificate of analysis
CAS#:	5326-23-8
Solubility:	In acetone: not indicated
Stability of test compound:	Expiry date: 09.07.2012, when stored at +5 ± 5°C in original container in the dark

Control

Oral Test:	a) 50 % (w/w) aqueous sugar solution (in tap water); b) 50% (w/w) sugar solution (45% water, 5% acetone, 50% sugar)
Contact Test:	a) Tap water with 0.5% Adhäsit* (applied after anesthetization with CO_2) b) Acetone (applied after anesthetization with CO_2) * (Adhäsit improves spreading of the test droplet on the water-repellent hairs on the thorax of bees)

Wetting Agent

Name:	Adhäsit
Batch No.:	0150207
Analytical content of active ingredient:	100 g/L Marlopon (nominal)
Manufacturer:	Spiess-Urania Chemicals GmbH, Heidenkampsweg 77, 20097 Hamburg, Germany
Storage:	Expiry Date: 12/2011, when stored in original container, at room temperature (20 ± 5°C), in the dark
Target Amount in this Study:	0.5%

Reference Item

The information concerning the reference item according to the substance container label and data sheet:

Name:	Perfekthion EC (BAS 152 11 I)
Manufacturer:	BASF AG, Agricultural Center Limburgerhof, D-67114 Limburgerhof
Batch No.:	90924-06
Analytical content of active ingredient:	nominal: Dimethoate: 400 g/L analyzed: Dimethoate: 414.8 g/L according to certificate of analysis
Certificate of Analysis Study Code:	346282_32
Type of formulation:	EC
Aggregate State at Room Temperature:	Liquid
Colour:	Blue
Density:	1.074 g/cm^3
Solubility:	In water: emulsifiable
Stability:	Expiry date: October 07, 2011 Storage: in original container, in refrigerator (≤10 °C), in the dark

2. Test organisms

Species:	*Apis mellifera* L.
Common name:	Honey bee
Age or developmental stage at test start:	Adult female worker bees
Source:	Honey bee colonies, disease-free and queen-right, bred by IBACON

B. Study design and methods

1. In life dates: August 16 to 19, 2010

2. Experimental treatments:

Test units were stainless steel chambers of 10 cm x 8.5 cm x 5.5 cm (length x width x height), the front side was a removable glass sheet, the bottom was perforated with 98 ventilation holes (Ø 1 mm), the inner walls were lined with filter paper.

10 bees were used per replicate unit, 5 replicates per treatment group (i.e. 50 individuals per treatment group).

Exposure time for both tests was 48 hours.

Food was commercial ready-to-use syrup (Apiinvert; 30% Saccharose, 31% Glucose, 39% Fructose).

Bees in the oral test were starved for 20 minutes prior to test start.

Bees in the contact test were anesthetized for *ca.* 20 seconds with CO_2 until they were completely immobilized immediately before application (only in the contact test).

Control:

Contact test:	a) CO_2/tap water + Adhäsit[5];	b) CO_2/acetone
Oral test:	a) Aqueous sugar solution;	b) Aqueous sugar solution + acetone

Test item:

Contact Test:

Nominal dosage	100 µg a.i./bee

Oral Test:

Nominal dosage	100 µg a.i./bee
Measured dosage	107.1 µg a.i./bee

Toxic reference item:

Contact test:

Nominal dosage	0.30, 0.20, 0.15 and 0.10 µg Dimethoate per bee

Oral Test:

Nominal dosage	0.30, 0.15, 0.08 and 0.05 µg Dimethoate per bee
Measured dosage	0.30, 0.15, 0.08 and 0.05 µg Dimethoate per bee

[5] Adhäsit was used to improve the spreading of the test droplet on the bee body. Adhäsit is non-toxic to honey bees.

Application of the test item in the contact test:

Bees were anaesthetized with CO_2 in the contact test. A single 5 µL droplet of the test item 6-chloronicotinic acid in an appropriate carrier (acetone) was placed on the dorsal bee thorax using a Burkard - Applicator. For the controls, one 5 µL droplet of a) tap water with 1% Adhäsit and b) pure acetone were used. The reference item was also applied in a 5 µL droplet (dimethoate made up in acetone).

A 5 µL droplet was chosen in deviation to the guideline recommendation of 1 µL, since a higher volume ensured a more reliable dispersion of the test item; IBACON experience has proven that higher volumes are suitable and no adverse effects on the outcome of the study are to be expected.

Application of the test item in the oral test:

Appropriate amounts of 6-chloronicotinic acid or reference item dilutions in acetone were mixed with syrup (ready-to-use syrup; Apiinvert, Südzucker, D-97195 Ochsenfurt; composition of the sugar component: 30% Saccharose, 31% Glucose, 39% Fructose) in order to achieve the required test concentrations in a final dilution of 50% syrup solution (45% water, 50% syrup and 5% acetone (w/w)). For the controls, the same proportion of syrup, water and acetone was used (solvent control) and similarly, 50% aqueous syrup solution was used for the negative control. The treated food was offered in syringes, which were weighed before and after introduction into the cages (duration of uptake was 3 hours 45 minutes for the test item treatments). After a maximum of 3 hours 45 minutes, the uptake was complete and the syringes were removed, weighed and replaced by ones containing fresh, untreated food.

The target dose levels (*e.g.* 100 µg a.i./bee nominal) would have been obtained if 20 mg/bee of the treated food was ingested. In practice, higher dose levels were obtained as the bees had a higher or lower uptake of the test solutions than the nominal 20 mg/bee.

3. Observation and measurements:

The number of dead bees was determined after 4 hours (first day); 24 and 48 hours (contact and oral test). Behavioural abnormalities (vomiting, apathy, intensive cleaning) were assessed after 4 hours (first day); 24 and 48 hours (contact and oral test).

Result evaluation:

Results obtained from the bees treated with test item were compared to those obtained from the toxic standard and the controls.

The contact and oral LD_{50} of the reference item were estimated according to moving average computations (Thompson and Weil, 1952).

If necessary, the LD_{50} calculation was carried out taking into account the mortality data corrected by control mortality using Abbott's formula (1925).

The software used to perform the statistical analysis was ToxRat Professional, Version 2.10.05, ® ToxRat Solutions GmbH.

RESULTS AND DISCUSSION

A. Environmental Parameters

Measurements of climatic parameters during the test are summarized as follows:

Test environment:	Incubator
Test temperature:	24 - 25°C
Relative air humidity:	61 to 86 %
Light intensity:	Darkness (except during observation)
Ventilation:	Ventilation to avoid possible accumulation of pesticide vapour
Recording:	Test conditions were continuously recorded with electronic data logger and documented in the raw data

B. Biological Findings

Observations:

Oral Test:

In the oral toxicity test, the maximum nominal test level of 6-chloronicotinic acid (100 µg a.i./bee) corresponded to an actual intake of 107.1 µg a.i./bee. This dose level led to no mortality after 48 hours.

Also no mortality occurred in the solvent control and in the water control (50% sugar solution), respectively.

No test item related behavioural abnormalities occurred.

Table: Mortality and behavioural abnormalities of the bees in the oral toxicity test

	after 4 hours		after 24 hours		after 48 hours	
consumed dosage	mortality mean %	behav. abnorm. mean %	mortality mean %	behav. abnorm. mean %	mortality mean %	behav. abnorm. mean %
test item µg a.i./bee						
107.1	0.0	0.0	0.0	0.0	0.0	0.0
water	0.0	0.0	0.0	0.0	0.0	0.0
solvent	0.0	0.0	0.0	0.0	0.0	0.0
reference item µg a.i./bee						
0.30	26.0	36.0	98.0	0.0	100.0	0.0
0.15	2.0	12.0	82.0	0.0	86.0	0.0
0.08	0.0	0.0	8.0	2.0	20.0	0.0
0.05	0.0	0.0	0.0	0.0	2.0	0.0

results are averages from five replicates (ten bees each) per dosage/control
Table 6 for details; behav. abnorm. = behavioural abnormalities
water = water control; solvent = solvent control

Contact Test:

At the end of the contact toxicity test (48 hours after application), there was 0.0% mortality at 100.0 µg a.i./bee. No mortality occurred in the water control (water + 0.5% Adhäsit) and there was 2.0% mortality in the solvent control (acetone).

No remarked reactions to exposure of the test item were noted in any of the test bees throughout the duration of the study.

Table: Mortality and behavioural abnormalities of the bees in the contact toxicity test

dosage	after 4 hours mortality mean %	after 4 hours behav. abnorm. mean %	after 24 hours mortality mean %	after 24 hours behav. abnorm. mean %	after 48 hours mortality mean %	after 48 hours behav. abnorm. mean %
test item µg a.i./bee						
100.0	0.0	0.0	0.0	0.0	0.0	0.0
water	0.0	0.0	0.0	0.0	0.0	0.0
solvent	0.0	0.0	0.0	0.0	2.0	0.0
reference item µg a.i./bee						
0.30	0.0	22.0	86.0	4.0	94.0	0.0
0.20	0.0	2.0	76.0	4.0	76.0	6.0
0.15	0.0	0.0	2.0	0.0	12.0	6.0
0.10	0.0	0.0	0.0	0.0	2.0	0.0

results are averages from five replicates (ten bees each) per dosage/control
Table 4 for details; behav. abnorm. = behavioural abnormalities;
water = CO_2/water-treated control; solvent = CO_2/solvent control

C. Validity Criteria

The validity criterion of control mortality <10% is fulfilled; the validity criterion regarding the performance of the toxic reference is fulfilled for both contact and oral toxicity test, respectively.

D. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

Contact toxicity test:

Since no mortality occurred in the 100.0 µg a.i./bee treatment group, the contact LD_{50} can be considered as > 100.0 µg a.i./bee.

Oral toxicity test:

There was no mortality in the 107.1 µg a.i./bee treatment group, therefore the oral LD_{50} can be considered as > 107.1 µg a.i./bee.

CONCLUSION

The toxicity of 6-chloronicotinic acid was tested in both an acute contact and oral toxicity test on honey bees. The LD_{50} (48h) of the test item was determined to be > 100.0 µg a.i./bee in the contact toxicity test and > 107.1 µg a.i./bee in the oral toxicity test, respectively. No test item related behavioural abnormalities occurred either in the contact or in the oral toxicity test.

Report:	**KIIA 8.7.1/06; Schmitzer, S. (2010)**
Title:	Effects of 6-chloro-picolylalcohol (Acute Contact and Oral) on Honey Bees (*Apis mellifera* L.) in the Laboratory
Report No:	50911035
Document No:	M-361234-01-2
Guidelines:	**OECD Guideline 213** **OECD Guideline 214**
Deviations:	**For the contact test, a 5 µL droplet was chosen (for any of the treatments) in deviation to the guideline recommendation of 1 µL**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of BYI-02960 CHMP (6-chloro-picolylalcohol, 6-CPA) on the honey bee (*Apis mellifera*) after oral or contact exposure.

In the oral limit test 50 honey bees (adult female worker bees) were exposed for 48 hours to a single dose of 106.7 µg a.i. per bee by feeding (value based on the actual intake of the test item). For the contact limit test 50 worker bees per treatment were exposed for 48 hours to a single dose of 100.0 µg a.i. per bee for topical application.

In addition, a negative control (water/sugar (oral test); tap water (contact test)) and a toxic reference (Dimethoate; 400 g/L nominal) at nominal rates of 0.30, 0.15, 0.08 and 0.05 µg dimethoate/bee, respectively, were tested.

Mortality and abnormal behaviour were recorded 4, 24 and 48 hours after test start, respectively.

The LD_{50} (48h) of the test item was determined to be > 100.0 µg a.i./bee in the contact toxicity test and > 106.7 µg a.i./bee in the oral toxicity test, respectively. No test item related behavioural abnormalities occurred neither in the contact nor in the oral toxicity test.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	6-chloro-picolylacohol (= 6-chloro-3-pyridinemethanol)
Type:	Pure metabolite
Chemical state and description:	Solid, yellowish
Batch No.:	M06773
Purity:	98.9% w/w according to certificate of analysis
CAS#:	21543-49-7
Solubility:	In water: soluble
Stability of test compound:	Expiry date: Oct. 2009, when stored at room temperature (10 to 30°C) in original container in the dark

Control

Oral Test:	50 % (w/w) aqueous sugar solution (in tap water)
Contact Test:	Tap water with 0.5% Adhäsit* (applied after anesthetization with CO_2) * (Adhäsit improves spreading of the test droplet on the water-repellent hairs on the thorax of bees)

Wetting Agent

Name:	Adhäsit
Batch No.:	0150207
Analytical content of active ingredient:	100 g/L Marlopon (nominal)
Manufacturer:	Spiess-Urania Chemicals GmbH, Heidenkampsweg 77, 20097 Hamburg, Germany
Storage:	Expiry Date: 12/2009, when stored in original container, at room temperature (10 to 30°C), in the dark
Target Amount in this Study:	0.5%

Reference Item

The information concerning the reference item according to the substance container label and data sheet:

Name:	Perfekthion EC (BAS 152 11 I)
Manufacturer:	BASF AG, Agricultural Center Limburgerhof, D-67114 Limburgerhof
Batch No.:	FRE-000627
Analytical content of active ingredient:	nominal: Dimethoate: 400 g/L analyzed: Dimethoate: 422.4 g/L according to certificate of analysis
Certificate of Analysis Study Code:	346282_14
Type of formulation:	EC
Aggregate State at Room Temperature:	Liquid
Colour:	Blue
Density:	1.076 g/cm^3
Solubility:	In water: emulsifiable
Stability:	Expiry date: October 31, 2009 Storage: in original container, in refrigerator (4 ± 4 °C), in the dark

2. Test organisms

Species:	*Apis mellifera carnica* L.
Common name:	Honey bee
Age or developmental stage at test start:	Adult female worker bees
Source:	Honey bee colonies, disease-free and queen-right, bred by IBACON

B. Study design and methods

1. In life dates: May 25 to 27, 2009

2. Experimental treatments:

Test units were stainless steel chambers of 10 cm x 8.5 cm x 5.5 cm (length x width x height), the front side was a removable glass sheet, the bottom was perforated with 98 ventilation holes (Ø 1 mm), the inner walls were lined with filter paper.

10 bees were used per replicate unit, 5 replicates per treatment group (i.e. 50 individuals per treatment group).

Exposure time for both tests was 48 hours.

Food was commercial ready-to-use syrup (Apiinvert; 30% Saccharose, 31% Glucose, 39% Fructose).

Bees in the oral test were starved for 15 minutes prior to test start in all treatment groups.

Bees in the contact test were anesthetized for *ca.* 20 seconds with CO_2 until they were completely immobilized immediately before application (only in the contact test).

Control:

Contact test: CO_2/tap water + Adhäsit[6]

Oral test: Aqueous sugar solution

Test item:

Contact Test:

Nominal dosage 100 µg a.i./bee

Oral Test:

Nominal dosage 100 µg a.i./bee

Measured dosage 106.7 µg a.i./bee

Toxic reference item:

Contact test:

Nominal dosage 0.30, 0.20, 0.15 and 0.10 µg Dimethoate per bee

Oral Test:

Nominal dosage 0.30, 0.15, 0.08 and 0.05 µg Dimethoate per bee

Measured dosage 0.29, 0.16, 0.08 and 0.05 µg Dimethoate per bee

Application of the test item in the contact test:

Bees were anaesthetized with CO2 in the contact test. A single 5 µL droplet of the test item 6-chloro-picolylacohol in an appropriate carrier (tap water + 0.5% Adhäsit) was placed on the dorsal bee thorax using a Burkard - Applicator. For the control, one 5 µL droplet of tap water containing 0.5% Adhäsit was used. The reference item was also applied in a 5 µL tap water (dimethoate made up in tap water containing 0.5% Adhäsit).

A 5 µL droplet was chosen in deviation to the guideline recommendation of 1 µL, since a higher volume ensured a more reliable dispersion of the test item; IBACON experience has proven that higher volumes are suitable and no adverse effects on the outcome of the study are to be expected.

Application of the test item in the oral test:

Aqueous stock solutions of the test item and reference item were prepared in such a way that they had the respective target concentration of the test item once they were subsequently mixed with sugar syrup at a ratio of 1 + 1. After mixing of these test solutions with ready-to-use sugar syrup (composition of the sugar component: 30 % saccharose, 31 % glucose, 39 % fructose) the final concentration of sugar syrup in the test item solutions offered to the bees was 50 %. For the controls water and sugar syrup was used at the same ratio (1 + 1). The treated food was offered in syringes, which were weighed before and after introduction into the cages (duration of uptake was 2.5 hours for the test item treatments). After a maximum of 2.5 hours, the food uptake was complete and the syringes containing the treated food were removed, weighed and replaced by ones containing fresh, untreated food.

[6] Adhäsit was used to improve the spreading of the test droplet on the bee body. Adhäsit is non-toxic to honey bees.

The target dose levels (*e.g.* 100 µg a.i./bee nominal) would have been obtained if 20 mg/bee of the treated food was ingested. In practice, higher dose levels were obtained as the bees had a higher uptake of the test solutions than the nominal 20 mg/bee.

3. Observation and measurements:

The number of dead bees was determined after 4 hours (first day); 24 and 48 hours (contact and oral test). Behavioural abnormalities (vomiting, apathy, intensive cleaning) were assessed after 4 hours (first day); 24 and 48 hours (contact and oral test).

Result evaluation:

Results obtained from the bees treated with test item were compared to those obtained from the toxic standard and the controls.

The contact and oral LD_{50} of the reference item were estimated according to moving average computations (Thompson and Weil, 1952).

If necessary, the LD_{50} calculation was carried out taking into account the mortality data corrected by control mortality using Abbott's formula (1925).

The software used to perform the statistical analysis was ToxRat Professional, Version 2.10, ® ToxRat Solutions GmbH, © 2009.

RESULTS AND DISCUSSION

A. Environmental Parameters

Measurements of climatic parameters during the test are summarized as follows:

Test environment:	Incubator
Test temperature:	25°C
Relative air humidity:	42 to 74 %
Light intensity:	Darkness (except during observation)
Ventilation:	Ventilation to avoid possible accumulation of pesticide vapour
Recording:	Test conditions were continuously recorded with electronic data logger and documented in the raw data

B. Biological Findings

Observations:

Oral Test: In the oral toxicity test, the maximum nominal test level of 6-chloro-picolylacohol (100 µg a.i./bee) corresponded to an actual intake of 106.7 µg a.i./bee. This dose level led to 2% mortality (1 of 50 bees) after 48 hours.

No mortality occurred in the water control (50% aqueous sugar solution).

No test item related behavioural abnormalities occurred.

Table: Mortality and behavioural abnormalities of the bees in the oral toxicity test

	after 4 hours		after 24 hours		after 48 hours	
	mortality	behav. abnorm.	mortality	behav. abnorm.	mortality	behav. abnorm.
ingested dose	mean	mean	mean	mean	mean	mean
µg a.i./bee	%	%	%	%	%	%
test item						
106.7	2.0	0.0	2.0	0.0	2.0	0.0
water	0.0	0.0	0.0	0.0	0.0	0.0
reference item						
0.29	78.0	18.0	100.0	0.0	100.0	0.0
0.16	8.0	86.0	94.0	0.0	96.0	0.0
0.08	0.0	4.0	38.0	6.0	50.0	0.0
0.05	0.0	2.0	2.0	0.0	4.0	0.0

results are averages from five replicates (ten bees each) per dosage/control
; water = water/sugar treated control
behav. abnorm. = behavioural abnormalities

Contact Test: At the end of the contact toxicity test (48 hours after application), there was 0.0% mortality at 100.0 µg a.i./bee. Also no mortality occurred in the water control (water + 0.5% Adhäsit).

No behavioural abnormalities attributed to exposure of the test item to the bees occurred during the experimental time of 48 hours.

There were behavioural abnormalities consistent with the observed toxicity in the reference item test.

Table: Mortality and behavioural abnormalities of the bees in the contact toxicity test

	after 4 hours		after 24 hours		after 48 hours	
	mortality	behav. abnorm.	mortality	behav. abnorm.	mortality	behav. abnorm.
dose	mean	mean	mean	mean	mean	mean
µg a.i./bee	%	%	%	%	%	%
test item						
100.0	0.0	0.0	0.0	0.0	0.0	0.0
water	0.0	0.0	0.0	0.0	0.0	0.0
reference item						
0.30	16.0	18.0	98.0	0.0	100.0	0.0
0.20	4.0	6.0	60.0	14.0	78.0	0.0
0.15	0.0	0.0	36.0	4.0	48.0	0.0
0.10	2.0	0.0	10.0	0.0	10.0	0.0

results are averages from five replicates (ten bees each) per dosage/control

behav. abnorm. = behavioural abnormalities; water = CO_2/water-treated control

C. Validity Criteria

The validity criterion of control mortality <10% is fulfilled; the validity criterion regarding the performance of the toxic reference is fulfilled for both contact and oral toxicity test, respectively.

D. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

Oral toxicity test:

Since only one bee was found dead during the entire experiment (2% mortality), the oral LD_{50} can be considered as > 106.7 µg a.i./bee.

Contact toxicity test:

Since no mortality occurred in the 100.0 µg a.i./bee group, the contact LD_{50} can be considered as > 100.0 µg a.i./bee.

CONCLUSION

The toxicity of BYI 02960-CHMP (6-chloro-picolylacohol) was tested in both an acute contact and oral toxicity test on honey bees. The LD_{50} (48h) of the test item was determined to be > 100.0 µg a.i./bee in the contact toxicity test and > 106.7 µg a.i./bee in the oral toxicity test, respectively. No test item related behavioural abnormalities occurred either in the contact or in the oral toxicity test.

IIA 8.7.2 Acute contact toxicity

Acute contact toxicity has been evaluated together with acute oral toxicity in the respective studies. Therefore, the study results for both tests are presented together under Point IIA 8.7.1 above.

IIA 8.7.3 Toxicity of residues on foliage to honey bees

This is not an EC data requirement. However, a residue study on foliage has been conducted with the formulation BYI 02960 SL 200G following the provisions of OPPTS No. 850.3030. Please refer to the Annex III document, KIIIA1 10.4.3/01, Porch & Krueger, 2011 M-413084-01-1..

IIA 8.7.4 Bee brood feeding test

A bee brood semi-field study has been conducted with the formulated product BYI 02960 SL 200 G, the study is summarised in the Annex III for the formulation (see report KIIIA1 10.4.7/06, Rentschler, 2012; M-427438-01-1).

IIA 8.8 Effects on non-target terrestrial arthropods

In accordance with the recommendations of the Terrestrial Guidance Document, the toxicity of BYI 02960 to non-target arthropods was determined using the representative lead formulation (BYI 02960 SL 200 G), for completeness these studies are summarized in this Annex II.

IIA 8.8.1 Effects on non-target terrestrial arthropods, artificial substrates

Laboratory tests on artificial substrate (glass plates) have been conducted with the BYI 02960 SL 200 on the standard species *Aphidius rhopalosiphi* and *Typhlodromus pyri.* The summaries are presented below.

IIA 8.8.1.1 Parasitoid

Report:	**KIIA 8.8.1.1/01; Jans, D. (2010)**
Title:	Toxicity to the parasitoid wasp *Aphidius rhopalosiphi* (DESTEPHANI-PEREZ) (Hymenoptera: Braconidae) using a laboratory test; BYI 02960 SL 200 (g/L)
Report No:	CW09/079
Document No:	M-366965-01-2
Guidelines:	**MEAD-BRIGGS ET AL. (2000), CANDOLFI ET AL. (2001)**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The objective of this laboratory study was to investigate the lethal and sublethal toxicity of BYI 02960 SL 200 (Sample description: FAR01438-00 (Batch ID: 2009-001253; Material No.: 79718845; Specification No.: 102000021884)) on the parasitoid wasp *Aphidius rhopalosiphi* when exposed on a glass surface.

The test item was applied on glass plates at nominal rates of 10, 20, 40, 80 and 160 g a.i./ha, respectively, and effects on 60 adults (4 replicates with 15 wasps per test group) of the parasitoid wasp *Aphidius rhopalosiphi* were assessed during 24 h after exposure. The control was treated with deionized water (200 L/ha). Dimethoate (0.04 g a.i./ha in 200 L water/ha) was used as a toxic reference item. The study was repeated a second time with lower application rates because all tested rates in the first trial showed 100% mortality after 24 h of exposure. In the second study trial, the test item was applied at nominal rates of 0.5, 1.1, 2.2, 4.7 and 10 g a.i./ha, respectively and mortality was assessed during 48 h after exposure.

At the lowest dose rate of 0.5 g a.i./ha, 85% corrected mortality was observed. At all higher test item rates 100% mortality occurred. The LR_{50} was calculated to be <0.5 g a.i./ha.

Due to the high mortality in the second trial, no assessment of reproductive capacity was performed. Mortality was used to determine the endpoint.

MATERIAL AND METHODS

A. Materials

1. Test material:

Test item:	BYI 02960 SL 200
Specification No.:	102000021884
Type:	Formulated product (soluble (liquid) concentrate)
Chemical state and description:	Clear brown liquid
Batch No.:	2009-001253
Material number:	79718845
Sample description:	FAR01438-00
Nominal content of active ingredient:	BYI 02960: 200 g/L
Analytical content of active ingredient:	BYI 02960: 17.0% w/w, 199.8 g/L according to certificate of analysis
Density:	1.175 g/mL
Stability of test compound:	Approved until 20.03.2010 (storage at +2 °C to +30 °C)

2. Vehicle and/or positive control:

Solvent:	No solvent used; deionized water was used as diluent for the test item and for the reference item
Reference item:	Dimethoate EC 400 (analytical content of a.i.: 428.5 g/L.)

3. Test organism

Species:	*Aphidius rhopalosiphi*
Common name:	Parasitoid wasp
Age:	48 h
Source of test organism	*Aphidius rhopalosiphi* used for testing were supplied by Katz Biotech AG, D–15837 Baruth, Germany

B. Study design and methods

1. In life dates November 30 to December 9, 2009

2. Design of the test

Number of test groups:	7 (control, test and reference item)
Number of application rates:	Test item: 5 Reference item: 1
Number of replicates per test group:	4 (one replicate = one exposure unit)
Number of larvae/per replicate:	15

A soluble concentrate formulation of BYI 02960 SL 200 G was tested. The test item was applied at rates of 10, 20, 40, 80 and 160 g a.i./ha on glass plates and the effects on the parasitoid wasp *Aphidius rhopalosiphi* were compared to those of a deionised water treated control. A toxic reference (active substance: dimethoate) applied at 3.0 g a.i /ha was included to indicate the relative susceptibility of the test organisms and the test system.

Mortality of 60 adults (4 replicates with 15 wasps per test group) was assessed 2 and 24 h after exposure.

In a second study trial with lower application rates of 0.5, 1.1, 2.2, 4.7 and 10 g a.i./ha mortality was assessed 2, 24 and 48 h after exposure.

3. Observation and measurements

Mortality was assessed by recording the condition of the test animals 2, 24 and 48 h (only in second study trial) after application:

- live (alive and apparently unaffected)
- affected (showing reduced co-ordination or any abnormal behaviour)
- moribund (unable to walk, but still moving legs or antennae)
- dead (no longer moving)

4. Statistical analysis

The computer program SAS (Version 9.1.3, 2002-2003) was used to perform the statistical analyses.

The mortality data were analysed for significance using the Fisher Exact test (one-sided with Bonferroni-Holm adjustment; $\alpha = 0.05$), which is a distribution-free test method and does not require testing for normality or homogeneity prior analysis.

RESULTS AND DISCUSSION

A. Environmental Conditions

Wasps were kept under conditions which are summarized as follows:

Test temperature:	19.5-21.5°C (first trial) 19.5-22.0°C (second trial)
Relative humidity:	60 - 70 % in the first trial (deviations: decreases <2h to 55%) 60 - 78 % in the second trial
Photoperiod:	16 hours light / 8 hours dark
Light intensity	511 - 1009 Lux (first trial) 489 – 661 Lux (second trial)

B. Biological Findings

A summary of effects of BYI 02960 SL 200 on mortality of *Aphidius rhopalosiphi* exposed on glass plates is given in the table below:

Table Effects of BYI 02960 SL 200 (g/L) on mortality of *Aphidius rhopalosiphi*

Test item		BYI 02960 SL 200 (g/L)		
Test organism		*Aphidius rhopalosiphi*		
Exposure on:		Glass plates		
Trial 1				
		Mortality **after 24 hours** [%]		
Treatment	g a.i./ha	Uncorr.	Corr.	P-Value(*)
Control	0	1.7		
Test item	10	100	100	<0.001 sign.
Test item	20	100	100	<0.001 sign.
Test item	40	100	100	<0.001 sign.
Test item	80	100	100	<0.001 sign.
Test item	160	100	100	<0.001 sign.
Reference item	0.04	100	100	
* Fisher`s Exact test (one-sided), p-values are adjusted according to Bonferroni-Holm n.d. not detected, n.sign. not significant, sign. significant				
Trial 2				
		Mortality **after 48 hours** [%]		
Treatment	g a.i./ha	Uncorr.	Corr.	P-Value(*)
Control	0	0		
Test item	0.5	85.0	85.0	<0.001 sign.
Test item	1.1	100	100	<0.001 sign.
Test item	2.2	100	100	<0.001 sign.
Test item	4.7	100	100	<0.001 sign.
Test item	10.0	100	100	<0.001 sign.
Reference item	0.04	100	100	
LR_{50}: <0.5 g a.i./ha				
* Fisher`s Exact test (one-sided), p-values are adjusted according to Bonferroni-Holm n.d. not detected, n.sign. not significant, sign. significant				

Mortality

In the first study trial 1.7% of the wasps introduced were dead after 24 h of exposure in the control group. In all rates of the test item and the reference item all wasps were dead.

In the second study trial all wasps were found alive in the control group after 48 h of exposure. In the lowest test item rate of 0.5 g a.i./ha 85% of the wasps died whereas in all other rates of BYI 02960 SL 200 g/L as well as in the reference item group all wasps were dead.

C. Validity Criteria

The validity criteria for the laboratory method using glass plates of mortality ≤13% in the control group, ≥50% corrected mortality in the toxic reference are fulfilled.

D. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

LR_{50}: <0.5 g a.i./ha

CONCLUSION

The effects of BYI 02960 SL 200 residues on the survival of the parasitoid wasp *Aphidius rhopalosiphi* in a laboratory test on glass plates can be quantified as an LR_{50} of < 0.5 g a.i./ha.

IIA 8.8.1.2 Predatory mites

Report:	**KIIA 8.8.1.2/01; Jans, D. (2010)**
Title:	Toxicity to the predatory mite *Typhlodromus pyri* SCHEUTEN (Acari, Phytoseiidae) using a laboratory test; BYI 02960 SL 200 g/L
Report No:	CW09/073
Document No:	**M-366957-01-2**
Guidelines:	**BLUEMEL ET AL. (2000); CANDOLFI ET AL. (2001)**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The test item BYI 02960 SL 200 (Sample description: FAR01438-00 (Batch ID: 2009-001253; Material No.: 79718845; Specification No.: 102000021884) was tested under laboratory conditions via residual contact exposure of protonymphs of the predatory mite *Typhlodromus pyri* to spray residues with rates of 2, 4, 9, 19 and 40 g a.i./ha, respectively in 200 L deionized water/ha applied on glass plates. The control was treated with deionized water (200 L/ha). Dimethoate EC 400 (4 g a.i./ha in 200 L water/ha) was used as a toxic reference item.

Mortality of 100 mites (5 replicates of 20 individuals per test group) was assessed 1, 4 and 7 days after exposure by counting the number of living and dead mites. The number of escaped mites was calculated as the difference from the total number exposed.

At the test item rates of 2 and 4 g a.i./ha a corrected mortality of 6.3% each was observed. At the higher rates of 9, 19 and 40 g a.i./ha, a corrected mortality of 25.0, 50.0 and 89.6%, respectively, occurred.

The LR_{50} was calculated to be 17 g a.i./ha. (95% CI: 13 to 21 g a.i./ha).

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960 SL 200
Type:	Formulated product (soluble (liquid) concentrate)
Chemical state and description:	Clear brown liquid
Specification No	102000021884
Batch No.:	2009-001253
Material number:	79718845
Sample description:	FAR01438-00
Nominal content of active ingredient:	BYI 02960: 200 g/L
Analytical content of active ingredient:	BYI 02960: 17.0% w/w, 199.8 g/L according to certificate of analysis
Density:	1.175 g/mL
Stability of test compound:	Approved until 2010 March (storage at +2 °C to +30 °C)

2. Vehicle and/or positive control

Solvent	No solvent used; deionized water was used as diluent for the test item and for the reference item
Negative control:	deionized water
Positive control:	Dimethoate EC 400 (428.5 g/L)

3. Test organism

Species	Predatory mite *Typhlodromus pyri*
Age	Protonymphs
Source of test organism	Eggs of the predatory mite were supplied by Katz Biotech AG, D-15837 Baruth, Germany

B. Study design and methods

1. In life dates: January 14 to February 18, 2010

2. Design of the test

Number of test groups:	7 (control, test and reference item)
Number of application rates:	Test item: 5 Reference item: 1
Number of replicates per test group:	5 (one replicate = one exposure unit)
Number of larvae/per replicate:	20

The test item (soluble concentrate formulation of BYI 02960 SL 200 (g/L)) was applied onto glass plates at rates of 2, 4, 9, 19 and 40 g a.i./ha and the effects on the predatory mite *Typhlodromus pyri* were compared to those of a deionised water treated control. A toxic reference (active substance: dimethoate) applied at 4 g a.i./ha was included to indicate the relative susceptibility of the test organisms and the test system.

3. Observation and measurements

Mortality of 100 mites (5 replicates of 20 individuals per test group) was assessed 1, 4 and 7 days after exposure by counting the number of living and dead mites. The number of escaped mites was calculated as the difference from the total number exposed.

4. Statistics

The computer program SAS (Version 9.1.3, 2002-2003) was used to perform the statistical analyses.

The mortality data were analysed for significance using the Fisher Exact test (one-sided with Bonferroni-Holm adjustment; $\alpha = 0.05$), which is a distribution-free test method and does not require testing for normality or homogeneity prior analysis.

The reproduction data were tested for normal distribution using the Shapiro-Wilk test and for homogeneity using the Levene test.

As the reproduction data in this study were not normally distributed the Wilcoxon test (one-sided with Bonferroni-Holm adjustment; $\alpha = 0.05$) was used.

The LR_{50} value was calculated using Probit analysis.

RESULTS AND DISCUSSION

A. Environmental Conditions

Mites were kept under conditions which are summarized as follows:

Test temperature:	25.0-26.0°C
Relative humidity:	65 - 71% (deviations < 2 h down to 50 % due to handling)
Photoperiod:	16 hours light / 8 hours dark
Light source	686 - 1446 Lux

B. Biological Findings

The mortality / escaping rate in the control exposure units up to day 7 after treatment was 4.0%.
A summary of effects of BYI 02960 SL 200 g/L and the toxic reference on mortality of *Typhlodromus pyri* exposed on glass cover slides is given below:

Table Effects of BYI 02960 SL 200 (g/L) on mortality of *Typhlodromus pyri*

Test item		BYI 02960 SL 200 (g/L)		
Test organism		*Typhlodromus pyri*		
Exposure on:		Glass cover slides		
		Mortality after 7 days [%]		
Treatment	g a.i./ha	Uncorr.	Corr.	P-Value(*)
Control	0	4.0		
Test item	2	10.0	6.3	0.164 n.sign.
Test item	4	10.0	6.3	0.164 n.sign.
Test item	9	28.0	25.0	<0.001 sign.
Test item	19	52.0	50.0	<0.001 sign.
Test item	40	90.0	89.6	<0.001 sign.
Reference item	4	80.0	79.2	
LR_{50}: 17 g a.i./ha; 95 % Confidence Interval: (13 - 21) (calculated with Probit analysis)				
* Fisher`s Exact test (one-sided), p-values are adjusted according to Bonferroni-Holm n.sign. not significant, sign. significant				

C. Validity Criteria

The validity criteria of mortality ≤ 20% in the control group and ≥ 50% corrected mortality in the toxic reference are fulfilled (laboratory method with glass plates (BLUEMEL ET AL., 2000)).

D. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

LR_{50}: 17 g a.i./ha (95% Confidence Interval: 13 – 21)

CONCLUSION

The effects of BYI 02960 SL 200 residues on the survival of the predatory mite *Typhlodromus pyri* in a laboratory test on glass plates can be quantified as an LR_{50} of 17 g a.i./ha .

IIA 8.8.1.3 Ground dwelling predatory species

Based on the results of the studies reported under 8.8.1.1 and 8.8.1.2, no tests with additional species relevant to the use pattern of the product are required.

IIA 8.8.1.4 Foliage dwelling predatory species

Based on the results of the studies reported under 8.8.1.1 and 8.8.1.2, no tests with additional species relevant to the use pattern of the product are required.

IIA 8.8.2 Effects on non-target terrestrial arthropods in extended lab/semi-field test

Extended laboratory tests have been conducted with the formulated product BYI 02960 SL 200 G and are filed in the corresponding Annex III document, at point IIIA1 10.5.2.

In addition, aged-residue studies have been conducted and are filed in the corresponding Annex III document, at point IIIA1 10.5.3.

IIA 8.8.2.1 Parasitoid

Please refer to point IIA 8.8.2.

IIA 8.8.2.2 Predatory mites

Please refer to point IIA 8.8.2.

IIA 8.8.2.3 Ground dwelling predatory species

Please refer to point IIA 8.8.2.

IIA 8.8.2.4 Foliage dwelling predatory species

Please refer to point IIA 8.8.2.

IIA 8.8.2.5 Other terrestrial invertebrates

Please refer to point IIA 8.8.2.

IIA 8.9 Effects on earthworms

To assess the impact of BYI 02960 on earthworms, laboratory studies were conducted with the technical substance BYI 02960, the formulation BYI 02960 SL 200 G, and the major soil metabolites BYI 02960-difluoroacetic acid and 6-chloronicotinic acid. Acute and chronic laboratory earthworm studies are available for the active substance and both metabolites. The earthworm reproduction study

for the active substance was performed, in accordance with guidelines, with the formulated product BYI 02960 SL 200 G.

Additionally studies on soil organisms *Folsimia* and *Hypoaspis* have been performed and are reported under point 8.14.

IIA 8.9.1 Acute toxicity to earthworms

Report:	**KIIA 8.9.1/01; Leicher, T. (2010)**
Title:	BYI 02960 (tech.): acute toxicity to earthworms (*Eisenia fetida*) tested in artificial soil
Report No:	LRT/Rg-A-131/09
Document No:	M-363742-01-2
Guidelines:	**OECD-Guideline No. 207**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239; purity 96.2% w/w) on survival and growth of earthworms (*Eisenia fetida andrei*).

Adult earthworms (more than two months old, four replicates of 10) were exposed in an artificial soil system with peat content of 10% over a period of 14 days to concentrations of 5.6, 10, 18, 32, 56, 100 mg test item / kg dry soil (1st run) and 178, 316, 562 and 1000 mg test item / kg dry soil (2nd run). In addition a water control was tested.

Mortality and sublethal behavioural effects were used to determine the endpoints.

The 14-day-LC_{50} was 192.9 mg a.i./kg dry soil, the 14-day-NOEC was determined to be < 5.6 mg a.i./kg dry soil.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Origin Batch No.:	2009-000239 (Batch code: BYI 02960-01-03)
Sample description:	TOX 08508-00
CAS name:	2(5H)-furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difluoroethyl)amino]-
CAS#:	951659-40-8
IUPAC name:	4-[[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Stability of test compound:	Expiry date: 16.01.2011, when stored at +25 ± 5°C

2. Test solutions

Test item mixed with:	Artificial soil, 10 % peat
Method of preparation:	Each application mixture (5 g) was transferred separately to artificial soil (595 g dry weight) and mixed
Controls:	Water control
Reference substance	Chloroacetamide A.R.

3. Test organisms

Species:	*Eisenia fetida andrei*
Common name:	Earthworm
Source:	In-house lab culture; strain of Prof. Graff, Forschungsanstalt für Landwirtschaft, 38104 Braunschweig
Age at study initiation:	More than two months old
Feeding during test:	None
Weight at test start:	0.35 g (both runs)
Maintenance of culture:	
Temperature:	22 ± 2°C
Photoperiod:	12 hours light, 12 hours dark
Food:	Dried cattle manure at 14 day intervals

B. Study design and methods

1. In life dates November 3, 2009 to January 26, 2010

2. Design of biological test

The adult worms used in this study were more than two months old. On the day prior to the beginning of the study, they were transferred from the breeding substrate to an artificial soil (without test item) under the test conditions for acclimatization. Ten earthworms were placed in a randomized procedure in each test container. The average weight was 0.35 g (first and second run).

Earthworms (*Eisenia fetida andrei;* more than two months old) were exposed to BYI 02960 (purity 96.2 %) in an artificial soil system over a period of 14 days. Nominal concentrations were 5.6, 10, 18, 32, 56, 100 mg test item/kg dry soil (1st run) and 178, 316, 562 and 1000 mg test item/kg dry soil (2nd run). In addition, a water control was tested.

Each jar (glass jar; 1.5 L) filled with 595 g dry weight test soil (equivalent to 803 g wet weight) served as one replicate, 10 worms were used per replicate. The test was conducted with 4 replicates per treatment level. In the controls 8 (four in each run) replicates were tested. The test was conducted at 20 ± 2°C and 531 to 586 lux (over both runs) at constant light. The artificial soil contained 10 % peat, 20 % kaolinite clay, 69.7 % quartz sand and 0.3 % calcium carbonate.

3. Analytical verification

Not applicable.

4. Observation and measurements

Seven days after the start of the study, the number of surviving earthworms was determined and returned to the test containers. After 14 days, the weight, abnormal behaviour, observed symptoms as well as the number of surviving earthworms were determined.

Mortality of worms, intoxication symptoms and physical-chemical soil parameters were assessed as indicated below in the result section.

5. Statistical analysis

The LC_{50}-values and the 95 percent confidence limits were calculated by Probit-Analysis according to "Maximum-Likelihood" Method (D.J. Finney, 1978). The statistic software used was ToxRatPro Version 2.09.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Table: Properties of the test soil

Parameter	Start of study		End of study	
First run				
pH value	5.74	5.73	5.70	5.63
water content in the artificial soil (%)	26.4	26.6	25.9	25.8
WHC_{max}	67.3	61.7	---	---
WHC_{max} (mean)	64.5		64.5 *)	
water content as % of WHC_{max}	53.0	56.1	54.1	53.9
Second run				
pH value	5.63	5.64	5.67	5.63
water content in the artificial soil (%)	24.9	25.0	24.6	24.7
WHCmax	57.6	57.3	---	---
WHC_{max} (mean)	57.4		57.4 *)	
water content as % of WHC_{max}	57.6	58.0	56.8	56.9

*) taken into account the WHC_{max} from the start of the study

B. Biological Findings

Observations on mortality, immobilisation and sublethal intoxication symptoms are listed as follows:

Table: Effects of BYI 02960 on mortality and body weight change of *Eisenia fetida andrei*

Concentration of test item (nominal) [mg/kg dry soil]	% mortality (mean ± SD) day 7	% mortality (mean ± SD) day 14	% weight alteration of the survivors (mean ± SD) day 14
control	0	0	-3 ± 3
5.6	0	0	-12 ± 3 *
10	0	0	-13 ± 3 *
18	0	0	-17 ± 3 *
32	0	0	-20 ± 3 *
56	0	0	-23 ± 3 *
100	0	3 ± 5	-25 ± 2 *
178	5 ± 6	23 ± 19	-25 ± 2 *
316	68 ± 15	95 ± 6	-38 ± 15 *
562	98 ± 5	100	n.a.
1000	100	100	n.a.

* statistically different from control, Williams-Test ($\alpha = 0.05$, one-sided smaller)

No sublethal behavioural changes were observed. No morphological effects were observed.

C. Validity Criteria

The validity criterion of control mortality less than 10% is fulfilled.

D. Test with toxic reference substance

Reference substance:	Chloroacetamide A.R.
Date of most recent test:	18 NOV 2008
Result: LC_{50}	13.2 mg/kg

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

14-day-results	
NOEC (no-observed-effect-concentration)	< 5.6 mg a.i./kg dry soil
LOEC (lowest-observed-effect-concentration)	5.6 mg a.i./kg dry soil
LC_{50}	**192.9 mg a.i./kg dry soil** (95% confidence limits: 143.3 to 276.9 mg /kg)

CONCLUSION

The acute effect of BYI 02960 on earthworms (*Eisenia fetida andrei*) can be quantified as a 14-day-LC_{50} of 192.9 mg a.i./kg dry soil. The highest concentration with no mortality and no sublethal behavioural effects can be set to < 5.6 mg a.i./kg dry soil (the lowest concentration tested).

Report:	**KIIA 8.9.1/02; Leicher, T. (2010):**
Title:	BYI 02960-difluoroacetic acid: acute toxicity to earthworms (*Eisenia fetida*) tested in artificial soil
Report No:	LRT/Rg-A-135/10
Document No:	M-368835-01-2
Guidelines:	**OECD-Guideline No. 207**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of BYI 02960-difluoroacetic acid (Batch code: BCS-AA56716; TOX 08889-00; purity 95.8% w/w) on survival and growth of earthworms (*Eisenia fetida andrei*).

Adult earthworms (more than two months old, four replicates of 10) were exposed in an artificial soil system with peat content of 10 % over a period of 14 days to concentrations of 31.3, 62.5, 125, 250, 500 and 1000 mg test item/kg dry soil. In addition a quartz sand control was tested.

Immobilisation and sublethal behavioural effects were used to determine the endpoints.

The 14-day-LC_{50} was > 1000 mg/kg dry weight soil, the 14-day-NOEC was determined to be 31.3 mg/kg dry weight soil.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	Difluoroacetic acid (Code: BCS-AA56716)
Type of test material:	Substance technical (pure metabolite)
Chemical state and description:	Colourless liquid
Origin Batch number:	BCOO 5984-1-1 (Batch code: BCS-AA56716-01-01
Material number:	BCS-AA56716
Sample description:	TOX 08889-00
CAS#:	381-73-7
Purity:	95.8% w/w
Stability:	Expiry date: 29.07.2010 , when stored at +25 ± 5°C

2. Test solutions

Test item mixed with:	Quartz sand
Method of preparation:	Application mixtures of 10 g each were transferred separately to artificial soil (595 g dry weight) and mixed thoroughly using a laboratory mixer.
Controls:	Water control

3. Test organisms

Species:	*Eisenia fetida andrei*
Common name:	Earthworm
Source:	In-house lab culture; strain of Prof. Graff, Forschungsanstalt für Landwirtschaft, 38104 Braunschweig, Germany
Age at study initiation:	More than two months old
Feeding during test:	None
Weight at test start:	0.32 g
Maintenance of culture:	
Temperature:	22 ± 2°C
Photoperiod:	12 hours light, 12 hours dark
Food:	Dried cattle manure at 14 day intervals

B. Study design and methods

1. In life dates February 24 to March 11, 2010

2. Design of biological test

The adult worms used in this study were more than two months old. On the day prior to the beginning of the study, they were transferred from the breeding substrate to an artificial soil (without test item) under the test conditions for acclimatization. Ten earthworms were placed in a randomized procedure in each test container. The average weight was 0.32 g.

Earthworms (*Eisenia fetida andrei*; more than two months old) were exposed to difluoroacetic acid (code: BCS-AA56716; purity 95.8 % w/w) in an artificial soil system over a period of 14 days. Concentrations were 31.3, 62.5, 125, 250, 500 and 1000 mg test item / kg dry soil. In addition a quartz sand control was tested. Each replicate consisted of a jar (glass; 1.5 L) filled with 595 g dry weight test soil (equivalent to 803 g wet weight). 10 worms were used per replicate. The test was conducted with 4 replicates per treatment level. The test was conducted at 20 ± 2°C and 529 lux at day 0, 547 lux at day 7 and 559 lux at day 14 at constant light. The artificial soil contained 10% peat, 20% kaolinite clay, 69.6% quartz sand and 0.4% calcium carbonate.

3. Observation and measurements

Seven days after the start of the study, the number of surviving earthworms and after 14 days, the weight, abnormal behaviour, observed symptoms as well as the number of surviving earthworms were determined. Biological data and physical-chemical soil parameters were assessed as indicated below in the result section.

4. Analytical verification

Not applicable

5. Statistical analysis

The LC_{50}-values and the 95 percent confidence limits were calculated by Probit-Analysis according to "Maximum-Likelihood" Method (D.J. Finney, 1978). The statistic software used was ToxRatPro Version 2.09.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Table: Properties of the test soil

Parameter	Start of study		End of study	
pH value	6.19	6.25	6.00	6.00
water content in the artificial soil (%)	25.9	26.0	25.2	25.3
WHC_{max}	66.4	64.5	---	---
WHC_{max} (mean)	65.4		65.4 *)	
water content as % of WHC_{max}	53.3	53.5	50.7	51.1

*) taken into account the WHC_{max} from the start of the study

B. Biological Findings

Observations on immobilisation and sublethal intoxication symptoms are listed as follows:

Table: Effects of BCS-AA56716 on mortality and body weight chance of *Eisenia fetida andrei*

Concentration of test item (nominal) (mg/kg dry soil)	% mortality (mean ± SD) day 7	% mortality (mean ± SD) day 14	% weight alteration of the survivors (mean ± SD) day 14
control	0	0	+6 ± 3
31.3	0	0	+5 ± 3
62.5	0	0	-2 ± 4 *
125	0	0	-4 ± 4*
250	2.5 ± 5	2.5 ± 5	-6 ± 3*
500	0	0	-8 ± 4*
1000	0	0	-11 ± 3*

* statistically different from control, Williams-Test ($\alpha = 0.05$, one-sided smaller)

No morphological and behavioural effects were observed.

C. Validity Criteria

The validity criterion of control mortality less than 10% is fulfilled.

D. Test with toxic reference substance

Reference substance:	Chloroacetamide A.R.
Date of most recent test:	February, 2009
Result:	LC_{50}: 13.2 mg Chloroacetamide A.R. /kg dry weight soil (95 % confidence limits: 12.0 – 14.5 mg /kg).

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

LC_{50} (14 day)	**> 1000 mg/kg dry weight soil**
NOEC	31.3 mg/kg dry weight soil
LOEC	62.5 mg/kg dry weight soil

CONCLUSION

The acute effect of BYI 02960-difluoroacetic acid (BCS-AA56716) on earthworms (*Eisenia fetida andrei*) can be quantified as a 14-day-LC_{50} of > 1000 mg/kg dry weight soil. The highest concentration with no mortality and no sublethal behavioural effects (NOEC) can be set to 31.3 mg/kg dry weight soil.

The following study was performed for the registration of acetamiprid and is the property of Nippon Soda Co. Ltd**,** access to the study has been granted. The study has been evaluated during the Annex I inclusion of acetamiprid, therefore only a very short summary of the study conclusion is repeated here. Note- the test compound IC-0 is identical to 6-CNA.

Report:	**KIIA 8.9.1/03; Wetton, P.M. (1999)**
Title:	IC-0: Acute toxicity to earthworms (*Eisenia foetida*)
Report No:	C007758 (Study report number: 282/575)
Document No:	M-196591-01-1
Guidelines:	**OECD-Guideline No. 207 (1984)**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of 6-chloronicotinic acid (Lot No. MF-4267; code: BYI 02960-6-CNA (IC-0); purity 99.7%) to earthworms (*Eisenia foetida*) in artificial soil.

Following a preliminary range-finding study, 60 earthworms (six replicates of 10 worms) were exposed to a single concentration of 1000 mg/kg of soil for a period 14 days. The number of mortalities was determined after 7 and 14 days of exposure. A positive control study using chloroacetamide, conducted approximately every 6 months, is reported for reference purposes.

Mortality and body weight change were used to determine the endpoints.

The 14-Day LC_{50} for the test material to earthworms (*Eisenia foetida*) based on nominal test concentrations was greater than 1000 mg/kg. Correspondingly the No Observed Effect Concentration (NOEC) was 1000 mg/kg.

The result of the positive control study gave a 14-Day LC_{50} for chloroacetamide (positive control) of 23 mg/kg with 95% confidence limits of 22 - 24 mg/kg.

IIA 8.9.2 Sublethal effects on earthworms

To investigate sublethal effects of BYI 02960 on earthworms a chronic study on earthworms was performed with the formulated product BYI 02960 SL 200G instead of technical substance. The summary of this study is provided in the Annex II as representative for the toxicity to the active substance. Additionally studies on metabolites are included in this section.

Report:	**KIIA 8.9.2/01; Leicher T.; 2010**
Title:	BYI 02960 SL 200 G: Effects on survival, growth and reproduction on the earthworm *Eisenia fetida* tested in artificial soil
Report No:	LRT-RG-R-76/09
Document No:	M-392964-01-2
Guidelines:	**ISO 11268-2, 1998 (E) and OECD 222 (2004)**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of BYI 02960 SL 200 G, Specification No. 102000021884 (Sample description: FAR01438-00, Batch ID: 2009-001253; Material No.: 79718845); (purity 199.8 g BYI 02960/L = 17.0% w/w) on growth and reproduction of earthworms (*Eisenia fetida andrei*).

Earthworms (approximately 7 month old, 8 x 10 animals for the control group and 4 x 10 animals per test concentration of the treatment group) were exposed in an artificial soil system over a period of 56 days to nominal concentrations of 8.9, 15.8, 28.1, 50.0 and 89.0 mg product/kg dry weight soil. In addition a water control was tested. The test item was mixed into the soil. After 28 days the number of surviving animals and their weight alteration was determined. They were then removed from the artificial soil. After further 28 days, the number of offspring was determined. The overall NOEC was determined to be 8.9 mg product/kg dry weight soil, based on reproduction.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960 SL 200 G
Type of test material:	Formulated product
Specification No.:	102000021884
Material number:	79718845
Sample description:	FAR01438-00
Batch No.:	2009-001253
Nominal content of active ingredient:	BYI 02960: 200 g/L
Analytical content of active ingredient:	BYI 02960: 17.0% w/w, 199.8 g/L according to certificate of analysis
Density:	1.175 g/mL at 20°C
Stability:	Expiry date: 2010-03-20, when stored at 25±5 °C

2. Vehicle and/or positive control

Test item mixed with:	Water
Controls:	Water control as negative control

3. Test organisms

Species:	*Eisenia fetida andrei*
Common name:	Earthworm
Source:	In-house lab culture; strain of Prof. Graff, Forschungsanstalt für Landwirtschaft, 38104 Braunschweig, Germany
Age at study initiation:	Approximately 7 month old
Feeding during test:	Dried cow manure
Weight at test start:	340 to 350 mg (mean figures per treatment level)
Maintenance of culture:	
Temperature:	22 ± 2°C
Photoperiod:	12 hours light, 12 hours dark
Food:	Dried cattle manure at 14 day intervals

B. Study design and methods

1. In life dates: 1 October, 2009 to 25 March, 2010

2. Design of biological test

Earthworms (*Eisenia fetida andrei*; approximately 7 month old. , 8 x 10 animals for the control group and 4 x 10 animals per test concentration of the treatment group) were exposed to BYI 02960 SL 200 G; (purity 199.8 g BYI 02960/L = 17.0% w/w) in an artificial soil system with 10 % peat over a period of 8 weeks. The test item was mixed into the soil. Nominal concentrations were 8.9, 15.8, 28.1, 50.0 and 89.0 mg product/kg dry weight soil, respectively. In addition, a water control was tested. Each jar (plastic boxes; ca. 16.5 cm x 12 cm x 6 cm (length x width x height)) served as one replicate filled with 500 g artificial soil (dry weight). The depth of the soil layer was approximately 5 cm. The surface area of the soil was 200 cm^2. The test was conducted at 20 ± 2°C and 518 to 543 lux at 16 h light and 8 h dark. The artificial soil contained 10% peat, 1% food, 20% kaolinite clay, 68.65% quartz sand and 0.35% calcium carbonate.

3. Observation and measurements

Food (dried cattle manure) was amended weekly during the first four weeks. After a period of 4 weeks the adult earthworms were removed from the test vessels and the survivors were counted and their fresh weight was measured. From these data mortality and biomass effects were determined. The test vessels were incubated for another 4 weeks under identical climatic conditions. From these the reproduction was determined by counting the number of offspring hatched from the cocoons after this additional test period per test vessel.

4. Statistical analysis

The homogeneity of variances of the data (body weight change and number of surviving juveniles) was checked by Cochran's test. The homogeneity hypothesis was accepted. The normal distribution of the data was tested by Kolmogorov-Smirnov test. The normality hypothesis of the data was accepted.

The data were statistically evaluated by means of a Williams multiple sequential t-test.

The statistical software package ToxRatPro Version 2.09 @ was used for the calculation.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

The soil-pH was 6.42 to 6.50. The moisture of the artificial soil was adjusted to nominal 37.5 g water/100 g dry weight artificial soil (nominal 27.3 % soil moisture) corresponding to approximately 53 % of the maximum water holding capacity of the artificial soil.

Prior to the start of the test the mean soil moisture was 20.9 %. At day 0 the mean soil moisture was 27.0 % and at day 56 it was 25.3 %.

B. Biological Findings

No mortality of adult earthworms was observed after 28 days of exposure at the test concentrations 8.9, 28.1, 50.0, and 89mg test item/kg dry weight soil. One worm died during 28 days in the concentration 15.8mg test item/kg dry weight soil Effects on survival, changes in body weight and number of juveniles are listed as follows:

Table: Effects of BYI 02960 SL 200 G on mortality, body weight change and reproduction of Eisenia fetida andrei

Nominal concentration of test item [mg/kg dry soil]	Mortality after 28 days (%)	Mean body weight change (%) from day 0 to day 28	Mean number of juveniles after 56 days	% juveniles compared to control
control	0	44.6 ± 8.9	220.0 ± 22.7	-
8.9	0	48.1 ± 8.5	205.3 ± 19.7	93.3
15.8	2.5	52.0 ± 3.1	190.0 ± 13.5 *	86.4
28.1	0	52.9 ± 7.7	188.8 ± 12.8 *	85.8
50	0	43.8 ± 9.1	162.8 ± 27.3 *	74.0
89	0	45.4 ± 4.8	130.0 ± 28.8 *	59.1

* Significant according to Williams Multiple Sequential t-test, one-sided smaller, $\alpha = 0.05$

C. Validity Criteria

The validity criteria of the test according to the guideline were fulfilled:

Validity criteria	Recommended	Obtained
Mortality of the adults in the control	≤ 10 %	0 %
Mean change in growth of the adult earthworms in the control during the exposure period of four weeks	> -20 %	+ 44.6 %
Mean rate of reproduction of juveniles in the control	≥ 30 earthworms per control vessel	220 earthworms per control vessel
Coefficient of variance of reproduction in the control	≤ 30 %	10.3 %

D. Test with toxic reference substance

Reference substance:	Derosal fluessig (Carbendazim 360 g/L)
Date of most recent test:	15 JAN 2009
Result:	NOEC: 1.25 mg a.i./kg dry weight soil

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

4-week figures	
NOEC (growth)	≥ 89 mg product/kg dws
LOEC (growth)	> 89 mg product/kg dry weight soil
8-week figures	
NOEC (reproduction):	8.9 mg product/kg dws
LOEC (reproduction):	15.8 mg product/kg dws

CONCLUSION

The chronic effect of BYI 02960 SL 200 G on earthworms (*Eisenia fetida andrei*) can be quantified as an overall-NOEC of 8.9 mg product/kg dry weight soil.

Report:	**KIIA 8.9.2/02; Leicher, T. (2010)**
Title:	BYI 02960 Difluoroacetic acid: Effects on survival, growth and reproduction on the earthworm *Eisenia fetida* tested in artificial soil with 10% peat
Report No:	LRT-Rg-R-81/10
Document No:	M-398061-01-2
Guidelines:	**ISO 11268-2: 1998(E), OECD 222: 2004**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the chronic effects of BYI 02960-difluoroacetic acid (Origin Batch no. BCOO 5984-1-1; TOX 08889-00; purity 95.8% w/w) on survival, growth and reproduction of earthworms (*Eisenia fetida andrei)*.

Adult *Eisenia fetida* (approx. 5 months old, 8 x 10 animals for the control group and 4 x 10 animals per test concentration of the treatment group) were exposed in an artificial soil (with 10% peat content) to the nominal test concentrations of 11, 20, 35, 62 and 110 mg test item/kg dry weight artificial soil. The test item was mixed into the soil. After 28 days the number of surviving animals and their weight alteration was determined. They were then removed from the artificial soil. After further 28 days, the number of offspring was determined.

No statistically significant different values for the growth relative to the control were observed at any test concentrations. A statistically significant different value for the number of juveniles per test vessel relative to the control was observed only at the highest test concentration of 110 mg test item/kg dry weight artificial soil.

Overall, based on the biological and statistical significance of the effects observed on growth and reproduction, it is concluded, that the NOEC for this study is 62 mg test item/kg dry weight artificial soil. Thus, the overall LOEC is determined to be 110 mg test item/kg dry weight artificial soil.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960-difluoroacetic acid (BCS-AA56716)
Type of test material:	Substance, technical (pure metabolite)
Chemical state and description:	Colourless liquid
Origin Batch number:	BCOO 5984-1-1 (Batch code: BCS-AA56716-01-01)
Sample description:	TOX 08889-00
Purity:	95.8% w/w
Stability of test compound:	Expiry date: 29.07.2010, when stored at +25 ± 5°C

2. Test solutions

Test item mixed with:	Quartz sand
Method of preparation:	Test item plus 5 g quartz sand were thoroughly mixed into the artificial soil using a laboratory mixer
Controls:	Water control

3. Test organisms

Species:	*Eisenia fetida andrei*
Common name:	Earthworm
Source:	In-house lab culture; strain of Prof. Graff, Forschungsanstalt für Landwirtschaft, 38104 Braunschweig, Germany
Weight at test start:	0.25 to 0.45 g
Maintenance of culture:	
Temperature:	22 ± 2°C
Photoperiod:	12 hours light, 12 hours dark
Food:	Dried cattle manure

B. Study design and methods

1. In life dates March 17 to May 12, 2010

2. Design of biological test

Principles of the testing procedure: Adult *Eisenia fetida* (approx. 5 months old) were exposed in an artificial soil (with 10 % peat content) to BYI 02960-difluoroacetic acid (purity 95.8%) at nominal test concentrations of 11, 20, 35, 62 and 110 mg test item/kg dry weight artificial soil. The test item was mixed into the soil. After 28 days the number of surviving animals and their weight alteration was determined. They were then removed from the artificial soil. After further 28 days, the number of offspring was determined.

Each replicate consisted of a jar (plastic boxes; ca. 16.5 cm x 12 cm x 6 cm; length x width x height) filled with 500 g soil. The depth of the soil layer was 5 cm. The surface area of the soil was approximately 200 cm^2. 10 worms were used per replicate. The test was conducted with 4 replicates per treatment level. In the controls 8 replicates were tested. The test was conducted at 20 ± 2°C and 579, 524 and 500 Lux at day 0, 28 and 56, respectively at a 16 h light - 8 h dark photoperiod. The artificial soil contained 10% peat, 20% kaolinite clay, 68.65% quartz sand and 0.35% calcium carbonate.

3. Observation and measurements

Food (dried cattle manure) was amended weekly during the first four weeks. After 4 weeks mortality of adult worms, their body weight change and intoxication symptoms were assessed as indicated below in the result section. The test vessels were incubated for another 4 weeks under identical climatic conditions. Then the juveniles were counted.

4. Analytical verification

Not applicable

5. Statistical analysis

The U-test after Bonferroni-Holm (two-sided, $\alpha = 0.05$) was used to determine significant differences of growth between control and treatments.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Prior to the start of the test the mean soil moisture was 20.2%. At day 0 the mean soil moisture was 27.15% and at day 56 it was 26.7%.

Table: pH values of soil

	mg test item/kg dry weight artificial soil	pH value
Control	----	6.46
Difluoroacetic acid	11	6.37
Difluoroacetic acid	20	6.43
Difluoroacetic acid	35	6.40
Difluoroacetic acid	62	6.39
Difluoroacetic acid	110	6.37

B. Biological Findings

Observations on immobilization, changes in body weight, number of juveniles and sublethal intoxication symptoms are listed as follows:

Table: Effects of on mortality, body weight change and reproduction of *Eisenia fetida andrei*

Concentration of test item [mg/kg dry soil]	% mortality (after 28 days)	% body weight change (after 28 days) mean ± SD	number of juveniles/test vessel (after 56 days) mean ± SD	Number of juveniles/surviving adult (after 56 days) mean ± SD
control	0	66.6 ± 7.2	264.8 ± 30.6	26.5 ± 3.1
11	0	61.8 ± 3.6	259.8 ± 28.2	26 ± 2.8
20	0	63.3 ± 13.4	273.5 ± 27.4	27.4 ± 2.7
35	0	60.4 ± 7.0	282.3 ± 45.8	28.2 ± 4.6
62	0	61.5 ± 5.5	230.3 ± 22.0	23.0 ± 2.2
110	0	60.1 ± 3.5	190.0 ± 32.0*	19.0 ± 3.2

No statistically significant differences to the control for body weight (U-test, Bonferroni-Holm, $\alpha = 0.05$)
* statistically significantly different compared to the control (Williams Multiple Sequential t-test, one-sided smaller, $\alpha = 0.05$)

No sublethal behavioural changes were observed.

C. Validity Criteria

The validity criterion of more than 30 juveniles in each control replicate is fulfilled. The validity criterion of control variation regarding reproduction of less than 30% is fulfilled. The validity criterion of control adult mortality less than 10% is fulfilled.

D. Test with toxic reference substance

Reference substance:	Derosal fluessig (Carbendazim 360 g/L)
Date of most recent test:	January to March 2009
Result:	NOEC = 1.25 mg a.i./kg dry weight soil

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

No worm died after 28 days of exposure at the control group. No statistically significant differences for the growth relative to the control were observed at any test concentration. No statistically significant different values for the number of juveniles per test vessel relative to the control were observed at the test concentrations of 11, 20, 35 and 62 mg test item/kg dry weight artificial soil.

A statistically significant different value for the number of juveniles per test vessel relative to the control was observed at the highest test concentration of 110 mg test item/kg dry weight artificial soil.

Based on these observations the following are derived.

NOEC (mortality):	≥110 mg/kg dry weight soil
NOEC (growth):	>110 mg/kg dry weight soil
NOEC (reproduction):	**62 mg/kg dry weight soil**
LOEC (reproduction)	110 mg/kg dry weight soil

CONCLUSION

The chronic overall NOEC for difluoroacetic acid on earthworms (*Eisenia fetida andrei*) is NOEC of 62 mg/kg dry weight soil.

Report:	**KIIA 8.9.2/03; Leicher, T. (2011)**
Title:	6-chloronicotinic acid (AE F161089): Effects on survival, growth and reproduction on the earthworm *Eisenia fetida* tested in artificial soil with 5% peat
Report No:	LRT-Rg-R-101/11
Document No:	M-413562-02-2
Guidelines:	**ISO 11268-2: 1998 (E)** **OECD 222: April 13, 2004**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the chronic effects of 6-chloronicotinic acid (Batch code: AE F161089 00 1B99 0001; purity 98.8% w/w) to earthworms (*Eisenia fetida andrei*).

Earthworms (8 month old, 10 worms used per replicate) were exposed in an artificial soil system with a peat content of 5% over a period of 4 weeks to nominal concentrations of 100 mg/kg (first run; limit test) and 9.5, 16.8, 30.0, 53.4 and 95.0 mg/kg dry weight soil (2nd run), respectively. In addition, a water control was tested.

The test item was mixed into the soil. After 28 days the number of surviving animals and their weight alteration was determined. They were then removed from the artificial soil. After further 28 days, the number of offspring was determined.

Immobilisation and body weight change in the adults and number of juveniles after another 4 weeks as well as sublethal behavioural effects were used to determine the endpoints.

Overall, based on the biological and statistical significance of the effects observed on growth and reproduction at 100 mg/kg in the first run, it is concluded, that the NOEC for this study is 95.0 mg test item/kg dry weight artificial soil.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	6-chloronicotinic acid (6-CNA, AE F161089)
Type of test material:	Substance, technical (pure metabolite)
Chemical state and description:	Beige powder
Origin Batch number:	M12653 (Batch code: AE F161089 00 1B99 0001)
Material number:	AE F161089
CAS#:	5326-23-8
Purity:	98.8% w/w
Stability of test compound:	Expiry date: 09.07.2013, when stored at + 5 ± 5 °C

2. Test solutions

test item mixed with:	Quartz sand
Concentration of vehicle:	5 % peat in 500 g dry weight artificial soil
Controls:	Water control

3. Test organisms

Species:	*Eisenia fetida andrei*
Common name:	Earthworm
Source:	In-house lab culture
Age at study initiation:	8 month (range less than 4 weeks)
Feeding during test:	Maximum 5 g dry manure moistened with 6mL water once per week during first 4 weeks
Weight at test start:	Range: 0.25 to 0.44 g per worm
Maintenance of culture:	
Temperature:	22 ± 2°C
Photoperiod:	12 hours light, 12 hours dark
Food:	Dried cattle manure at 14 day intervals

B. Study design and methods

1. In life dates November 18, 2010 to June 15, 2011

2. Design of biological test

Earthworms (*Eisenia fetida andre*i; 8 month (range less than 4 weeks)) were exposed to 6-chloronicotinic acid (code: AE F161089; purity 98.8% w/w) in an artificial soil system over a period of 8 weeks. Concentrations were 100 mg/kg (first run; limit test) and 9.5, 16.8, 30.0, 53.4 and 95 mg/kg dry weight soil, respectively (2nd run), mixed into the soil. Due to a statistical significance of the treated

group in the limit test, compared to the control group, a second run was necessary. In addition, a water control was tested. Each replicate consisted of a jar (non-re-usable plastic boxes; ca. 16.5 cm x 12 cm x 6 cm; l x w x h) filled with 500 g artificial soil with 5 % peat. The depth of the soil layer was 5 cm. The surface area of the soil was approximately 200 cm^2. 10 worms were used per replicate. The test was conducted with 4 (8 in the 1st run; limit test) replicates per treatment level. In the controls 8 replicates were tested. The test was conducted at 20 ± 2 °C and 477 – 588 lux at 16 h light: 8 h dark.

3. Observation and measurements

After 4 weeks mortality of adult worms, their body weight change and intoxication symptoms were assessed as indicated below in the result section. After removal of the adults the test vessels were incubated for another 4 weeks under identical climatic conditions. Then the juveniles were counted.

4. Statistical analysis

The homogeneity of variances of the data was checked by Cochran's test. The normal distribution of the data was tested by Kolmogorov-Smirnov test. The normally distributed data of the limit test were statistically evaluated by a Pairwise Mann-Whitney U-test Procedure, two – sided, α = 0.05. The data of the dose-response test were statistically evaluated by means of a Williams multiple sequential t-test, two – sided, α = 0.05.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

The soil-pH was 6.0 ± 0.5. The water content was 56.8 to 62.8% (1st run) and 56.2 to 64.6% (2nd run), respectively, of the maximum water holding capacity.

B. Biological Findings

Observations on immobilization, changes in body weight, number of juveniles and sublethal intoxication symptoms are listed as follows:

Table: Effects of AE F161089 on mortality, body weight change and reproduction of *Eisenia fetida andrei*

Concentration of test item (mg/kg dry soil)	28 days	0-28 days	56 days
	% mortality	% body weight change (mean ± SD)	number of juveniles/test vessel (mean ± SD)
control (1st run)	0	72.8 ± 4.3	211.9 ± 30.2
100	0	50.5 * ± 19.8	107.4 **± 36.1
control (2nd run)	0	59.17 ± 8.25	215.1 ± 29.2
9.5	0	55.82 ± 10.00	208.0 ± 36.4
16.8	0	56.01 ± 2.66	199.0 ± 55.3
30	0	49.94 ± 7.45	222.3 ± 34.9
53.4	0	49.52 ± 6.48	180.0 ± 34.4
95	0	53.35 ± 4.43	186.0 ± 16.8

* statistical significance compared to the control (Pairwise Mann-Whitney U-Test Procedure, two-sided, α = 0.05)

** statistical significance compared to the control (STUDENT t-test for homogeneous variances, one-sided smaller, α = 0.05)

No sublethal behavioural changes were observed.

C. Validity Criteria

The validity criteria were fulfilled.

Validity criteria	Recommended	Limit-Test	Dose-response-Test
Mortality of the adults in the control	≤ 10%	0	0
Mean change in growth of the adult earthworms in the control during the exposure period of four weeks	should not exceed - 20 %	72.8%	59.2%
Rate of reproduction of juveniles (no of earthworms per control vessel)	≥ 30	211.9	215.1
Coefficient of variance of reproduction in the control	≤ 30%	14.2	13.6

D. Test with toxic reference substance

Reference substance: Carbendazim SC 360 G (Derosal flüssig)
Date of most recent test: JAN to MAR 2010

Test object	*Eisenia fetida*			
Reference test item	Control	Carbendazim SC 360 G		
mg t.i./kg dry weight artificial soil	---	1.25	2.5	5.0
Mortality of adult earthworms after 28 days [%]	0	0	0	0
Body weight change of adults from day 0 to day 28 [%]	+ 44.6	+ 55.4 *	+ 44.2	+ 32.2 *
Standard Deviation	± 8.9	± 4.6	± 6.6	± 2.8
Statistical comparison to the control	---	s.	n.s.	s.
Mean number of offspring/test vessel after 56 days	220.0	247.5	157.5 **	20.8 **
Standard Deviation	± 22.7	± 24.3	± 5.4	± 15.6
Statistical comparison to the control	---	n.s.	s.	s.

* Result of a Williams multiple sequential t-test, two-sided, $\alpha = 0.05$
** Result of a Williams multiple sequential t-test, one-sided smaller, $\alpha = 0.05$
s. mean value statistically significantly different compared to the control ($p < 0.05$)
n.s. mean value not statistically significantly different compared to the control ($p \geq 0.05$)

The results of the reference test item indicated that the test system was sensitive to the reference test item.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

NOEC (growth): 95 mg/kg dry weight soil
NOEC (reproduction): 95 mg/kg dry weight soil
LOEC (growth) 100 mg/kg dry weight soil
LOEC (reproduction) 100 mg/kg dry weight soil

CONCLUSION

The chronic effect of 6-chloronicotinic acid (AE F161089) on earthworms (Eisenia fetida andrei) can be quantified as an overall-NOEC of 95 mg/kg dry weight soil.

IIA 8.10 Effects on soil microbial activity

IIA 8.10.1 Nitrogen transformation

Report:	**KIIA 8.10.1/01; Frommholz, U. (2009)**
Title:	BYI 02960 a.s.: Determination of effects on nitrogen transformation in soil
Report No:	FRM-N-130/09
Document No:	M-359803-01-2
Guidelines:	**OECD guideline 216, 2000**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of BYI 02960 (Origin Batch No: 2009-000239; Batch code: BYI 02960-01-03; TOX 08508-00; purity 96.2% w/w) to the nitrogen turnover of soil microflora.

Rates of 0.3 and 3.0 mg/kg a.i./ha (corresponding to 0.4 and 4.0 mg a.i./ kg dry weight soil) were applied on loamy sand soil. After the amendment with Lucerne-grass-green meal the nitrogen turnover was measured at day 0, and after 7, 14 and 28 days of incubation.

The deviation from the control did not exceed 25% after 28 days. BYI 02960 has negligible effects on nitrogen turnover of soil microflora.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Origin Batch number:	2009-000239 (Batch code: BYI 02960-01-03)
Sample description:	TOX 08508-00
CAS name:	2(5H)-furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difluoroethyl)amino]-
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Stability of the test item:	Expiry date: 16.01.2011, when stored at +25 ± 5°C

2. Test solutions

Test item mixed with:	Loamy sand: 72 % sand, 17.4 % silt, 10.4 % clay
Method of preparation:	Sieved soil (2 mm) was treated with either 10 g ground quartz sand/kg dry weight soil
Controls:	Water control

3. Test soil

Soil nomenclature	Loamy sand
Collection depth	0 to 20 cm
Lot Nr.:	3650
Source:	Laacherhof, Germany (Bayer CropScience experimental farm)
Date of collection:	10 AUG 2009
Storage temperature:	4 ± 2 °C
Particle size distribution (% w/w):	
630 – 2000 µm	0.046
200 – 630 µm	0.477
63 – 200 µm	0.199
20 – 63 µm	0.076
6.3 – 20 µm	0.061
2.0 – 6.3 µm	0.037
< 2.0 µm	0.104
Soil properties:	
biomass (mg microbial C/kg dry soil):	902
% microbial C of organic carbon:	5.7
% nitrogen	0.06
pH:	6.28 to 6.42
Cation exchange capacity (meq/100 g dry weight soil):	6.63
History of soil:	
Plant protection products not used since:	2000
Fertilisers not used since:	2000
Crops:	Grass

B. Study design and methods

1. In life dates September 4 to October 16, 2009

2. Design of biological test

Rates of 0.3 and 3.0 mg/kg a.i./ha (corresponding to 0.4 and 4.0 mg a.i./ kg dry weight soil) were applied on loamy sand. After the amendment with Lucerne-grass-green meal (consisting of 40.6% C_{total}, 0.05% C_{inorg}, 2.5% N) the nitrogen turnover was measured at day 0, and after 7, 14 and 28 days of incubation. In addition a water control was tested. Each replicate consisted of a jar (brown glass bottles; 0.5 L) filled with 300 g dry weight test soil. The test was conducted with 3 replicates per treatment level. The test was conducted at 20 ± 2°C.

3. Observation and measurements

At day 0, and after 7, 14 and 28 days of incubation subsamples [moist samples (equivalent to 10 g dry weight)] were taken from each jar. The content of ammonium, nitrite and nitrate was measured with a Bran + Lübbe Autoanalyzer 3.

4. Statistical analysis

All calculations were performed using Microsoft Excel 2003.

The percentage differences in the quantities of nitrate-N formed between control soils and treated soils were expressed as absolute values and determined as follows:

((treatment rates – control rates)/control rates) x 100 % = % difference.

Rates were expressed in “mg nitrate-N/kg dry weight soil/day”.

Homogeneity of variances was determined by Cochran’s Test, $\alpha = 0.05$. Depending on the results the appropriate T-tests were performed. In the T-test, the values of nitrate-N/kg dry weight soil/time

interval/day from control soils and treated soils were compared. The statistical calculations were carried out using ToxRatPro 2.09 (Ratte 2002).

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

The soil-pH was 7.19. The water content was 40 to 50% of the maximum water holding capacity before the start of the study.

B. Biological Findings

During the 28-day test, 0.4 mg BYI 02960 a.i./kg dry weight soil caused a temporary stimulation of the daily nitrate rates at the time interval 0-7 days after treatment in a loamy sand soil amended with Luzerne-grass-green meal. At the end of the test (14-28 day interval), differences in the nitrate-N rates between control soil samples and treated soil samples are <25 % and meet the trigger values of above mentioned guideline for a termination of the study.

Table: Effects of BYI 02960 on nitrogen turnover of the soil microflora in loamy sand (transformation per time interval and day)

Time Interval (days)	BYI 02960 a.s. mg nitrogen (N)/kg dws/time interval/day (mean ± SD)										
				Application rates of test item							
	Control			0.4 mg/kg dry weight soil				4.0 mg/kg dry weight soil			
	Nitrate-N			Nitrate-N			% difference to control	Nitrate-N			% difference to control
0-7	0.45	±	0.13	0.63	±	0.37	39 n.s.	0.56	±	0.22	24 n.s.
7-14	3.52	±	0.10	3.51	±	0.19	0 n.s.	4.01	±	0.16	14 *
14-28	1.75	±	0.03	1.71	±	0.05	2 n.s.	1.76	±	0.04	1 n.s.

* = Statistically significant difference to the control (Student-t Test, two-sided, α = 0.05).
n.s. = No statistically significant difference to the control (Student-t Test, two-sided, α = 0.05).

C. Validity Criteria

The validity criterion of control variation of less than 15% is fulfilled.

D. Test with toxic reference substance

A reference test with sodium chloride conducted 2009 demonstrated that 16 g NaCl/kg dry weight soil had distinct and long-term (> 28 days) influence on microbial mineralization of nitrogen.

CONCLUSION

BYI 02960 has negligible effects on nitrogen turnover of soil microflora when applied at 300 g and 3 kg a.i./hectare.

Report:	**KIIA 8.10.1/02; Frommholz, U. (2011)**
Title:	6-chloronicotinic acid (AE F161089): Determination of effects on nitrogen transformation in soil
Report No:	FRM-N-156/11
Document No:	M-408028-01-2
Guidelines:	**OECD guideline 216, 2000**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of 6-chloronicotinic acid (Batch code: AE F161089 00 1B99 0001; purity 98.8% w/w) to the nitrogen turnover of soil microflora.

A rate of 1 kg test item/ha (corresponding to 1.33 mg test item /kg dry weight soil) was applied on silty sand. After the amendment of Lucerne-grass-green meal the nitrogen turnover was measured at day 0, and after 7, 14 and 28 days of incubation.

The deviation from the control did not exceed 25% after 28 days. 6-chloronicotinic acid (AE F161089) has negligible effects on nitrogen turnover of soil microflora.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	6-chloronicotinic acid (BYI 02960-6-CNA, AE F161089)
Type of test material:	Substance, technical (pure metabolite)
Chemical state and description:	Beige powder
Origin Batch number:	M 12653 (Batch code: AE F161089 00 1B99 0001)
Material number:	AE F161089
CAS#:	5326-23-8
Purity:	98.8% w/w
Storage conditions:	Expiry date: 09.07.2012, when stored at 5 ± 5 °C

2. Test solutions

Test item mixed with:	Silty sand
Method of preparation:	Sieved soil (2 mm) was treated with either 10 g ground quartz sand/kg dry weight soil
Controls:	Water control

3. Test soil

Soil nomenclature	Silty sand
Collection depth	0 to 20 cm
Lot Nr.:	F2.30110
Source:	Germany/Rheinland-Pfalz/Offenbach
Date of collection:	05 JAN 2011
Storage temperature:	4 ± 2 °C
Particle size distribution (% w/w):	
63 – 2000 µm	0.623
2 – 63 µm	0.287
< 2.0 µm	0.08
Soil properties:	
% organic carbon:	0.83
Biomass (mg microbial C/kg dry soil):	231
% of soil organic carbon content:	2.78
Mineralized nitrogen (mg/100g dry weight soil)	84
Cation exchange capacity (meq/100 g dry weight soil):	12.4
pH:	7.77
Cation exchange capacity (meq/100 g dry weight soil):	12.4
History of soil:	
Plant protection products not used since:	2006
Fertilisers not used since:	2006
Crops:	Fallow land since 2006

B. Study design and methods

1. In life dates March 17 to April 21, 2011

2. Design of biological test

A rate of 1 kg test item/ha (corresponding to 1.33 mg test item /kg dry weight soil) was applied on silty sand soil. After the amendment of Lucerne-grass-green meal (consisting of 40.6% Ctotal, 0.05% Cinorg, 2.5% N) the nitrogen turnover was measured at day 0, and after 7, 14 and 28 days of incubation. In addition, a water control was tested. Each replicate consisted of a jar (brown glass bottles; 0.5 L) filled with 300 g dry weight test soil. The test was conducted with 3 replicates per treatment level. The test was conducted at 20 ± 2°C.

3. Observation and measurements

At day 0, and after 7, 14 and 28 days of incubation subsamples (moist samples (equivalent to 10 g dry weight)) were taken from each jar. The content of ammonium, nitrite and nitrate was measured with a Bran + Lübbe Autoanalyzer 3.

4. Statistical analysis

All calculations were performed using Microsoft Excel 2003.

The percentage differences in the quantities of nitrate-N formed between control soils and treated soils were expressed as absolute values and determined as follows:

((treatment rates – control rates)/control rates) x 100 % = % difference.

Rates were expressed in “mg nitrate-N/kg dry weight soil/day”.

Homogeneity of variances was determined by Cochran’s Test, $\alpha = 0.05$. Depending on the results the appropriate T-tests were performed. In the T-test, the values of nitrate-N/kg dry weight soil/time

interval/day from control soils and treated soils were compared. The statistical calculations were carried out using ToxRatPro 2.10 (Ratte 2010).

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

The soil-pH was 7.08 to 7.13. The water content was 41.94 to 44.58% of the maximum water holding capacity.

B. Biological Findings

The deviation from the control was not significant at any time interval and did not exceed 25% after 28 days.

Table: Effects of AE F161089 on nitrogen turnover of the soil microflora in silty sand given as deviation from the control

Time Interval (days)	Application rate		
	6-chloronicotinic acid		
	control	1.33 mg/kg dry weight soil	
	Nitrate-N[1)]	Nitrate-N[1)]	% difference to control
0-7	-1.75 ± 0.15	-1.88 ± 0.04	**7** n.s.
7-14	2.03 ± 0.09	1.91 ± 0.04	**6** n.s.
14-28	0.88 ± 0.16	0.89 ± 0.03	**1** n.s.

[1)] Rate: Nitrate N mg/kg dry weight soil/time interval/day
n.s. = No statistically significant difference to the control (Student-t Test, two-sided, $\alpha = 0.05$).

C. Validity Criteria

The validity criterion of control variation of less than 15% is fulfilled.

D. Test with toxic reference substance

A reference test with Sodium chloride conducted in 2011 showed that 16 g NaCl/kg dry weight soil had distinct and long-term (> 28 days) influences on microbial mineralization of nitrogen.

CONCLUSION

6-chloronicotinic acid (AE F161089) has negligible effects on nitrogen turnover of soil microflora when applied at a rate of 1 kg test item/ha.

IIA 8.10.2 Carbon mineralization

Report:	**KIIA 8.10.2/01; Schulz, L. (2011)**
Title:	BYI 02960 a.s.: Effects on the activity of soil microflora (Carbon transformation test)
Report No:	11 10 48 058 C
Document No:	M-417194-01-2
Guidelines:	**OECD guideline 217, 2000**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of BYI 02960 (Origin Batch No: 2009-000239; Batch code: BYI 02960-01-03; TOX 08508-01; purity 96.2% w/w) to the carbon turnover of soil microflora.

Rates of 0.3 and 3 kg a.i./ha (corresponding to 0.4 and 4.0 mg a.i./kg soil dry weight) were applied on sandy loam (USDA nomenclature). After the amendment of 2000 mg glucose/kg dry weight to soil

subsamples at day 0, and after 7, 14 and 28 days of incubation the carbon turnover was measured during a period of at least 12 hours.

The deviation from the control did not exceed 25% after 28 days.

BYI 02960 has negligible effects on carbon turnover of soil microflora when applied at 0.3 and 3 kg a.i/ha.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239 (Batch code: BYI 02960-01-03)
Sample description:	TOX 08508-01
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[[(6-chloropyridin-3-yl)methyl](2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Stability of test compound:	Expiry date: 14.01.2013, when stored at +10 to +30 °C

2. Test solutions

Test item mixed with:	Quartz sand
Method of preparation:	Sieved soil (2 mm) was treated with either 10 g ground quartz sand/kg dry weight soil
Controls:	Water control

3. Test soil

Soil nomenclature	Sandy loam (USDA nomenclature) loamy sand (DIN nomenclature)
Collection depth	0 to 20 cm
Lot Nr.:	40603
Source:	Wassergut Ganitz, Germany
Date of collection:	12 JUL 2011
Storage temperature:	4 ± 2 °C
Particle size distribution (% w/w):	
Sand 50 – 2000 µm	53
Silt (2 – 50 µm)	36.8
Clay (< 2 µm)	10.2
Soil properties:	
% organic carbon:	1.39
% humus:	2.39
Biomass (mg microbial C/kg dry soil):	33.04
% microbial C of organic carbon:	2.38
% nitrogen	0.13
pH:	6.4
History of soil:	
Plant protection products not used since:	1991
Fertilisers not used since:	2003
Crops:	Fallow land

B. Study design and methods

1. In life dates August 26 to September 23, 2011

2. Design of biological test

Rates of 0.3 and 3 kg a.i./ha (corresponding to 0.4 and 4.0 mg a.i./kg soil dry weight) were applied on sandy loam (USDA nomenclature) /loamy sand (DIN nomenclature) soil. After the amendment of 2000 mg glucose/kg dry weight to soil subsamples at day 0, and after 7, 14 and 28 days of incubation the carbon turnover was measured during a period of at least 12 hours. In addition a water control was tested. Each replicate consisted of a jar (stainless steel vessels; 4 L) filled with 550 g dry weight test soil. The test was conducted with 3 replicates per treatment level. The test was conducted at 19.6 to 22.0°C.

3. Observation and measurements

At day 0, and after 7, 14 and 28 days of incubation subsamples (moist samples; equivalent to 100 g dry weight) were amended with 2000 mg glucose/kg dry weight. The carbon-dioxide production was measured with a respirometer (BSB digi SELUTEC) over a period of at least 12 hours.

4. Statistical analysis

The cumulative O_2 consumption was calculated using regression analysis over 12 hours.

The coefficients of determination were greater than 0.99 in all replicates and on all days of measurement.

Standard deviation and coefficient of variation were calculated.

The percent deviation (D) from control was calculated for each sampling date by the following equation:

$$D\,[\%] = \left(\frac{C_t}{C_c} - 1 \right) * 100\,\%$$

C_t = O_2 released in the treated group in mg O_2/kg soil d.w./h

C_c = O_2 released in the control group in mg O_2/kg soil d.w./h

Statistical evaluation of the test results (2-sided Student-t-test for homogeneous variances at 5 % significance level) was performed.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

The soil-pH was 6.2 to 6.3. The water content was 46.63 to 49.26 of the maximum water holding capacity.

B. Biological Findings

No adverse effects of BYI 02960 on carbon transformation in soil were observed at either test concentrations (0.40 mg/kg dry soil or 4.00 mg/kg dry soil) after 28 days. Only negligible deviations from control of +0.1 % (test concentration 0.40 mg/kg dry soil) and -0.9 % (test concentration 4.00 mg/kg dry soil) were measured at the end of the 28-day incubation period.

Table: Effects on carbon transformation in soil after treatment with BYI 02960 a.i.

Days after application	Control	0.40 mg test item/kg soil dry weight equivalent to 0.3 kg test item/ha		4.00 mg test item/kg soil dry weight equivalent to 3 kg test item/ha	
	O_2 consumption [mg/kg soil d.w./h]	O_2 consumption [mg/kg soil d.w./h]	Deviation from control [%][1]	O_2 consumption [mg/kg soil d.w./h]	Deviation from control [%][1]
0	12.54	12.51	-0.2	12.26*s	-2.2
7	11.27	11.28	+0.1	11.27	±0.0
14	10.61	10.95	+3.2	10.34	-2.5
28	9.64	9.66	+0.1	9.56	-0.9

The calculations were performed with unrounded values.
1) based on O_2 consumption; - = inhibition; + = stimulation
*s statistically significantly different to control (Student-t-test for homogeneous variances, 2-sided, $p \leq 0.05$)

Table: Variation in control replicates on carbon turnover of the soil microflora in sandy loam (USDA nomenclature) given as % c.v.

	day 0	day 7	day 14	day 28
% c.v.	0.7	0.7	1.7	2.2

The deviations can be regarded as negligible

C. Validity Criteria

The validity criterion of control variation of less than 15% is fulfilled.

D. Test with toxic reference substance

A reference test with Dinoterb conducted 05 JAN 2011 to 02 FEB 2011 revealed inhibitions of 27.8, 45.5 and 47.8% at 6.8, 16 and 27 mg/kg soil dry weight, respectively.

CONCLUSION

BYI 02960 has negligible effects on carbon turnover of soil microflora when applied at up to 3 kg a.i./hectare.

IIA 8.10.3 Rates of recovery following treatment

Studies on recovery following treatment are not required as BYI 02960 is not intended for use in products for soil sterilisation and has no long-term effects on soil micro-organisms

IA 8.11 Effects on marine and estuarine organisms

For the European registration of an active substance inclusion of an active tests on marine and estuarine organisms are not a data requirement. However, acute and chronic studies have been conducted as requirements for registration in USA and Canada.

The data from marine fish (*Cyprinodon variegatus*), the shrimp *Americamysis bahia* and the oyster *Crassostrea virginica* are summarized in the following subchapters.

IIA 8.11.1 Marine or estuarine organisms acute toxicity LC50/EC50

Report:	**KIIA 8.11.1/01; Banman, C.S. & Lam, C.V. (2009)**
Title:	Acute Toxicity of BYI 02960 Technical to the Sheepshead Minnow (*Cyprinodon variegatus*) Under Static Conditions
Report No:	EBRVP034
Document No:	M-357479-01-1
Guidelines:	**OECD Test Guideline 203:** **EPA-FIFRA § 72-3** **OPPTS 850.1075**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** Some data (screening for contaminants in water) was not performed according to GLP, as described in the study report

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of BYI 02960 (Origin Batch No: 2009-000239; Batch code: BYI 02960-01-03; TOX 08508-00; purity 96.2% w/w) to sheepshead minnow (*Cyprinodon variegatus*).

Cyprinodon variegatus (10 fish per treatment level) were exposed in a static system over a period of 96 hours to nominal concentrations of 5.00, 10.0, 20.0, 40.0 and 80.0 mg a.i./L (corresponding to analytically verified concentrations of 5.6, 10.4, 21.0, 40.4 and 83.9 mg a.i./L; 101 to 112% of nominal). In addition, a water control and solvent control were tested.

Mortality and sublethal behavioural effects were used to determine the endpoints. Based on analytical findings the biological endpoints are reported as mean measured figures.

The 96-hour-LC_{50} was > 83.9 mg a.i./L, the 96-hour-NOEC was determined to be 83.9 mg a.i./L the highest concentration tested.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Origin Batch number:	2009-000239 (Batch code: BYI 02960-01-03)
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difluoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Stability of test compound:	Expiry date: 16.01.2011, when stored at +25 ± 5°C
Water solubility:	Approximately 80 mg a.i./L under test conditions

2. Test solutions

Vehicle:	Dimethylformamide (DMF)
Concentration of vehicle:	0.1 mL/L
Method of preparation:	Sonicated for 20 minutes
Controls:	Water control and solvent control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Cyprinodon variegatus*
Common name:	Sheepshead minnow
Lot Nr.:	ABS073008
Source:	Aquatic Bio Systems, Inc., Fort Collins, CO
Feeding during test:	None
Length at test start:	25.2 ± 1.6 mm (range: 22.5 to 29.0 mm)
Weight at test start:	0.24 ± 0.05 g (0.17 to 0.33 g)
Static loading:	0.08 g fish/L
Maintenance of culture:	
Temperature:	22 ± 1.0 °C
Photoperiod:	16/8 hour light/dark photoperiod
Food:	Tetramin flake food and brine shrimp
Period of maintenance prior to study initiation:	At least 14 days
Mortality during acclimatisation period:	No mortalities during 48 hours prior to testing, no treatments for disease

B. Study design and methods

1. In life dates April 20 to 24, 2009

2. Design of biological test

Cyprinodon variegatus were exposed to BYI 02960; (purity 96.2%) in a static system over a period of 96 hours. Nominal concentrations were 5.00, 10.0, 20.0, 40.0 and 80.0 mg a.i./L. In addition a water control and solvent control were tested. Each vessel (glass aquaria; 38 L (49.5 x 25.4 x 30.5 cm)) filled with 30 L synthetic sea water served as one replicate, 10 fish were used per replicate. Toxicology Laboratory consists of artificial sea salts (HW Marinemix Professional from Hawaiian Marine Imports) mixed with blended soft water to produce a salinity of approximately 17 parts per thousand (‰). Length of fish at test start was 25.2 ± 1.6 mm (range: 22.5 to 29.0 mm). Body weight of fish at test start was 0.24 ± 0.05 g (0.17 to 0.33 g). The static biological loading was 0.08 g fish/L. The test was conducted with one replicate per treatment level.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at day 0 and 4 from all concentrations. LC-MS/MS was used as analytical method. The limit of quantification (LOQ) was 0.3 mg a.i./L. The range of linearity was 0.001 to 0.5 mg/L.

4. Observation and measurements

Mortality of fish, intoxication symptoms and physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

Not applicable.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	21.8 to 22.6 °C (mean: 22.1 °C)
pH:	8.0 to 8.2
Dissolved oxygen (mg/L):	6.6 to 7.7 mg/L
Dissolved oxygen (% saturation):	83 to 97 %
Photoperiod:	16 hours light / 8 hours dark
Light source	Cool white fluorescents
Light/dark transition period:	30 minutes
Light intensity:	745 to 1016 lux
Salinity:	17 ± 1 ‰

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of 5.6, 10.4, 21.0, 40.4 and 83.9 mg a.i./L; 101 to 112 % of nominal calculated as arithmetic mean. Biological results are reported as mean measured. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960

Nominal Test Concentration (mg a.i./L)	Day 0 (New)		Day 4 (Old)		Mean Measured (mg a.i./L)	Mean S D	Mean % Nominal
	Measured (mg a.i./L)	% Nominal	Measured (mg a.i./L)	% Nominal			
Control	<0.30	NA	<0.30	NA	<0.30	NA	NA
Solvent Control	<0.30	NA	<0.30	NA	<0.30	NA	NA
5	5.6	113	5.6	111	5.6	0.04	112
10	10.7	107	10.2	102	10.4	0.34	104
20	20.9	105	21.1	106	21	0.17	105
40	40	100	40.8	102	40.4	0.51	101
80	83.4	104	84.5	106	83.9	0.82	105

C. Biological Findings

Mortality was observed as listed below

Table: Effect of BYI 02960 on mortality of *Cyprinodon variegatus*

Exposure time	24 h		48 h		72 h		96 h	
Measured test concentration (mg a.i./L)	no. of dead	% dead	no. of dead	% dead	no. of dead	% dead	no. of dead	% dead
Control	0	0	0	0	0	0	0	0
solvent control	0	0	0	0	0	0	0	0
5.6	0	0	0	0	0	0	0	0
10.4	0	0	0	0	0	0	0	0
21.0	0	0	0	0	0	0	0	0
40.4	0	0	0	0	0	0	0	0
83.9	0	0	0	0	0	0	0	0

No sublethal behavioural changes were observed.

D. Validity Criteria

The validity criterion of control mortality less than 10% was fulfilled. The validity criterion of oxygen saturation above 60% is fulfilled.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

96-hour-figures:	
LC_{50}:	**> 83.9 mg a.i./L**
lowest concentration with effect (LOEC):	> 83.9 mg a.i./L
highest concentration with no effect (NOEC):	83.9 mg a.i./L
highest concentration with no mortality (NOLEC):	83.9 mg a.i./L

CONCLUSION

The acute effect of BYI 02960 on sheepshead minnow (*Cyprinodon variegatus*) can be quantified as a 96-hour-LC_{50} of > 83.9 mg a.i./L, the highest concentration tested.

Report:	**KIIA 8.11.1/02; Gallagher, S.P., Kendall, T.Z. & Krueger, H.O. (2009)**
Title:	BYI 02960: A 96-Hour Shell Deposition Test with the Eastern Oyster (*Crassostrea virginica*)
Report No:	EBRVP023
Document No:	M-361668-01-1
Guidelines:	**OPPTS 850.1025**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** **Periodic screening of saltwater for potential contaminants was not performed under GLP**

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of BYI 02960 (Origin Batch No: 2009-000239; Batch code: BYI 02960-01-03; TOX 08508-00; purity 96.2% w/w) on shell deposition of the Eastern Oyster (*Crassostrea virginica*).

Oysters (mean valve height of 35.1 ± 2.7 mm; range: 30.2 to 40.1 mm, 20 per treatment level) were exposed in a flow through system over a period of 96 hours to nominal concentrations of 0.94, 1.9, 3.8, 7.5, 15 and 30 mg a.i./L (corresponding to analytically verified concentrations of 0.90, 1.8, 3.6, 7.3, 15 and 29 mg a.i./L; 95 to 97% of nominal). In addition a saltwater control was tested. The test was conducted at concentrations near the functional limit of solubility of BYI 02960 in a saltwater system.

Shell deposition, mortality and sublethal behavioural effects were used to determine the endpoints. Based on analytical findings the biological endpoints are reported as mean measured figures. There were no mortalities or clinical signs of toxicity observed at any concentration tested.

The 96-hour-EC_{50} was > 29 mg a.i./L, the 96-hour-NOEC was determined to be 29 mg a.i./L.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Origin Batch number:	2009-000239 (Batch code: BYI 02960-01-03)
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difluoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Stability of test compound:	Expiry date: 16.01.2011, when stored at +25 ± 5°C°C

2. Test solutions

Vehicle:	Dimethylformamide (DMF)
Concentration of vehicle:	0.1 mL/L
Method of preparation:	Mixed at least 20 times
Controls:	Saltwater control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Crassostrea virginica*
Common name:	Eastern oyster
Source:	Circle C Oysters, Ridge, Maryland
Examination and preparation of oysters:	Oysters showed no evidence of spawning or parasitation. Prior to testing, 3 to 5 mm of the new peripheral shell growth of each oyster was removed by grinding the shell to a blunt edge using a fine-grit grinding wheel
Size at study initiation:	mean valve height of 35.1 ± 2.7 mm (range: 30.2 to 40.1 mm)
Feeding during test:	Concentrated volumes of algal suspension (approximately 2.9 to 5.8 10^9 cells per oyster per day) supplied with a peristaltic pump
Maintenance of culture:	
Temperature:	21 to 23 ºC
Food:	suspension of marine microalgae (Reed Mariculture, Campbell, CA)

B. Study design and methods

1. In life dates August 13 to 21, 2009

2. Design of biological test

Crassostrea virginica (mean valve height of 35.1 ± 2.7 mm; range: 30.2 to 40.1 mm) were exposed to BYI 02960 (purity 96.2 %) in a flow through system over a period of 96 hours. Nominal concentrations were 0.94, 1.9, 3.8, 7.5, 15 and 30 mg a.i./L. In addition a saltwater control and a solvent control were tested. Each replicate consisted of one vessel (glass aquaria; 54 L) filled with 27 L (depth of water: 15.6 cm) natural seawater filtered and diluted in order to adjust salinity to 20‰. 20 oysters were used per replicate. Oysters showed no evidence of spawning or parasitation. Prior to testing, 3 to 5 mm of the new peripheral shell growth of each oyster was removed by grinding the shell to a blunt edge using. The test was conducted with one replicate per treatment level.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at 0, 48 and 96 hours from all concentrations. BYI 02960 (purity: 99.4%) served as analytical standard. High-performance liquid chromatography (HPLC) was used as analytical method. The limit of quantification (LOQ) was 0.400 mg a.i./L.

4. Observation and measurements

Oysters were inspected visually at approximately 6, 24, 48, 72 and 96 hours after test initiation to determine the numbers of mortalities and the numbers of individuals exhibiting sub-lethal signs of toxicity. At the end of the test, the longest finger of new shell growth on each oyster was measured to the nearest 0.1 mm using calipers. Shell deposition, mortality and intoxication symptoms of the oysters as well as physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

There were no statistically significant differences ($p > 0.05$) between the negative and solvent control groups, using an appropriate t-test. Therefore, the control data were pooled for comparisons among the BYI 02960 treatment groups. Shell growth inhibition was calculated for each treatment group as the percent reduction in mean shell growth relative to the mean control shell growth. The shell deposition data were evaluated for normality and homogeneity of variance using the Chi-Square test and Levene's test, respectively. Since the data passed the assumptions of normality and homogeneity, the data in the treatment groups were compared to the pooled control data using analysis of variance (ANOVA) and Bonferroni's t-test to identify any significant differences.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	19.2 to 21.2 °C
pH:	8.1 to 8.2
Dissolved oxygen (mg/L):	6.9 to 7.4 mg/L
Dissolved oxygen (% saturation):	> 86 % of saturation
Photoperiod:	16 hours light and 8 hours darkness
Light source	Fluorescent bulbs
Light/dark transition period:	30 min
Light intensity:	578 lux at water surface
Salinity:	20‰

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of 0.90, 1.8, 3.6, 7.3, 15 and 29 mg a.i./L, respectively, (95 to 97% of nominal) calculated as arithmetic mean.

Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960

Nominal Concentration (mg a.i./L)	Day 0 (New)		Day 2		Day 4		Mean Measured (mg a.i./L)	Mean Percent Nominal
	Measured (mg a.i./L)	Percent Nominal	Measured (mg a.i./L)	Percent Nominal	Measured (mg a.i./L)	Percent Nominal		
Control	< LOQ[#]		< LOQ		< LOQ			
solvent control	< LOQ		< LOQ		< LOQ			
0.94	0.894	95.1	0.911	96.9	0.888	94.5	0.9	96
1.9	1.83	96.1	1.81	95.5	1.78	93.8	1.8	95
3.8	3.61	94.9	3.65	96.0	3.56	93.6	3.6	95
7.5	7.32	97.6	7.3	97.4	7.28	97.1	7.3	97
15	15.0	99.9	14.8	99.0	14.9	99.3	15	100
30	29.1	97.0	29.4	98.1	29.3	97.5	29	97

[#]LOQ = 0.4 mg a.i/L

C. Biological Findings

Observations on mortality, signs of toxicity and mean shell deposition and growth inhibition are summarised below:

There were no mortalities among oysters in any treatment or control group during the test.

All oysters appeared normal throughout the 96-hour exposure period.

Table: Effect of BYI 02960 on shell deposition of *Crassostrea virginica*

Mean measured concentration (mg a.i./L)	Shell Deposition[A] (mean ± SD) (mm)	Mean percent reduction[B]
Negative Control	3.2 ± 1.4	--
Solvent Control	2.6 ± 1.4	--
Pooled Control	2.9 ± 1.4	--
0.9	2.8 ± 1.1	2.2
1.8	2.8 ± 1.6	2.9
3.6	3.3 ± 1.6	-13
7.3	2.5 ± 1.1	13
15	3.5 ± 1.4	-19
29	2.5 ± 1.1	13

[A] Mean and standard deposition for 20 oysters
[B] No significant difference from pooled control using Bonferroni t-test ($_p > 0.05$)

There was no significant difference in shell deposition between the treatment groups and the controls.

D. Validity Criteria

The validity criterion of control mortality less than 10% is fulfilled. The validity criterion of control shell growth > 2mm is fulfilled. The validity criterion of oxygen saturation above 60% is fulfilled.

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

	Based on mean measured concentrations
NOEC:	29 mg a.i./L
96-hour EC_{50} for Shell Deposition:	> 29 mg a.i./L

CONCLUSION

The acute effect of (BYI 02960) on *Crassostrea virginica* can be quantified as a 96-hour-EC_{50} of > 29 mg a.i./L. The NOEC was determined to be 29 mg a.i./L.

Report:	**KIIA 8.11.1/03; Gallagher, S.P., Kendall, T.Z. & Krueger, H.O. (2009)**
Title:	BYI 02960: A 96-Hour Static Acute Toxicity Test with the Saltwater Mysid (*Americamysis bahia*)
Report No:	149A-236
Document No:	M-364620-01-1
Guidelines:	**EPA OPP 72-3(b)** **EPA OPPTS 850.1035**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** **Screening of saltwater for contaminants was not performed under GLP, as detailed in the study report**

EXECUTIVE SUMMARY

The aim of the study was to determine the acute effects of BYI 02960 (Sample description: TOX 08508-00 (Batch ID: 2009-000239); purity 96.2% w/w) to *Americamysis bahia*.

Juvenile *Americamysis bahia* (< 24 hours old, 20 per treatment level) were exposed in a static system over a period of 96 hours to nominal concentrations of 0.13, 0.22, 0.36, 0.60 and 1.0 mg a.i./L, , (corresponding to analytically verified concentrations of 0.12, 0.21, 0.35, 0.58 and 0.98 mg a.i./L, respectively).

In addition a water control was tested. Two replicate test chambers were maintained in each treatment and control group, with 10 saltwater mysids in each test chamber, for a total of 20 mysids per test concentration.

Mortality and sublethal behavioural effects were determined by visual interpretation. Based on analytical findings the biological endpoints are reported as mean measured figures.

The 96-hour-EC_{50} was 0.26 mg a.i./L (95% confidence limits: 0.12 - 0.58 mg a.i./L), the 96-hour-NOEC was determined to be 0.12 mg a.i./L.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Description:	Beige powder
Specification No.:	102000022313
Batch number:	2009-000239
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Stability:	Expiry date: 16.01.2011, when stored at +25 ± 5°C

2. Test solutions

Vehicle:	None
Method of preparation:	The stock solution was stirred with a wire whisk for approximately 1-2 minutes and sonicated for approximately 35 minutes.
Controls:	Water control
Evidence of undissolved material:	None

3. Test organisms

Species:	*Americamysis bahia*
Common name:	Mysid shrimp
Source:	Reed Mariculture, Inc., Campbell, California
Age at study initiation:	< 24 hours old
Feeding during test:	Live brine shrimp nauplii (Artemia sp.)
Maintenance of culture:	
Temperature:	25.6 to 27.0°C
Photoperiod:	16 hours of light and 8 hours of darkness
Food:	Live brine shrimp nauplii (Artemia sp.)

B. Study design and methods

1. In life dates August 31 to September 4, 2009

2. Design of biological test

Americamysis bahia (< 24 hours old) were exposed to BYI 02960; purity 96.2%) in a static system over a period of 96 hours. Nominal concentrations were 0.13, 0.22, 0.36, 0.60 and 1.0 mg a.i./L. In addition a water control was tested. Each vessel (glass beakers; 2 L, filled with 1.5 L test solution) served as one replicate. 10 mysids were used per replicate. The test was conducted with two replicates per treatment level. Saltwater mysids were impartially assigned to exposure chambers at test initiation. Observations of mortality and other signs of toxicity were made at 18, 24, 48, 72 and 96 hours (± 1 hour) after test initiation. The cumulative percent mortality observed in the treatment groups was used to determine LC50 values at 24, 48, 72 and 96 hours.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at 0, 48 and 96 hours from all concentrations. BYI 02960 (purity: 99.4%) served as analytical standard. High-performance liquid chromatography (HPLC) was used as analytical method. The limit of quantification (LOQ) was 0.05 mg a.i./L.

4. Observation and measurements

Mortality, intoxication symptoms and physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

The probit method was used to calculate the 24-hour LC_{50} and the 95% confidence interval. Nonlinear interpolation was used to calculate the 48, 72 and 96-hour LC_{50} values and binominal probability was used to calculate the 95% confidence intervals. Due to the method used to calculate the 96-hour LC_{50} value, the slope of the dose response curve could not be calculated. The no-mortality concentration and NOEC were determined by visual interpretation of the mortality and observation data.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	25 ± 2 °C (range: 23.2 to 26.2 °C)
pH:	8.1 to 8.2
Dissolved oxygen (mg/L):	6.5 to 7.4 mg/L
Dissolved oxygen (% saturation):	> 89%
Photoperiod:	16 hours of light and 8 hours of darkness
Light source	fluorescent light bulbs
Light/dark transition period:	30 min
Light intensity:	798 lux
Salinity:	20 ‰

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of 0.12, 0.21, 0.35, 0.58 and 0.98 mg a.i./L calculated as arithmetic mean. Biological results are reported as mean measured. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960

Nominal Conc. (mg a.i/L)	Day 0		Day 2		Day 4		Mean Measured (mg a.i./L)	Mean Percent Nominal
	Measured (mg a.i./L)	Percent Nominal	Measured (mg a.i./L)	Percent Nominal	Measured (mg a.i./L)	Percent Nominal		
Control	-	-	-	-	-	-	-	-
0.13	0.128	98.3%	0.122	94.0%	0.123	94.4%	0.12	92%
0.22	0.214	97.3%	0.209	95.0%	0.21	95.2%	0.21	95%
0.36	0.352	97.8%	0.347	96.4%	0.349	97.0%	0.35	97%
0.60	0.566	94.3%	0.573	95.6%	0.587	97.8%	0.58	97%
1.00	0.97	97.0%	0.981	98.1%	-	-	0.98	98%

C. Biological Findings

The single mortality in the 0.12 mg a.i./L treatment group was a mysid that was missing and assumed to be dead at the 72-hour observation period, this was considered to be incidental to treatment and was therefore was excluded from the LC_{50} calculations.

Signs of toxicity (loss of equilibrium and lethargy) were noted in the 0.21, 0.35, 0.58 and 0.98 mg a.i./L treatment groups, but all surviving mysids were normal in appearance and behavior at test termination.

Observations are summarised below as follows:

Table: Effect of BYI 02960 on mortality of *Americamysis bahia*

Mean meas. conc. (mg a.i./L)	Cumulative % mort.	Observation period							
		24 hours		48 hours		72 hours		96 hours	
		No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.	No. Dead	Obs.
control	0	0	10 AN	0	10 AN	0	10 AN	0	10 AN
		0	10 AN	0	10 AN	0	10 AN	0	10 AN
0.12	5	0	10 AN	0	10 AN	1	9 AN	1	9 AN
		0	10 AN	0	10 AN	0	10 AN	0	10 AN
0.21	60	0	10 AN	0	10 AN	2	8 AN	3	7 AN
		0	10 AN	2	8 AN	6	3AN, 1N	9	1 AN
0.35	50	2	8 AN	3	7 AN	7	3 AN	8	2 AN
		0	8 AN, 2N	0	10 AN	0	10 AN	2	8 AN
0.58	100	8	2 C	10	-	10	-	10	-
		8	2 C	10	-	10	-	10	-
0.98	100	10	-	10	-	10	-	10	-
		10	-	10	-	10	-	10	-

Observations: AN = appear normal; N = loss of equilibrium; C = lethargy

D. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived, based on mean measured concentrations:

48-Hour LC_{50}:	0.42 mg a.i./L
72-Hour LC_{50}:	0.38 mg a.i./L
96-Hour LC_{50}:	**0.26 mg a.i./L**
95% Confidence Limits:	0.12 and 0.58 mg a.i./L
No-Mortality Concentration:	0.12 mg a.i./L
No-Observed-Effect Concentration:	0.12 mg a.i./L

CONCLUSION

The acute effect of BYI 02960 on *Americamysis bahia* (formerly *Mysidopsis bahia*) can be quantified as a 96-hour-LC_{50} of 0.26 mg a.i./L (95% confidence limits: 0.12 - 0.58 mg a.i./L).

The following chronic test is on mysid shrimps is also included in this section as there is no specific dossier point for the chronic toxicity on marine species.

Report:	**KIIA 8.11.1/04; Claude, M.B., Kendall, T.Z. & Krueger, H.O. (2011)**
Title:	BYI 02960: A Flow-Through Life-Cycle Toxicity Test with the Saltwater Mysid (*Americamysis bahia*)
Report No:	EBRVP038
Document No:	M-420783-01-1
Guidelines:	**OPPTS Number 850.1350: Mysid Chronic Toxicity Test ASTM Standard E 1191-03a: Standard Guide for Conducting Life-Cycle Toxicity Tests with Saltwater Mysid**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the chronic effects of BYI 02960 (Sample description: TOX 08508-01 (Batch ID: 2009-000239); purity 96.2%) on the survival, reproduction and growth of the mysid shrimp (*Americamysis bahia*).

Fifteen neonates (<24 h old) of *Americamysis bahia* per replicate were exposed in a flow-through system over a period of 28 days to nominal concentrations of 4.6, 8.0, 13.9, 24.2 and 42 µg a.i./L (corresponding to analytically verified concentrations of 4.2, 7.8, 13.2, 23.6 and 40 µg a.i./L; (91 to 98% of nominal). In addition a water control and solvent control were tested. On Day 14 of the test, after mysids attained sexual maturity, male and female adults were paired in each treatment and control group, with a maximum of five reproductive pairs per replicate. Reproduction of the paired mysids was monitored through termination on Day 28. Observations for mortality and signs of toxicity were conducted daily throughout the test. At test termination, the total body lengths and dry weights of all surviving first-generation mysids were measured.

Observations of the effects of BYI 02960 on mortality, reproduction and growth were used to determine the no-observed-effect concentration (NOEC), the lowest-observed-effect concentration (LOEC), and the maximum acceptable toxicant concentration (MATC). Based on analytical findings the biological endpoints are reported as mean measured figures.

The NOEC was determined to be 13.2 µg a.i./L. The corresponding LOEC was 23.6 µg a.i./L and the MATC 18.0 µg a.i./L.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Batch number:	2009-000239
Sample description:	TOX 08508-01
CAS#:	951659-40-8
Purity:	96.2% w/w

2. Test solutions

Vehicle:	Dimethylformamide (DMF)
Concentration of vehicle:	0.1 mL/L
Controls:	Water control and solvent control
Water media type:	Natural seawater filtered and diluted with fresh water to 20 ‰ and UV-sterilized

3. Test organisms

Species:	Americamysis bahia (formerly Mysidopsis bahia)
Common name:	Mysid shrimp
Source:	Inhouse-culture
Age at study initiation:	< 24 hours old
Feeding during test:	Live brine shrimp (Artemia salina) nauplii, ≤ 48 hours old (post-hydration), twice daily
Maintenance of culture:	
Temperature:	25.1 to 27.3°C
Photoperiod:	16 hours of light and 8 hours of darkness
Food:	Live brine shrimp nauplii enriched with Algamac 3050. Mysids also periodically supplemented with Skeletonema

B. Study design and methods

1. In life dates: April 15 to May 13, 2011

2. Design of biological test

Less than 24 hours old neonates of *Americamysis bahia* were exposed to BYI 02960 (purity 96.2 %) in a flow-through system over a period of 28 days. Nominal concentrations were 4.6, 8.0, 13.9, 24.2 and 42 µg a.i./L. In addition a water control and solvent control were tested. Flow through was achieved with a continuous flow diluter mixing 25 µL stock solution per minute into 250 µL dilution water per minute. Mysids were separated in retention chambers until they could be sexed and separated pairwise into the pairing chambers. The test was conducted with 4 replicates per treatment level. During the first 14 days the juveniles were kept in 9 L glass aquaria containing 2.5 L test solution. In these tanks the juveniles of each replicate were enclosed in 2 L compartments with two nylon mesh covered holes on opposite sides. The flow rate was 18 volume changes per day.

During the last 14 days of the test adult mysids were selected for pairing. Each pair was kept in 10 cm diameter petri dishes with sides of nylon mesh screen. The petri dishes were placed in 19 L glass aquaria containing 17.5 L test solution. The flow rate was 5 volume changes per day.

3. Method of analytical verification

For analytical verification of the test item concentrations samples were taken at days -2, 0, 6, 14, 15, 21 and 28, respectively, from all concentrations. BYI 02960 (purity 99.4%) served as analytical standard. HPLC was used as analytical method. The limit of quantification (LOQ) was 2.00 µg a.i./L.

4. Observation and measurements

Immobilisation, growth and reproduction of mysids and physical-chemical water parameters were assessed as indicated below in the result section.

5. Statistical analysis

Survival data was considered to be discrete-variable data, while reproduction and growth data were considered continuous-variable data. Discrete-variable data were analyzed using Chi-square and Fisher's Exact tests to identify treatment groups that showed a statistically significant difference from the pooled control ($p < 0.05$). All continuous-variable data were evaluated for normality using the Shapiro-Wilk's test and for homogeneity of variance using Levene's test ($p = 0.01$). The data for all parameters passed the assumptions of normality and homogeneity of variance. Those treatment means that were significantly different from the pooled control means were identified using Dunnett's test ($p < 0.05$). All statistical tests were performed using a personal computer with SAS (3) software.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measurements of physical and chemical parameters of the test solutions are summarized as follows:

Test temperature:	within the 25 ± 2°C range
pH:	7.9 to 8.1
Dissolved oxygen (mg/L):	5.0 mg/L
Dissolved oxygen (% saturation):	>68%
Photoperiod:	14 hours light/10 hours dark
Light/dark transition period:	120 minutes
Salinity:	19 to 21‰

B. Analytical Findings

Analytical verification of test solutions revealed measured concentrations of 4.2, 7.8, 13.2, 23.6 and 40 µg a.i./L (91 to 98 % of nominal) calculated as arithmetic mean. Biological results are reported as mean measured. Detailed analytical results are presented in the following table:

Table: Nominal and measured concentrations of BYI 02960

Nominal Concentration (µg a.i./L)	Measured Concentration (µg a.i./L)							
	day 0	day 6	day 14	day 15	day 21	day 28	Mean	Percent Nominal (%)
control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ		
solvent control	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ		
4.6	3.5	4.5	4.4	4.3	4.5	4.4	4.2	91
8.0	7.3	8.0	7.8	7.6	7.9	8.0	7.8	98
13.9	12.2	14.3	12.6	13.1	13.3	13.7	13.2	95
24.2	22.8	23.6	23.9	22.7	24.4	24.0	23.6	98
42	37.8	42.4	40.2	39.1	41.7	41.4	40.0	95

C. Biological Findings

Observations on mortality, growth and reproduction are listed as follows:

Table: Effect of BYI 02960 on mortality and reproduction of *Americamysis bahia*

Mean Measured Concentration (µg a.i./L)	% juvenile survival to pairing on day 14	% adult survival to test termination on day 28	mean number of young produced per reproductive day ± SD
control	100	83.0	0.396 ± 0.119
solvent control	95.0	92.5	0.450 ± 0.167
pooled control	97.5	88.0	0.423 ± 0.137
4.2	85.0	86.1	0.358 ± 0.250
7.8	93.3	92.3	0.267 ± 0.145
13.2	78.3*[1]	84.6	0.240 ± 0.090
23.6	95.0	88.2	0.156 ± 0.157**
40.0	93.3	81.3	0.173 ± 0.113**

* Statistically significant decrease in survival in comparison to the pooled control using Fisher's Exact test ($p < 0.05$).
"Statistically significant decrease in reproduction in comparison to the pooled control using Dunnett's test ($p < 0.05$)
While the decrease in survival was statistically significant in comparison to the pooled control, it was not considered to be treatment-related since the difference was slight and was not dose-responsive.

Table: Effect of BYI 02960 on growth of *Americamysis bahia*

Mean Measured Concentration (µg a.i./L)	Length at day 28 (mm)		Body weight at day 28 (mg)	
	Male	Female	Male	Female
control	8.15 ± 0.244	8.38 ± 0.297	1.02 ± 0.105	1.38 ± 0.204
solvent control	8.14 ± 0.169	8.17 ± 0.192	1.00 ± 0.102	1.15 ± 0.091
pooled control	8.15 ± 0.195	8.28 ± 0.257	1.01 ± 0.097	1.27 ± 0.191
4.2	7.85 ± 0.297	8.25 ± 0.203	0.928 ± 0.096	1.38 ± 0.224
7.8	7.89 ± 0.268	8.32 ± 0.163	0.889 ± 0.105	1.24 ± 0.137
13.2	8.00 ± 0.363	8.32 ± 0.202	0.943 ± 0.121	1.24 ± 0.086
23.6	8.01 ± 0.217	8.16 ± 0.214	0.917 ± 0.043	1.13 ± 0.150
40.0	8.16 ± 0.038	8.34 ± 0.110	0.923 ± 0.045	1.21 ± 0.060

D. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

28 day-figures:	
NOEC (adult survival):	40 µg a.i./L
NOEC (reproduction):	13.2 µg a.i./L
NOEC (growth in terms of total length and weight):	40 µg a.i./L
LOEC (lowest observed effect concentration)	23.6 µg a.i./L
highest concentration with no effect (overall **NOEC**):	**13.2 µg a.i./L**
Maximum acceptable toxicant concentration (MATC)	18.0 µg a.i./L

CONCLUSION

Reproduction, measured as mean number of young produced per reproductive day, was the most sensitive biological endpoint measured. Therefore, the chronic effect of (BYI 02960) on *Americamysis bahia* as the highest concentration with no effects on mortality, growth and reproduction can be set to 13.2 µg a.i./L. as the measured decrease in survival at 13.2 µg a.i./L, although found to be statistically significant in comparison to the pooled control, should not be considered treatment-related since the difference was slight and was not dose-responsive

IIA 8.11.2 Marine/Estuarine fish - salinity challenge

This is not an EC data hence; data/documents were not created and are not submitted.

IIA 8.12 Effects on terrestrial vascular plants

The summary of this study is presented below, as it is a core requirement. The test has been performed with the lead formulation BYI 02960 SL 200G.

Report:	**KIIA 8.12/01; Gosch H., 2010**
Title:	BYI 02960 SL 200 g/L – Effects on the vegetative vigour of eleven species of non-target terrestrial plants (Tier 1)
Report No:	VV10/002
Document No:	M-397734-01-2
Guidelines:	**OPPTS 850.4150 (1996); OECD Guideline 227 (2006)**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The purpose of this specific study is to evaluate the potential side effects of BYI 02960 SL 200 g/L (Sample description: TOX08854-00; Batch ID: 2009-001253; Material No.: 79718845; Specification No.: 102000021884-01) on the vegetative vigour of eleven non-target terrestrial plant species following a post-emergence 410 g a.i./ha application of the product onto the foliage of plants.

A total of eleven species were tested in this vegetative vigour test including seven dicotyledonous and four monocotyledonous species representing nine plant families.

At the 2-4 leaf stage, plants (except *Allium cepa*, which was treated at the 1-2 leaf stage) were sprayed once with BYI 02960 SL 200 g/L at an application rate of 410 g a.i./ha and a volume rate of 200 L/ha.

Each pot (replicate) contained 4 plants and there were 32 plants treated (i.e. 8 replicates). Control pots were treated with de-ionized water.

Following application, pots were grown and maintained under glasshouse conditions. Survival of the treated plants and visual phytotoxicity were recorded 7, 14 and 21 days after application and assessments were made against the water treated controls. The study was terminated 21 days after application.

Following a foliar application of BYI 02960 SL 200 g/L applied at 410 g a.i./ha (corresponding to 2.4 kg product/ha) to eleven terrestrial non-target plant species, no adverse effects on survival, visual phytotoxicity, growth, shoot length and shoot dry weight above 25% effect were observed in this vegetative vigour study. Only minimal responses were observed, typically within the range of natural variability.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960 SL 200 G
Type:	Formulated product (soluble (liquid) concentrate)
Chemical state and description:	Clear brown liquid
Specification No.:	102000021884-01
Material number:	79718845
Sample description:	TOX 08854-00
Batch No.:	2009-001253
Nominal content of active substance:	BYI 02960: 200 g/L
Analytical content of active substance:	BYI 02960: 17.0% w/w, 199.8 g/L according to certificate of analysis
Stability of test compound:	Expiry date: 20.03.2010, when stored at 25 ± 5°C in original container in the dark (also acceptable from +2 to +30°C)
Density:	1.174 g/mL

2. Vehicle and/or positive control

Negative control:	De-ionized water (volume rate: 200 L/ha)
Positive control:	None

3. Test organisms

Species (Common name):	Dicotyledonae: *Beta vulgaris* (sugar beet) *Brassica napus* (oilseed rape) *Cucumis sativa* (cucumber) *Fagopyrum esculentum* (buckwheat) *Glycine max* (soybean) *Lactuca sativa* (lettuce) *Lycopersicon esculentum* (tomato) Monocotyledonae: *Allium cepa* (onion) *Avena sativa* (oat) *Lolium perenne* (ryegrass) *Zea mays* (corn)
Source:	Different, mostly commercial, sources, see report, page 18
Test units:	Commercial plastic flower pots (13 cm diameter), 8 pots per treatment group, each prepared with 4 plants
Climatic conditions:	Glasshouse conditions
Photoperiod:	At least 16 hours light; natural daylight supplemented by artificial lighting
Temperature:	23 ± 8°C day, 18 ± 8°C night
Humidity:	70 ± 30%
Watering:	Irrigation by bottom watering via saucers standing below each pot
Details of Nutrient Medium:	Liquid fertilizer was added in the saucers on test day 7 for all tested species. *Cucumis sativus* was fertilized also on days 1 and 14.

B. Study design and methods

1. In life dates: February 25 to March 31, 2010

2. Experimental treatments:

A total of eleven plant species were tested in this vegetative vigour test including seven dicotyledonous and four monocotyledonous species representing nine plant families.

The following species were treated: *Beta vulgaris* (sugar beet), *Brassica napus* (oilseed rape), *Cucumis sativus* (cucumber), *Fagopyrum esculentum* (buckwheat), *Glycine max* (soybean), *Lactuca sativa*

(lettuce), *Lycopersicon esculentum* (tomato), *Allium cepa* (onion), *Avena sativa* (oat), *Lolium perenne* (ryegrass) and *Zea mays* (corn).

Each pot (replicate) contained 4 plants and there were 32 plants treated i.e. 8 replicates. Control pots were treated with de-ionized water.

The spray solution was applied once, at test initiation on the leaves and above-ground portions of plants. The blank control spray solution was 200 L deionized water/ha. The test item was dissolved in deionized water and was applied once with 200 L/ha using a spray chamber equipped with an overhead nozzle, with nozzle height set at 30 cm above the target area (highest leaf density).

Following application, pots were maintained under glasshouse conditions with a temperature control set at 23 ± 8ºC during day and 18 ± 8ºC at night with a 16 h photoperiod.

The blank control was 200 L deionized water/ha. Plants were exposed to the test item for 21 days.

3. Observation and measurements:

The parameters measured were survival, visual phytotoxicity, plant growth stage, shoot length and shoot dry weight. Observations were recorded 7, 14 and 21 days after application and assessments were made against the water treated controls.

4. Statistical analysis

Statistical analysis of data was performed to obtain significance for shoot length and shoot dry weight effects, carried out using the Pairwise Mann-Whitney-U-Test (one sided smaller; $p \leq 0.05$) by ToxRat statistics.

RESULTS AND DISCUSSION

A. Environmental Parameters

Pots were kept in glasshouses under conditions as described above (see material and methods).

B. Biological Findings

Analysis of BYI 02960 of the tested application rate revealed it to be 99% of nominal.

As a result of a foliar application of BYI 02960 SL 200 g/L with 410 g a.i./ha to eleven plant species, this study revealed a very low level of phytotoxicity. There were no adverse effects on surviving with any of the species tested.

There were limited phytotoxic symptoms in this study with slight chlorosis, necrosis and stunting in oilseed rape, cucumber, buckwheat, tomato and corn.

Buckwheat (*Fagopyrum esculentum*) was the most sensitive species for shoot dry weight, with 12% reduction, which was statistically significant.

A summary of the findings from a single application of 410 g a.i./ha to eleven plant species tested is summarised in the following table:

Species	Survival (% inhibition)	Phytotoxicity	Shoot Length (% reduction)	Shoot Dry Weight (% inhibition)
Dicotyledoneae				
Beta vulgaris	0	0	-2.3	6.5
Brassica napus	0	0 - 10%	-1.0	**7.0**
Cucumis sativus	0	10 - 20%	-2.1	4.5
Fagopyrum esculentum	0	10%	3.1	**12.0**
Glycine max	0	0	0.6	-11.1
Lactuca sativa	0	0	-4.0	0.7
Lycopersicon esculentum	0	10 - 20%	3.1	10.9
Monocotyledoneae				
Allium cepa	0	0	-2.8	-9.3
Avena sativa	0	0	2.1	-1.9
Lolium perenne	0	0	4.2	-3.0
Zea mays	0	0 - 10%	0.3	4.1

Bold figures for shoot dry weight are statistically significant (Pairwise Mann-Whitney-U-test, one sided smaller; $p \leq 0.05$)

C. Validity Criteria

This study can be considered valid as the validity criterion of at least 90% survival during the study period was achieved for the untreated controls of all species.

CONCLUSION

Following a foliar application of BYI 02960 SL 200 g/L applied at 410 g a.i./ha (corresponding to 2.4 kg product/ha) to eleven terrestrial non-target plant species, no adverse effects on survival, visual phytotoxicity, growth, shoot length and shoot dry weight above 25% effect were observed in this vegetative vigour study. Only minimal responses were observed, typically within the range of natural variability.

Report:	**KIIA 8.12/02; Gosch H., 2010**
Title:	BYI 02960 SL 200 g/L – Effects on the seedling emergence and growth of eleven species of non-target terrestrial plants (Tier 1)
Report No:	SE10/001
Document No:	M-397727-01-2
Guidelines:	**OPPTS 850.4100 (1996); OECD Guideline 208 (2006)**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The purpose of this specific study is to evaluate the potential phytotoxic effects of BYI 02960 SL 200 g/L (Sample description: TOX 08854-00; Batch ID: 2009-001253; Material No.: 79718845; Specification No.: 102000021884-01) on the seedling emergence and growth of eleven non-target terrestrial plant species following a pre-emergence application of the product onto the soil surface at a rate of 410 g a.i./ha.

A total of eleven species were tested in this seedling emergence and growth test including seven dicotyledonous and four monocotyledonous species representing nine plant families.

Five seeds of each species were sown in pots in the glasshouse. The soil surface of the pots was sprayed with BYI 02960 SL 200 g/L applied at 410 g a.i./ha and a volume rate of 200 L/ha. Each pot (replicate)

contained 5 seeds and there were 40 seeds treated (i.e. 8 replicates). Control pots were treated with de-ionized water.

Following application, pots were grown and maintained under glasshouse conditions. Emergence, survival of the emerged seedlings and visual phytotoxicity were recorded 7, 14 and 21 days after application and assessment were made against the water treated controls. The study was terminated 21 days after application.

Following a soil surface application of BYI 02960 SL 200 g/L applied at 410 g a.i./ha (corresponding to 2.4 kg product/ha) to eleven terrestrial non-target plant species, no adverse effects on emergence, seedling survival, visual phytotoxicity, growth, shoot length and shoot dry weight above 25% effect were observed in this seedling emergence and growth study. Only minimal responses were observed, typically within the range of natural variability.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960 SL 200 G
Type:	Formulated product (soluble (liquid) concentrate)
Chemical state and description:	Clear brown liquid
Specification No.:	102000021884-01
Material number:	79718845
Sample description:	TOX 08854-00
Batch No.:	2009-001253
Nominal content of active ingredient:	BYI 02960: 200 g/L
Analytical content of active ingredient:	BYI 02960: 17.0% w/w, 199.8 g/L according to certificate of analysis
Density:	1.174 g/mL
Stability of test compound:	Expiry date: 20.03.2010, when stored at 25 ± 5°C in original container in the dark (also acceptable from +2 to +30°C)

2. Vehicle and/or positive control

Negative control:	De-ionized water
Positive control:	None

3. Test organisms

Species (Common name):	Dicotyledonae: *Beta vulgaris* (sugar beet) *Brassica napus* (oilseed rape) *Cucumis sativa* (cucumber) *Fagopyrum esculentum* (buckwheat) *Glycine max* (soybean) *Lactuca sativa* (lettuce) *Lycopersicon esculentum* (tomato) Monocotyledonae: *Allium cepa* (onion) *Avena sativa* (oat) *Lolium perenne* (ryegrass) *Zea mays* (corn)
Source:	Different, mostly commercial, sources, see report, page 18
Test units:	Commercial plastic flower pots (10.5 cm diameter), 8 pots per treatment group, each prepared with 5 seeds
Climatic conditions:	Glasshouse conditions
Photoperiod:	At least 16 hours light; natural daylight supplemented by artificial lighting
Temperature:	23 ± 8°C day, 18 ± 8°C night
Humidity:	70 ± 30%
Watering:	Irrigation by bottom watering via saucers standing below each pot

B. Study design and methods

1. In life dates: February 25 to June 28, 2010

2. Experimental treatments:

A total of eleven plant species were tested in this seedling emergence and growth test including seven dicotyledonous and four monocotyledonous species representing nine plant families.
The following species were treated: *Beta vulgaris* (sugar beet), *Brassica napus* (oilseed rape), *Cucumis sativus* (cucumber), *Fagopyrum esculentum* (buckwheat), *Glycine max* (soybean), *Lactuca sativa* (lettuce), *Lycopersicon esculentum* (tomato), *Allium cepa* (onion), *Avena sativa* (oat), *Lolium perenne* (ryegrass) and *Zea mays* (corn).

Five seeds of each species were sown in pots maintained in the glasshouse. The soil surface of the pots were treated once with BYI 02960 SL 200 g/L using a laboratory track sprayer applied at 410 g a.i./ha and a volume rate of 200 L/ha. Each pot (replicate) contained 5 seeds and there were 40 seeds treated i.e. 8 replicates. Control pots were treated with de-ionized water.

Following application, pots were grown and maintained under glasshouse conditions with a temperature control set at 23 ± 8ºC during day and 18 ± 8ºC at night with a 16 h photoperiod.

The blank control was 200 L deionized water/ha. Seedlings were exposed to the test item for 21 days.

3. Observation and measurements:

The parameters measured were emergence, survival of the emerged seedlings, visual phytotoxicity, plant growth stage, shoot length and shoot dry weight. Observations were recorded 7, 14 and 21 days after application and assessments were made against the water treated controls.

4. Statistical analysis

Statistical analysis of data was performed to obtain significance for shoot length and shoot dry weight effects, carried out using the Pairwise Mann-Whitney-U-Test (one sided smaller; $p \leq 0.05$) by ToxRat statistics.

RESULTS AND DISCUSSION

D. Environmental Parameters

Pots were kept in glasshouses under conditions as described above (see material and methods).

E. Biological Findings

As a result of a soil application of BYI 02960 SL 200 g/L with 410 g a.i./ha, this study revealed a very low level of phytotoxicity. There were no adverse effects on surviving with any of the species tested.

There were limited phytotoxic symptoms in this study with slight necrosis, leaf deformation and stunting in Buckwheat, Soybean, Tomato, Oat and Corn (maize).

Buckwheat (*Fagopyrum esculentum*) was the most sensitive species for shoot length and shoot dry weight, with 13.8% and 19.7% reduction, respectively, which were both statistically significant.

A summary of the findings from a single application of 410 g a.i./ha to eleven plant species tested is summarised in the following table:

Plant Species	Emergence (% inhibition)	Survival (% inhibition)	Phytotoxicity	Shoot Length (% reduction)	Shoot Dry Weight (% inhibition)
Dicotyledoneae					
Beta vulgaris	15.4	6.1	0	1.8	-7.4
Brassica napus	2.8	0	0	-1.3	-10.5
Cucumis sativus	-2.9	0	0	-5.3	-8.6
Fagopyrum esculentum	2.6	0	0 - 40%	**13.8**	**19.7**
Glycine max	8.3	0	0 - 30%	7.0	10.7
Lactuca sativa	-2.6	0	0	2.9	1.1
Lycopersicon esculentum	7.9	0	0 - 20%	-2.0	-16.3
Monocotyledoneae					
Allium cepa	-3.3	-3.4	0	3.6	6.5
Avena sativa	7.5	0	0 - 20%	0.1	-1.0
Lolium perenne	-12.5	0	0	4.6	7.4
Zea mays	7.5	0	0 - 20%	4.2	13.1

Bold figures for shoot dry weight are statistically significant (Pairwise Mann-Whitney-U-test, one sided smaller; $p \leq 0.05$)

F. Validity Criteria

This study can be considered valid as the validity criteria of crop specific emergence and at least 90% survival of the emerged seedlings during the study period was achieved for the untreated controls of all species tested.

CONCLUSION

Following a soil surface application of BYI 02960 SL 200 g/L applied at 410 g a.i./ha (corresponding to 2.4 kg product/ha) to eleven terrestrial non-target plant species, no adverse effects on emergence, seedling survival, visual phytotoxicity, growth, shoot length and shoot dry weight above 25% effect were observed in this seedling emergence and growth study. Only minimal responses were observed, typically within the range of natural variability.

IIA 8.13 Effects on terr. vertebrates other than birds / wild mammal toxicity

Not a current data requirement, therefore no additional studies have been performed.

IIA 8.14 Effects on other non-target organisms believed to be at risk

Chronic studies on *Folsomia* and *Hypoaspis* have been conducted with the formulated product BYI 02960 SL 200G and are summarized below for the parent and for the metabolites DFA and 6-CNA.

Report:	**KIIA 8.14/01; Frommholz, U., 2009**
Title:	BYI 02960 SL 200 G: Influence on the Reproduction of the Collembola Species *Folsomia candida* tested in Artificial Soil with 5% Peat
Report No:	FRM-COLL-75/09
Document No:	M-359728-01-2
Guidelines:	**ISO 11267 (1999)**
Deviations:	**To fulfil the recommendations of the proposal for a new OECD guideline 5% peat instead of 10% peat in the artificial soil was tested.**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the chronic effects of BYI 02960 SL 200 G (Sample description: FAR01438-00 (Batch ID: 2009-001253; Material No.: 79718845; Specification No.: 102000021884; purity 199.8 g BYI 02960/L; 17.0% w/w) to springtails (*Folsomia candida*).

Ten springtails (10 to 12 days old) per replicate (5 replicates per treatment group) were exposed in an artificial soil system with a peat content of 5 % over a period of 14 days to nominal concentrations of 8.8, 13.2, 19.9, 29.8 and 44.6 mg test item/kg artificial soil dry weight corresponding to 1.5, 2.3, 3.4, 5.1 and 7.6 mg a.i./kg dry weight soil in the 1st run and 5.88, 7.06 and 8.47 mg test item/kg dry weight soil, corresponding to1.00, 1.20 and 1.44 mg a.i./kg dry weight soil in the 2nd run. Since the first test run on BYI 02960 SL 200 G did not provide a final result, a second test run was performed studying lower concentrations. In addition a water control was tested.

Mortality and reproduction were determined after 28 days.

The overall 28-day NOEC was determined to be 8.47 mg product/kg soil dry weight.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960 SL 200 G
Type of test material:	Formulated product (soluble (liquid) concentrate)
Chemical state and description:	Clear, dark brown, liquid
Specification No.:	102000021884
Sample description:	FAR01438-00
Batch no.:	2009-001253
Nominal content of active ingredient:	BYI 02960: 200 g/L
Analytical content of active ingredient:	BYI 02960: 17.0% w/w, 199.8 g/L according to certificate of analysis
Density:	1.175 g/mL at 20°C
Stability:	Expiry date: 2010-03-20 , when stored at room temperature

2. Vehicle and/or positive control

Test item mixed with:	Water
Controls:	Water control as negative control

3. Test organisms

Species:	*Folsomia candida*
Common name:	Springtail
Source:	In-house lab culture
Age at study initiation:	10 to 12 days old
Maintenance of culture:	
Temperature:	22 ± 2°C
Photoperiod:	Permanent dark
Food:	Once a week with bakers dry yeast

B. Study design and methods

1. In life dates June 30 to September 18, 2009

2. Design of biological test

Springtails (*Folsomia candida*; 10 to 12 days old) were exposed to BYI 02960 SL 200 G; (content of a.i.: 199.8 g BYI 02960/L; 17.0% w/w) in an artificial soil system with 5 % peat over a period of 4 weeks.

Since the first test run on BYI 02960 SL 200 G did not provide a final result, a second test run was performed studying lower concentrations.

10 Collembola (10-12 days old) per replicate (5 replicates per treatment group) were exposed to control (water treated), 8.8, 13.2, 19.9, 29.8 and 44.6 mg test item/kg artificial soil dry weight corresponding to 1.5, 2.3, 3.4, 5.1 and 7.6 mg a.s./kg dry weight soil in the first test run and 5.88, 7.06 and 8.47 mg test item/kg dry weight soil, corresponding to 1.00, 1.20 and 1.44 mg a.i./kg dry weight soil in the second test run. In addition, a water control was tested as negative control. Each jar (glass vessels; 140 mL; 5 cm in diameter) served as one replicate filled with 30 g wet weight test substrate. 10 springtails were used per replicate. The test was conducted with 5 replicates per treatment level. The test was conducted at 20 ± 2°C and 577 to 608 lux at 16 h light : 8 h dark. The artificial soil contained 5% peat, 20 % kaolinite clay, 74.8% quartz sand and 0.2% calcium carbonate.

3. Observation and measurements

After 28 days mortality of the adult springtails and the number of juveniles were assessed as indicated below in the result section.

4. Statistical analysis

The software used to perform the statistical analysis was ToxRat Pro 2.09, (Ratte, 2006). Data of reproduction were tested for normal distribution and homogeneity of variance using Kolmogorov - Smirnov -Test and Cochran's -Test ($\alpha = 0.05$) respectively. Data of reproduction were normally distributed and homogeneity of variances was given. Therefore William's T-test (one-sided-smaller, $\alpha = 0.05$) was used to determine NOEC and LOEC values.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Table: pH and water content of the artificial soil

Test item concentration[1)]	pH		Water content in %		WHC_{max}	
	Start	End	Start	End	Start	End
first test run					WHC_{max}[2)]	
control	5.64	5.36	20.59	18.82	48.21	43.09
8.8	5.74	5.37	20.47	18.49	47.85	42.16
13.2	5.69	5.34	20.78	18.45	48.74	42.06
19.9	5.69	5.35	20.57	18.60	48.13	42.46
29.8	5.67	5.33	20.53	18.03	48.01	40.89
44.6	5.69	5.32	19.96	19.38	46.35	44.69
second test run					WHC_{max}[3)]	
control	5.82	5.71	19.05	17.42	47.78	42.82
5.88	6.05	5.68	19.33	17.60	48.66	43.37
7.06	5.90	5.66	19.43	17.29	48.96	42.45
8.47	5.95	5.66	19.20	17.01	48.25	41.62

[1)] mg test item/kg soil dry weight
[2)] % WHC_{max} = percent of maximum water holding capacity of 53.8 g water per 100 g artificial soil dry weight
[3)] % WHC_{max} = percent of maximum water holding capacity of 49.25 g water per 100 g artificial soil dry weight

B. Biological Findings

Observations on mortality and number of juveniles are listed as follows:

Table: Effects of BYI 02960 SL 200 G on mortality and reproduction of Folsomia candida

Concentration (mg prod/kg. dws)	adult mortality (%)	mean number of juveniles ± SD	reproduction (as % of control)
control (2nd run)	4	1202 ± 101	-
5.88	14	1289 ± 104	107.3
7.06	14	1308 ± 97	108.9
8.47	12	1166 ± 121	97.0
control (1st run)	2	1340 ± 60	-
8.8	2	1111 ± 120 *	82.9*
13.2	6	934 ± 144 *	69.7*
19.9	12	680 ± 130 *	50.8*
29.8	14	427 ± 169 *	31.9*
44.6	44	196 ± 61 *	14.7*

* Significantly different from the control (William's t-Test, one-sided-smaller, $\alpha = 0.05$)

C. Validity Criteria

The validity criteria (< 20% control mortality; average reproduction rate > 100 juveniles per control vessel and variation of reproduction < 30% c.v.) are fulfilled.

D. Test with toxic reference substance

Reference substance:	Betosip, active ingredient: Phenmedipham (153 g/L)
Date of most recent test:	2009
Result:	NOEC 7.6 mg a.i./kg artificial soil dry weight

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

NOEC:	8.47 mg test item/kg dry weight soil (equivalent to 1.442 mg a.i./kg dws)
LOEC:	8.8 mg test item/kg dry weight soil (equivalent to 1.498 mg a.i./kg dws)

CONCLUSION

The chronic effect of BYI 02960 SL 200 G on springtails (*Folsomia candida*) can be quantified as an overall-NOEC of 8.47 mg product/kg dry weight soil, equivalent to 1.442 mg a.i./kg dry soil.

Report:	**KIIA 8.14/02, Frommholz, U. (2010)**
Title:	Metabolite BYI 02960-difluoroacetic acid: Influence on the reproduction of the collembolan species *Folsomia candida* tested in artificial soil
Report No:	FRM-COLL-85/10
Document No:	M-368675-01-2
Guidelines:	**OECD Guideline No. 232**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the chronic effects of difluoroacetic acid (Origin Batch No: BCOO 5984-1-1; Batch code: BCS-AA56716-01-01; TOX 08889-00; purity 95.8% w/w) on the reproduction of springtails (*Folsomia candida*).

Springtails (10 to 12 days old) were exposed in an artificial soil system with peat content of 5% over a period of 14 days to a concentration of 100 mg/kg. In addition a quartz sand control was tested.

Mortality and number of juveniles were used to determine the endpoints.

The overall 28-day NOEC was determined to be > 100 mg /kg soil dry weight.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960-difluoroacetic acid (DFA, code: BCS-AA56716)
Type of test material:	Substance, technical (pure metabolite)
Chemical state and description:	Colourless liquid
Origin Batch number:	BCOO 5984-1-1 (Batch code: BCS-AA56716-01-01)
Sample description:	TOX 08889-00
CAS#:	381-73-7
Purity:	95.8% w/w
Stability of test compound:	Expiry date: 29.07.2010, when stored at +25 ± 5°C

2. Test solutions

Test item mixed with:	Quartz sand
Controls:	Water control

3. Test organisms

Species:	*Folsomia candida*
Common name:	Springtail
Source:	In-house lab culture since April 2002; originally obtained from IBACON
Age at study initiation:	10 to 12 days old
Maintenance of culture:	
Temperature:	22 ± 2°C
Photoperiod:	Permanent dark
Food:	Once a week with bakers dry yeast

B. Study design and methods

1. In life dates March 26 to April 27, 2010

2. Design of biological test

Springtails (*Folsomia candida;* 10 to 12 days old) were exposed to difluoroacetic acid (code: BCS-AA56716; purity 95.8 %) in an artificial soil system over a period of 28 days. The concentration was 100 mg/kg (limit test) mixed into the soil. In addition, a water treated quartz sand control was tested. Each replicate consisted of a jar (glass; 140 mL) filled with 30 g wet weight soil. Ten collembola were used per replicate. The test was conducted with 8 replicates per treatment level. The test was conducted at 20 ± 2°C and 692 to 702 lux at 16 h light : 8 h dark. The artificial soil contained 5% peat, 20% kaolinite clay, 74.85% quartz sand and 0.15% calcium carbonate.

3. Observation and measurements

Food (2 mg granulated dry yeast) was amended weekly during four weeks. After 4 weeks mortality of and reproduction were assessed as indicated below in the result section.

4. Statistical analysis

The software used to perform the statistical analysis was ToxRat Pro 2.09 released November 08, 2006, (Ratte, 2006). Data of reproduction were tested for normal distribution and homogeneity of variance using Kolmogorov - Smirnov -Test and Cochran's -Test ($\alpha = 0.05$) respectively. Data of reproduction were normally distributed and homogeneity of variances was given. Therefore Student-t test (one-sided-smaller, $\alpha = 0.05$) was used to determine NOEC and LOEC values.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

The soil-pH was 5.57 to 5.65. The water content was 42.39 to 49.33% of the maximum water holding capacity.

B. Biological Findings

Observations on mortality and reproduction are listed as follows:

In the control group 2.5% of the adult *Folsomia candida* died which is below the allowed maximum of ≤ 20% mortality. An LC_{50} could not be calculated and is considered to be > 100 mg test item/kg artificial soil dry weight.

Concerning the number of juveniles statistical analysis (Student-t test, one-sided smaller, $\alpha = 0.05$) revealed no significant difference between control and the treatment group.

Table: **Effects of difluoroacetic acid on mortality, body weight change and reproduction of *Folsomia candida***

Concentration of test item (mg p.m./kg dry soil)	Adult mortality (%)	Mean number of juveniles ± SD	Reproduction (as % of control)
control	2.5	1337 ± 175	
100	1.3	1268 ± 146	95 % (n.s.)

C. Validity Criteria

Validity criteria	Recommended by the guideline	Obtained in this study
Mean adult mortality	≤ 20 %	2.5 %
Mean number of juveniles per replicate (with 10 colembolans introduced)	≥ 100	1337
Coefficient of variation calculated for the number of juveniles per replicate	≤ 30 %	13.1 %

All validity criteria were met. Therefore this study is valid.

D. Test with toxic reference substance

Reference substance:	Boric acid
Date of most recent test:	March, 2010
Result:	EC_{50}: 96 mg boric acid/kg soil dry weight $NOEC_{repro}$: 44 mg boric acid/kg soil dry weight

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

LC_{50} mortality	>100 mg test item/kg soil dry weight
$NOEC_{reproduction}$:	≥100 mg test item/kg soil dry weight
$LOEC_{reproduction}$	>100 mg test item/kg soil dry weight
Overall NOEC	**> 100 mg p.m./kg soil dry weight**

CONCLUSION

The chronic effect of difluoroacetic acid (BCS-AA56716) on springtails (*Folsomia candida*) can be quantified as an overall-NOEC of > 100 mg p.m./kg soil dry weight.

Report:	**KIIA 8.14/03, Frommholz, U. (2010)**
Title:	6-chloronicotinic acid (AE F161089): Influence on the reproduction of the collembolan species *Folsomia candida* tested in artificial soil
Report No:	FRM-Coll-111/11
Document No:	M-407861-01-2
Guidelines:	**OECD Guideline No. 232**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the chronic effects of 6-chloronicotinic acid (Origin Batch No: M12653; Batch code: AE F161089 00 1B99 0001; purity 98.8% w/w) on reproduction of springtails (*Folsomia candida*). Ten springtails (10 to 12 days old) per replicate were exposed in an artificial soil system with peat content of 5% over a period of 28 days to concentrations of 100 mg/kg (1st run) and 10, 17, 30, 52 and 90 mg test item/kg artificial soil dry weight (2nd run). Eight replicates per dose for the first run and 4 replicates for the second run were performed. In addition a water control was tested.

Mortality in the adults and number of juveniles were determined after 28 days. The overall 28-day NOEC was 90 mg /kg soil dry weight.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	6-chloronicotinic acid (BYI 02960-6-CNA, AE F161089)
Type of test material:	Substance, technical (pure metabolite)
Chemical state and description:	Beige powder
Origin Batch number:	M12653 (Batch code: AE F161089 00 1B99 0001)
CAS#:	5326-23-8
Purity:	98.8% w/w
Stability of test compound:	Expiry date: 09.07.2012, when stored at + 5 ± 5 °C

2. Test solutions

test item mixed with:	Quartz sand
Controls:	Water control

3. Test organisms

Species:	*Folsomia candida*
Common name:	Springtail
Source:	In-house lab culture
Age at study initiation:	10 to 12 days old
Maintenance of culture:	
Temperature:	22 ± 2°C
Photoperiod:	Permanent dark
Food:	Once a week with bakers dry yeast

B. Study design and methods

1. In life dates January 14 to April 15, 2011

2. Design of biological test

Springtails (*Folsomia candida;* 10 to 12 days old) were exposed to 6-chloronicotinic acid (code: AE F161089; purity 98.8% w/w) in an artificial soil system over a period of 28 days. Concentrations were 100 mg/kg (1st run) and 10, 17, 30, 52 and 90 mg test item/kg artificial soil dry weight (2nd run). In addition, a water control was tested. Each replicate consisted of a jar (glass; 140 mL) filled with 30 g wet weight. 10 springtails were used per replicate. The test was conducted with 8 (1st run) and 4 (2nd run) replicates per treatment level. In the controls, 8 replicates were tested. The test was conducted at

20 ± 2°C and 574 to 614 lux (1st run) and 567 to 590 lux (2nd run) at 16 h light : 8 h dark. The artificial soil contained 5% peat, 20% kaolinite clay, 74.8% quartz sand and 0.2% calcium carbonate.

Directly after the addition of the collembolans, they were fed with granulated dry yeast. Feeding was also done 14 days after test start.

3. Observation and measurements

The surviving adults and living juveniles were counted. Missing adults (compared to the number of initially placed test organisms) were considered to be dead, since dead collembolans cannot be extracted. Endpoints of the test were mortality of the adult collembolans in comparison to the initially placed test organisms expressed in % and the number of offspring hatched from the eggs and surviving until the end of the test period per test vessel (reproduction).

4. Statistical analysis

The analyses were done with the program ToxRat Professional 2.10 (Ratte, 2010). Data of reproduction were tested for normal distribution and homogeneity of variance using Kolmogorov - Smirnov -Test and Cochran's -Test ($\alpha = 0.05$) respectively. In the 1st test run data of reproduction were normally distributed and homogeneity of variances was given. Therefore Student's-t test, one-sided-smaller, $\alpha = 0.05$ was used to determine NOEC and LOEC values. In the 2nd test run data of reproduction were normally distributed and homogeneity of variances was given. Therefore William's-t test, one-sided-smaller, $\alpha = 0.05$ was used to determine NOEC and LOEC values.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

The soil-pH was 5.47 to 5.86. The water content was 42.94 to 47.98% of the maximum water holding capacity.

B. Biological Findings

Observations on mortality and number of juveniles are listed as follows:

Table: Effects of AE F161089 on mortality, body weight change and reproduction of *Folsomia candida*

Concentration (mg/kg dry soil)	**Adult mortality (%)**	**Mean number of juveniles** ± standard deviation	**Reproduction (as % of control)**
control (1st run)	13.8	1541.4 ± 165.6	
100	20.0	1258.9 ± 132.5	81.7*
control (2nd run)	2.5	1566.9 ± 166.0	
90	5.0	1485.8 ± 124.8	94.8 n.s.
52	5.0	1434.3 ± 155.0	91.5 n.s.
30	7.5	1573.5 ± 95.4	100.4 n.s.
17	5.0	1641.3 ± 51.4	104.7 n.s.
10	5.0	1583.0 ± 93.6	101.0 n.s.

* Significantly different to control (Student's t-test, one-sided smaller, $\alpha \leq 0.05$)

C. Validity Criteria

All validity criteria were met. Therefore this study is valid.

Validity criteria	Recommended by the guideline	Obtained in this study	
		1st run	2nd run
Mean adult mortality	≤ 20 %	13.8 %	2.5 %
Mean number of juveniles per replicate (with 10 collembolans introduced)	≥ 100	1541	1567
Coefficient of variation calculated for the number of juveniles per replicate	≤ 30 %	10.7 %	10.6 %

D. Test with toxic reference substance

Reference substance:	boric acid
Date of most recent test:	March 2011
Result:	EC_{50}: 91 mg boric acid/kg soil dry weight
	$NOEC_{repro}$: 44 mg boric acid/kg soil dry weight

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

LC_{50} (adult mortality)	> 100 mg p.m./kg soil dry weight
$LOEC_{reproduction}$	100 mg p.m./kg soil dry weight
$NOEC_{reproduction}$:	**90 mg p.m. /kg soil dry weight**

CONCLUSION

The chronic effect of 6-chloronicotinic acid (AE F161089) on springtails (*Folsomia candida*) can be quantified as an overall-NOEC of 90 mg p.m. /kg soil dry weight based on the slight but statistically significant effects seen at 100 mg p.m/kg..

Report:	**KIIA 8.14/04; Kratz A., 2009**
Title:	BYI 02960 SL 200 G: Influence on mortality and reproduction on the soil mite species *Hypoaspis aculeifer* tested in artificial soil with 5% peat
Report No:	Kra-HR-19/09
Document No:	M-358752-01-2
Guidelines:	**OECD Guideline No. 226 (2008)**
Deviations:	**To fulfil the recommendations of the proposal for a new OECD guideline 5% peat instead of 10% peat in the artificial soil was tested.**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the chronic effects of BYI 2960 SL 200 G, (Sample description: FAR01438-00 (Batch ID: 2009-001253; Material No.: 79718845; Specification No.: 102000021884); 17.0% w/w) to predatory soil mites (*Hypoaspis aculeifer*).

Ten mites (28 days old, after start of egg-laying) per replicate (4 replicates per treatment group and 8 control replicates) were exposed in an artificial soil system with a peat content of 5% over a period of 14 days to nominal concentrations of 100, 178, 316, 562 and 1000 mg test item/kg artificial soil dry weight. In addition, a water control was tested. Mortality of the adults and number of juveniles were used to determine the endpoints.

The overall 14-day NOEC was determined to be ≥ 1000 mg product/kg dry weight soil.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 2960 SL 200 G
Type of test material:	Formulated product (soluble (liquid) concentrate)
Chemical state and description:	Dark brown, clear liquid
Specification No.:	102000021884
Material number:	79718845
Sample description:	FAR01438-00
Batch No.:	2009-001253
Nominal content of active substance:	BYI 02960: 200 g/L
Analytical content of active substance:	BYI 02960: 17.0 % w/w, 199.8 g/L according to certificate of analysis
Solubility:	In water: soluble
Density:	1.175 g/mL at 20°C
Stability:	Expiry date: March 10, 2010, when stored at +25 ± 5 °C

2. Vehicle and/or positive control

Test item mixed with:	Water
Controls:	Water control as negative control

3. Test organisms

Species:	*Hypoaspis aculeifer*
Common name:	Soil mite
Source:	In-house lab culture
Age at study initiation:	28 days after start of egg-laying
Feeding during test:	Fed with cheese mites on days 0, 4, 7 and 10
Maintenance of culture:	
Temperature:	20 ± 2°C
Photoperiod:	Permanent dark
Food:	Cheese mites bred on brewer's yeast

B. Study design and methods

1. In life dates August 13 to September 14, 2009

2. Design of biological test

Predatory soil mites (Hypoaspis aculeifer; 28 days after start of egg-laying) were exposed to BYI 2960 SL 200 G; (content of a.i.: 17.0% w/w) in an artificial soil system over a period of 14 days. Cheese mites were amended twice a week.

10 fertilized female mites per replicate (4 replicates per treatment group) were exposed to concentrations of 100, 178, 316, 562 and 1000 mg test item/kg dry weight artificial soil. In addition a water control was tested in 8 replicates.

Each jar (glass vessels; 140 mL) served as one replicate filled with an amount equivalent to 20 g dry weight artificial soil (5% peat, 20% kaolinite clay, 74.8% quartz sand and 0.2% calcium carbonate). The depth of the soil layer was approximately 1.5 cm. The test was conducted at 20 ± 2°C and 575 to 585 lux at 16 h light; 8 h dark.

3. Observation and measurements

After 14 days mortality of adult mites and number of juveniles were assessed as indicated below in the result section.

4. Statistical analysis

The software used to perform the statistical analysis was ToxRat Pro 2.10 (released February 19, 2009); (Ratte, 2001-2009). Data of reproduction were tested for normal distribution and homogeneity of variance using Kolmogorroff-Smirnov Test and Cochran-Test ($\alpha = 0.05$), respectively. Data of reproduction were normally distributed and homogeneity of variances was given. Therefore Williams test (one-sided smaller, $\alpha = 0.05$) was used to determine NOEC and LOEC values.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Table: pH and water content of the artificial soil

Test item concentration 1)	pH		Water content in %			% of WHCmax2, 3)	
	Start	End	Start	End	% deviation	Start	End
Control	5.84	5.66	19.25	19.40	0.79	48.40	48.88
100	5.74	5.80	19.05	19.62	3.01	47.78	49.56
178	5.72	5.63	19.26	19.75	2.54	48.43	49.96
316	5.63	5.52	19.40	18.89	-2.59	48.86	47.29
562	5.56	5.48	19.43	19.37	-0.33	48.97	48.77
1000	5.62	5.51	19.04	19.32	1.48	47.75	48.62

1) mg test item/kg soil dry weight
2) % of WHC_{max} = percent of maximum water holding capacity of 49.25 g H_2O/100 dry weight artificial soil
3) The results represent rounded values calculated on the exact raw data

B. Biological Findings

Observations on mortality and number of juveniles are listed as follows:

Table: Effects of BYI 2960 SL 200 G on mortality and reproduction of *Hypoaspis aculeifer*

Concentration (mg test item/kg dws)	adult mortality (%)	mean number of juveniles	reproduction (as % of control)
control	1.3	361.9 ± 23.4	-
100	2.5	334.5 ± 44.8	92.4
178	5.0	377.3 ± 22.8	104.2
316	0.0	387.5 ± 23.1	107.1
562	0.0	384.3 ± 19.3	106.2
1000	0.0	382.0 ± 43.4	105.6

C. Validity Criteria

The validity criteria (< 20% control mortality; average reproduction rate > 100 juveniles per control vessel and variation of reproduction < 30% c.v.) are fulfilled.

D. Test with toxic reference substance

Reference substance:	dimethoate
Date of most recent test:	09 FEB 2009
Result:	LC_{50} of 3.86 mg a.i./kg dry weight artificial soil

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

LC_{50}:	>100 mg product/kg dws
LOEC (reproduction):	>1000 mg product/kg dws
NOEC (reproduction):	≥1000 mg product/kg dws

CONCLUSION

The chronic effect of BYI 2960 SL 200 G on predatory soil mites (*Hypoaspis aculeifer*) can be quantified as an overall-NOEC of ≥ 1000 mg product/kg dry weight soil.

Report:	**KIIA 8.14/05, Kratz, A. (2010)**
Title:	BYI 2960-DFA (BCS-AA56716): Influence on mortality and reproduction on the soil mite species *Hypoaspis aculeifer* tested in artificial soil with 5% peat
Report No.:	kra-HR-27/10
Document No.:	M-390091-01-2
Guidelines:	**OECD 226**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The aim of the study was to determine the effects of difluoroacetic acid (Origin Batch No: BCOO 5984-1-1; Batch code: BCS-AA56716-01-01; TOX 08889-00; Purity 95.8% w/w) on mortality and reproduction of predatory soil mites (*Hypoaspis aculeifer*).

Ten female mites per replicate (35 and 28 days after start of egg-laying in the 1st and 2nd run, respectively) were exposed in an artificial soil system with a peat content of 5 % over a period of 14 days to concentrations of 63, 125, 250, 500 and 1000 mg test item/kg dry weight artificial soil (1st run), respectively, and 1000 mg test item/kg dry weight artificial soil (2nd run).

Mortality of adults and number of juveniles were determined.

The overall 14 day- NOEC was > 1000 mg test item/kg dry weight artificial soil.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960-difluoroacetic acid (BCS-AA56716)
Type of test material:	Substance technical (pure metabolite)
Chemical state and description:	Colourless liquid
Origin Batch No.:	BCOO 5984-1-1 (Batch code: BCS-AA56716-01-01)
Sample description:	TOX 08889-00
CAS#:	381-73-7
Purity:	95.8% w/w
Storage conditions:	Expiry date: 29.07.2010, when stored at +25 ± 5 °C

2. Test solutions

Test item mixed with:	Quartz sand
Controls:	Water control

3. Test organisms

Species:	*Hypoaspis aculeifer*
Common name:	Soil mite
Source:	In-house lab culture
Age at study initiation:	35 and 28 days after start of egg-laying in the 1st and 2nd run, respectively
Feeding during test:	Fed with cheese mites on days 0, 3, 7 and 11 (1st run) and on days 0, 3, 7 and 11 (2nd run)
Maintenance of culture:	
Temperature:	20 ± 2°C
Photoperiod:	Permanent dark
Food:	Cheese mites bred on brewer's yeast

B. Study design and methods

1. In life dates March 5 to May 5, 2010

2. Design of biological test

Fertilized female predatory soil mites (*Hypoaspis aculeifer;* 35 and 28 days after start of egg-laying in the 1st and 2nd run, respectively) were exposed to difluoroacetic acid (code: BCS-AA56716; purity 95.8 % w/w) in an artificial soil system over a period of 14 days. Concentrations were 63, 125, 250, 500 and 1000 mg test item/kg dry weight artificial soil (1st run) and 1000 mg test item/kg dry weight artificial soil (2nd run). Since in the 1st test run in each concentration at least in one replicate more than 10 adult mites were found, the test was repeated as limit test with 1000 mg test item /kg dry weight artificial soil. In addition a water control was tested. Each replicate consisted of a jar (glass vessels; 140 mL) filled with equivalent to 20 g dry weight artificial soil. The depth of the soil layer was approximately 1.5 cm. Ten fertilized females mites were used per replicate. The test was conducted with 4 replicates per treatment level. In the controls 8 replicates were tested. The test was conducted at 20 ± 2°C and 400 to 800 Lux at 16 h light : 8 h dark. The artificial soil contained 5 % peat, 20 % kaolinite clay, 74.8 % quartz sand and 0.2 % calcium carbonate. After a period of 14 days, the surviving adults and the living juveniles were extracted by applying a temperature gradient using a MacFadyen-apparatus. Extracted mites were collected in a fixing solution (20 % ethylene glycol, 80 % deionised water; 2 g detergent/L fixing solution were added). All *Hypoaspis aculeifer* were counted under a binocular.

3. Observation and measurements

Cheese mites were amended twice a week. After a period of 14 days, the surviving adults and the living juveniles were extracted by applying a temperature gradient using a MacFadyen-apparatus. Extracted mites were collected in a fixing solution (20 % ethylene glycol, 80 % deionised water; 2 g detergent/L fixing solution were added). All *Hypoaspis aculeifer* were counted under a binocular.

4. Statistical analysis

The software used to perform the statistical analysis was ToxRat Pro 2.10 (released February 19, 2009); (Ratte, 2001-2009). Data of reproduction were tested for normal distribution and homogeneity of variance using Kolmogorroff-Smirnov Test and Cochran-Test ($\alpha = 0.05$), respectively. Data of

reproduction were normally distributed and homogeneity of variances was given. Therefore Williams test (one-sided smaller, $\alpha = 0.05$) was used to determine NOEC and LOEC values.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

Measured pH values and water holding capacity (WHC_{max}) of the artificial soil used in this study were as follows:

Table: pH and water content of the artificial soil

mg test item/kg dry weight artificial soil	pH		% water content			% of WHC_{max}	
	Start	End	Start	End	% deviation	Start	End
First run							
Control	6.25	5.80	17.66	17.16	- 2.8	43.54	42.07
63	6.09	6.14	17.98	17.69	- 1.6	44.51	43.63
125	5.81	5.71	20.55	19.11	- 7.0	52.52	47.96
250	5.61	5.57	19.31	18.68	- 3.2	48.58	46.65
500	5.56	5.47	20.14	18.25	- 9.4	51.21	45.34
1000	6.00	5.43	19.82	18.74	- 5.4	50.20	46.84
Second run							
Control	5.51	5.43	19.22	20.30	5.6	48.30	51.72
1000	5.50	4.87	19.63	19.12	- 2.6	49.61	48.00

B. Biological Findings

Biological observations are listed in the following table:

Table: Effects of BYI 02960-DFA (BCS-AA56716) on mortality, body weight change and reproduction of *Hypoaspis aculeifer*

Concentration (mg p.m./kg dry soil)	Adult mortality (%)	Mean number of juveniles ± SD	Reproduction (as % of control)
Control (1st run)	5	369.9 ± 44.3	-
Control (2nd run)	1.3	380.6 ± 37.7	-
63	-7.5	393.0 ± 50.1	106.3
125	2.5	356.5 ± 39.4	96.4
250	-10	436.0 ± 43.6	117.9
500	-7.5	377.3 ± 18.3	102.0
1000 (1st run)	5	393.3 ± 48.1	106.3
1000 (2ndt run)	0	391.9 ± 29.9	103.0

C. Validity Criteria

Validity criteria (control values)	Recommended by the guideline	Obtained in the 1st test run	Obtained in the 2nd test run
Mean adult female mortality	≤ 20 %	5.0 %	1.3 %
mean number of juveniles per replicate (with 10 adult females introduced)	≥ 50	369.9	380.6
coefficient of variation calculated for the number of juvenile mites per replicate	≤ 30 %	12.0 %	9.9 %

All validity criteria were met. Therefore both test runs of this study are valid.

D. Test with toxic reference substance

Reference substance:	Dimethoate
Date of most recent test:	03 FEB 2010
Result:	LC_{50} of 4.2 mg p.m./kg dry weight soil $NOEC_{repro}$: 3.2 mg p.m./kg dry weight soil

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

NOEC:	≥ 1000 mg test item/kg dry weight soil
LOEC:	> 1000 mg test item/kg dry weight soil

CONCLUSION

The chronic effect of difluoroacetic acid (BCS-AA56716) on predatory soil mites (*Hypoaspis aculeifer*) can be quantified as an overall-NOEC of ≥ 1000 mg test item/kg dry weight artificial soil.

Report:	**KIIA 8.14/06; Kratz, A. (2011)**
Title:	6-chloronicotinic acid (AE F161089): Influence on mortality and reproduction on the soil mite species *Hypoaspis aculeifer* tested in artificial soil
Report No.:	kra-HR-44/11
Document No.:	M-404434-01-2
Guidelines:	**OECD 226, 2008**
Deviations:	**None**
GLP:	**Yes (certified laboratory)** **The regular of the sensitivity of the test organisms was not performed according to GLP.**

EXECUTIVE SUMMARY

The aim of the study was to determine the chronic effects of 6-chloronicotinic acid (Batch code: AE F161089 00 1B99 0001; purity 98.8%w/w) on mortality and reproduction of predatory soil mites (*Hypoaspis aculeifer*).

Adult mites (28 days after start of egg-laying) were exposed in an artificial soil system with peat content of 5% over a period of 14 days to a concentration of 100 mg test item/kg dry weight artificial soil. In addition, a water control was tested. After a period of 14 days, the surviving adults and the living juveniles were extracted by applying a temperature gradient using a MacFadyen-apparatus. Extracted mites were collected in a fixing. All *Hypoaspis aculeifer* were counted under a binocular.

Mortality in the adults and number of juveniles were used to determine the endpoints. The overall 8-week NOEC was determined to be ≥ 100 mg p.m./kg soil dry weight.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	6-chloronicotinic acid (BYI 02960-6-CNA, AE F161089)
Type of test material:	Substance, technical (pure metabolite)
Chemical state and description:	Beige powder
Origin Batch number:	M12653 (Batch code: AE F161089 00 1B99 0001)
CAS#:	5326-23-8
Purity:	98.8% w/w
Stability of test compound:	Expiry date: 09.07.2012, when stored at + 5 ± 5 °C

2. Test solutions

Test item mixed with:	Quartz sand
Controls:	Water control

3. Test organisms

Species:	*Hypoaspis aculeifer*
Common name:	Soil mite
Source:	In-house lab culture
Age at study initiation:	28 days after start of egg-laying
Feeding during test:	fed with cheese mites on days 0, 3, 7 and 10
Maintenance of culture:	
Temperature:	22 ± 2°C
Photoperiod:	Permanent dark
Food:	Cheese mites bred on brewer's yeast

B. Study design and methods

1. In life dates January 14 to February 4, 2011

2. Design of biological test

Predatory soil mites *(Hypoaspis aculeifer*; 28 days after start of egg-laying) were exposed to 6-chloronicotinic acid (code: AE F161089; purity 98.8 %w/w) in an artificial soil system over a period of 14 days. One concentration of 100 mg test item/kg dry weight artificial soil was tested. In addition a water control was tested. Each replicate consisted of a jar (glass vessels; 140 mL) filled with the equivalent of 20 g dry weight artificial soil, the depth of the soil layer was approximately 1.5 cm. 10 fertilized females mites were used per replicate. The test was conducted with 8 replicates per treatment level. The test was conducted at 20 ± 2°C and 640 to 739 lux at 16 h light : 8 h dark. The artificial soil contained 5% peat, 20% kaolinite clay, 74.8% quartz sand and 0.17% calcium carbonate.

Directly after the addition of *Hypoaspis aculeifer*, they were fed with the cheese mite *Tyrophagus putrescentiae*. Cheese mites were bred on brewer's yeast in the laboratory. Feeding was also done 3, 7 and 10 days after test start. Between 0.050 and 0.090 mg food per test vessel was added per feeding date. Each test vessel was weighed for the determination of water loss. After 7 days the loss of water was determined by reweighing the test vessels. The missing amount of water was added.

3. Observation and measurements

After a period of 14 days, the surviving adults and the living juveniles per test vessel were extracted, applying a temperature gradient. For this purpose the content of each test vessel was carefully transferred to sieve vessels (mesh size approximately 0.8 mm). Each sieve vessel was put onto another vessel containing a fixing liquid. The vessels were positioned in MCFADYEN-Extractor. The

temperature was increased from approximately 25° to 40° C within two days. Extracted mites were collected in a fixing solution (20 % ethylene glycol, 80 % deionised water; 2 g detergent/L fixing solution were added). All *Hypoaspis aculeifer* (adult, females and juveniles) were counted under a binocular.

4. Statistical analysis

Concerning the number of juveniles statistical analysis (Welch-t test for inhomogeneous variances, one-sided smaller, $\alpha = 0.05$) revealed no significant differences between the control and treatment.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

The soil-pH was 5.59 to 6.81. The water content was 43.85 to 47.98% of the maximum water holding capacity.

B. Biological Findings

Observations on survival of adults and number of juveniles are listed as follows: The results indicate that there is no significant difference in the number of juvenile between the control and the treatment group.

Table: Effects of AE F161089 on mortality, body weight change and reproduction of *Hypoaspis aculeifer*

Concentration (mg a.i./kg dry soil)	adult mortality (mean of 8 replicates) (%)	mean number of juveniles ± Standard deviation	reproduction (% of control)
control	16.3	264.0 ± 54.5	-
100	21.3	252.0 ± 130.2	95.5

C. Validity Criteria

Validity criteria (for control replicates)	Recommended by the guideline	Obtained in this study
Mean adult female mortality	≤ 20 %	16.3 %
mean number of juveniles per replicate (with 10 adult females introduced)	≥ 50	264.0
coefficient of variation calculated for the number of juvenile mites per replicate	≤ 30 %	20.6 %

All validity criteria were met. Therefore this study is valid.

D. Test with toxic reference substance

Reference substance:	Dimethoate
Date of most recent test:	03 FEB 2010
Result:	LC_{50} of 4.2 mg a. s./kg dry weight artificial soil

E. Biological Endpoints Derived

LC_{50} (adult mortality)	> 100 mg p.m./kg soil dry weight
NOEC (reproduction):	≥ 100 mg p.m. /kg soil dry weight
LOEC (reproduction)	> 100 mg p.m./kg soil dry weight

CONCLUSION

The chronic effect of 6-chloronicotinic acid (AE F161089) on predatory soil mites (*Hypoaspis aculeifer*) can be quantified as an overall-NOEC of ≥ 100 mg p.m./kg soil dry weight.

IIA 8.14.1 Summary of preliminary data: biological activity & dose range finding

Insecticidal activity

PECgw calculations indicate that the metabolite difluoroacetic acid might occur at concentrations of>0.1 µg/L in groundwater under certain worst-case conditions. Therefore, as part of studies required to demonstrate the non-relevance a screening test has been performed for the metabolite difluoroacetic acid (DFA) to compare its efficacy on target insects in comparison with the parent compound BYI 02960. The results are briefly summarized below.

Report:	**KIIA 8.14.1/01; Voerste A., Malsam O. (2010)**
Title:	Determination of the insecticidal efficacy of the metabolite difluoroacetic acid (BCS-AA56716) compared to the parent compound BYI02960 (BCS-BZ89914)
Report No:	VAR 2010-01
Document No:	M-386333-01-1
Guidelines:	**No existing guideline**
Deviations:	**Not applicable**
GLP:	**Non-GLP**

EXECUTIVE SUMMARY

The insecticidal efficacy of BYI 02960 and its metabolite difluoroacetic acid (BCS-AA56716; Purity: 95.8% w/w)) has been determined by leaf disc and whole plant screening systems. As expected, the parent compound BYI 02960 (technical; Purity: 96.2% w/w) efficiently controlled the relevant target insects.

It could be demonstrated that the metabolite difluoroacetic acid does not have any insecticidal activity against any of the species tested, neither in leaf disc nor in whole plant test systems.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	Difluoroacetic acid (DFA, BCS-AA56716)
Type of test material:	Substance, technical (pure metabolite)
Origin Batch number:	BCOO5984-1-1 (Batch code: BCS-AA56716-01-01)
Purity:	95.8% w/w

2. Positive control

Test item:	BYI 02960
Type of test material:	Substance, technical
Origin Batch number:	2009-000239 (Batch code: BYI02960-01-03)
Purity:	96.2% w/w
Stability of test compound:	Not stated

3. Test organisms

A: Test organisms for the microtiter plate test:	*Phaedon cochleariae* PHAECO (Mustard beetle) *Myzus persicae* MYZUPE (Green peach aphid) *Nilaparvata lugens* NILALU (Brown plant hopper)
B: Test organisms for the greenhouse test:	*Myzus persicae* MYZUPE (Green peach aphid) *Aphis gossypii* APHIGO (Cotton aphid) *Bemisia tabaci* BEMITA (Silver leaf whitefly)

B. Study design and methods

1. In-life dates Not stated in the report

2. Experimental treatments

For this comparing study, two different standardised test systems have been used:

A - Microtiter plate testing:

A range of insects, covering relevant species that are known to be sensitive against BYI 02960 (mainly sucking insect segment), have been used in the actual study (mustard beetle, green peach aphid and brown plant hopper). It is generally accepted that such microtiter systems are suitable to describe the intrinsic insecticidal potential of a test compound.

Methodology of the microtiter plate test

For *Myzus* and *Phaedon* (leaf discs):

The cavities of 12-hole microtiter plates are filled up to half of its height with artificial insect diet (*Phaedon*) or agar gel (*Myzus*), respectively, and for each cavity a leaf disc (cabbage) of 2cm diameter is put on top.

In case of *Myzus*, the leaf discs are already infected with all instars of *Myzus* at times of spraying.

In case of *Phaedon*, two *Phaedon* larvae are put together in each cavity after spraying of the compounds.

The test compound BYI 02960 and its metabolite DFA (BCS-AA56716), respectively, are dissolved in a mixture of water, acetone, DMF and emulsifier (Emulsifier W), and four different concentrations are prepared. Each microtiter plate cavity is subsequently sprayed with one of the concentrations, resulting in application rates on the leaf discs of 500, 100, 20 and 4 g/ha. The leaf discs are allowed to dry afterwards for a certain period of time.

The incubation time for the *Phaedon* tests are 7 days, for the *Myzus* tests 6 days before the test evaluation is carried out. Efficacy is given in percentage of dead insects.

For Nilaparvata (whole plant):

A ca. 10 cm high tube is plugged into each cavity of the microtiter plate, and small rice plants are grown in perlite in these tubes over one week.

Test compound solutions (solvents water, acetone, DMF, emulsifier W, RME1, AMS2) are prepared in concentrations of 0.8, 0.16, 0.032, 0.0064 ppm. These solutions are then sprayed into the tube, so that the spray is covering the small rice plants evenly.

The plants are infested with plant hoppers afterwards and stored for 7 days until the evaluation is done. Efficacy is given in percentage of dead insects.

B - Greenhouse test:

BYI 02960 and its metabolite DFA (BCS-AA56716) were tested against a range of insects at application rates regarded as the relevant efficacy range for BYI02960 (70 - 100% efficacy).

Testing was done against the following relevant insect pests, all sensitive to BYI02960: Green peach aphid, Cotton aphid, Silver leaf whitefly.

Methodology of the greenhouse test

Spray test:

In each case, the parent compound and the metabolite, respectively, are dissolved in a mixture of water, DMF (Dimethylformamide), emulsifier W, RME and AMS and are immediately applied by spraying on a plant set, resulting in a complete coverage of the leaves with the test solution. At times of spraying, the plants are already infested with the respective insects.

Incubation time for the *Myzus* and Aphis tests were 6 days and for the whitefly tests 8 days.

Drench test (only *Myzus*):

The respective compounds are dissolved in a mixture of water, DMF, emulsifier W, RME (rape oil methylester) and AMS (Ammonium sulfate) and a specific volume (10 mL per 125 mL soil) of the solution is poured on the soil of a pot (7 cm diameter) in which a cabbage plant is growing. The plant is already infested with the respective insects at this time.

Incubation time before the evaluation of the test is 10 days.

RESULTS AND DISCUSSION

A. Evaluation of results / Validity Criteria

Results of the test systems applied have been analyzed for fulfilling of EU guidance and German guidance:

- **EU guidance document[7]:** Metabolite must have clearly less than 50% of parent compound efficacy
- **German guidance (UBA)[8]:** Efficacy of metabolite must be less than 30% of parent compound with parent efficacy being higher than 70%.

[7] **EU guidance (SANCO/221/2000-rev.l0):** Guidance Document on the Assessment of the Relevance of Metabolites in Groundwater of Substances

[8] **Umweltbundesamt (UBA):** Assessment of the Relevance of Metabolites in Groundwater in the Context of National Authorisation Procedure of Plant Protection Products (Draft 2003-09-19)

B. Biological Findings

A – Microtiter plate testing:

Table: Insecticidal efficacy of BYI 02960 and its metabolite in microtiter plate system (referring to A)

Microtiter plate test	Test object	MYZUPE	PHAEDON		Test object	NILALU
	Test plant	Chinese cabbage	Chinese cabbage		Test plant	Rice
	Application	Spray	Spray		Application	Spray
	Evaluation	6 d	7 d		Evaluation	7 d
	Conc. (g/ha)	Insecticidal efficacy (%)			Conc. (g/ha)	Efficacy (%)
BYI02960 (BCS-BZ89914)	500	**100**	**100**		0,8	**90**
	100	**100**	**100**		0,16	**50**
	20	**90**	**0**		0,032	**30**
	4	**70**	**0**		0,0064	**0**
Difluoroacetic acid (BCS-AA56716)	500	**0**	**0**		0,8	**0**
	100	**0**	**0**		0,16	**0**
	20	**0**	**0**		0,032	**0**
	4	**0**	**0**		0,0064	**0**

While BYI 02960 shows significant efficacy up to 100% at the higher application rates, clearly no insecticidal efficacy at all is exhibited by the metabolite DFA (BCS-AA56716).

Both, EU guidance and German guidance (UBA), are clearly fulfilled by these test results obtained.

B – Greenhouse efficacy:

In greenhouse, the efficacy of BY102960 and its metabolite has been determined in whole plant systems at concentrations between 20 and 0.032 ppm, regarded as the relevant efficacy range for BYI 02960 (70 - 100% efficacy).

Results are summarized in the following table:

Table Efficacy of BYI 02960 and its metabolite in greenhouse test on plants (referring to B)

Whole plant spray test	Test object	MYZUPE	APHIGO	BEMITA	Test object	MYZUPE
	Test plant	Bell pepper	Cotton	Cotton	Test plant	Cabbage
	Application	Spray	Spray	Spray	Application	Drench
	Evaluation	6 d	6 d	8 d	Evaluation	6 d
	Conc. (ppm)	Insecticidal efficacy (%)			Conc. (ppm)	
BYI02960 (BCS-BZ89914)	20			**98**	20	
	4	**100**	**100**	**98**	4	**100**
	0,8	**75**	**95**	**0**	0,8	**75**
	0.16	**0**	**0**	**0**	0.16	**0**
	0.032	**0**	**0**		0.032	**0**
Difluoroacetic acid (BCS-AA56716)	20			**0**	20	
	4	**0**	**0**	**0**	4	**0**
	0,8	**0**	**0**	**0**	0,8	**0**
	0.16	**0**	**0**	**0**	0.16	**0**
	0.032	**0**	**0**		0.032	**0**

The results presented in the table above demonstrate very clearly that the metabolite DFA (BCS-AA56716) does not show any insecticidal efficacy at all, in contrast to the parent compound BYI 02960.

CONCLUSION

Summarising the results of the two test systems, the following can be stated:
In all test systems it could be demonstrated in a very uniform manner that the metabolite difluoroacetic acid (BCS-AA56716) does not exhibit any substantial insecticidal efficacy in comparison to the parent compound BYI 02960.

IIA 8.14.2 Assessment of relevance to potential impact on non-target species

Risk assessments for all non-target species are performed in product specific Annex III dossiers.

IIA 8.15 Effects on biological methods for sewage treatment

Report:	**KIIA 8.15/01; Caspers, N. (2010)**
Title:	Activated Sludge, Respiration Inhibition Test with BYI 02960 (tech.)
Report No:	2010/0089/01
Document No:	M-377311-01-1
Guidelines:	**EC No. 440/2008 method C.11 (2008)** **OECD 209 (1984)**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The study was performed to assess the toxicity of BYI 02960 (tech.) to bacteria by measuring the respiration rate.

Activated sludge was exposed to BYI 02960 (Origin Batch No: 2009-000239; Batch code: BYI 02960-01-03; TOX 08508-00; purity 96.2% w/w) at nominal concentrations of 100, 180, 320, 560 and 1000 mg a.i./L, respectively. The respiration rate of each mixture was determined after aeration periods of 3 hours. A reference compound (3,5-Dichlorophenol) was tested at concentrations of 2.5, 5, 10, 20 and 40 mg/L, respectively.

After an incubation period of 3 hours, BYI 02960 (tech.) showed 23.3% respiration inhibition of activated sludge at the highest test item concentration of 1000 mg a.i./L.

Hence, the EC_{50} was higher than 1000 mg a.i./L. The EC_{10} was determined to be 472.5 mg a.i./L.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Specification no:	102000022313
Origin Batch number:	2009-000239 (Batch code: BYI 02960-01-03)
Sample description	TOX 08508-00
CAS name:	2(5H)-furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difluoroethyl)amino]-
CAS#:	951659-40-8
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Purity:	96.2% w/w
Stability of test compound:	Expiry date: 16.01.2011, when stored at +25 ± 5°C

2. Test organism and synthetic sewage feed

Type:	Mixed population of aquatic microorganisms (activated sludge)
Origin:	Aeration tank of a domestic sewage treatment plant (Municipal STP Cologne-Stammheim)
Date of collection:	14 June 2010
Storage:	Aeration of the activated sludge at 20°C ± 2°C, daily fed with synthetic medium
Synthetic sewage feed (per 1L 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 $7H_2O$ 2.8 g K_2HPO_4
pH:	7.5

B. Study design and methods

1. In life dates June 14 to 21, 2010

2. Design of biological test

10 mL of sludge suspension were dried in order to calculate the amount of sludge suspension to achieve a concentration of activated sludge of 2 g/L (dry weight) suspended solids. The calculated amount of sludge suspension was taken and filled up to a defined end volume with deionised water. The pH was measured and adjusted to pH 6-8.

8 mL of the synthetic medium and 100 mL of activated sludge were added to the dissolved test item. The mixture was filled up with deionised water to 250 mL and aerated at 20°C ± 2°C.

The exposure medium with the reference substance was prepared by adding 8 mL of the synthetic medium, 100 mL of activated sludge and a defined amount of the stock solution to achieve the test concentrations and was filled up to 250 mL with deionised water.

A control at test start and end (control 1, 2) was tested in the same way. The exposure medium to determine the physico-chemical oxygen consumption was prepared as well as the medium with the test item, but without activated sludge. To measure the oxygen consumption, 250 mL of sludge with test item (or control or reference compound) was incubated for 3 h in 300 mL closed Erlenmeyer flasks.

3. Observation and measurements

Oxygen consumption was measured and recorded after an aeration time of 3 hours. Thereafter, temperature and pH of the exposure medium were measured. 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).

The respiration rate for each concentration was determined graphically from the linear part of the curve of O_2-content versus time. The inhibitory effect of the test item at a particular concentration is expressed as a percentage of the mean of the respiration rates of two controls. An EC_{50} value was calculated from the respiration rates at different test item concentrations.

4. Statistical analysis

Respiration rates were analysed by probit analysis.

RESULTS AND DISCUSSION

A. Physical and Chemical Parameters

The pH was adjusted to 7±0.5 with HCl.

B. Biological Findings

After an incubation period of 3 hours, analysis of the respiration rates (by probit analysis) gave the following values:

Table: Effects of BYI 02960 (tech.) on respiration rate of activated sludge

Test item concentration (nominal) [mg/L]	Respiratory rate test item [mg/L x h]	Phys.-chem. O_2 consumption [mg/L x h]	Respiratory rate - phys - chem O_2 consumption [mg/L x h]	Inhibition [%]
100	26.6	0.0	26.6	0.0
180	26.0	0.0	26.0	0.0
320	25.7	0.0	25.7	0.0
560	23.3	0.0	23.3	8.6
1000	19.5	0.0*	19.5	23.3
Control mean	25.4	-	-	-
Control 1	24.9	-	-	-
Control 2	26.0	-	-	-

Comments: Test concentrations are given as nominal concentrations and were not confirmed by analytical methods.

* The physico-chemical oxygen consumption has been determined at 1000 mg/L test item concentration. As no physico-chemical oxygen consumption was observed at that test item concentration this observation also holds true for the lower test item concentrations.

Table: Selected effective concentrations (ECx) of BYI 02960 (techn.) and their 95%- and 99%-confidence limits (according to Fieller`s theorem)

Parameter	EC10	EC20	EC50
Value [mg a.i./L]	472.5	1015.2	n.d.
lower 95%-cl	259.9	595.8	n.d.
upper 95%-cl	963.9	5945.3	n.d.
lower 99%-cl	150.3	227.8	n.d.
upper 99%-cl	1666.5	15545.8	n.d.

n.d.: not determined due to mathematical reasons or inappropriate data
Computation of variances and confidence limits was adjusted to metric data ([redacted]ensen & Nyholm 1984). Slope function after Litchfield and Wilcoxon: 5.688

C. Validity Criteria

All validity criteria of the test method were met:

− respiratory rates of the 2 controls differed less than 15 % from each other

− the EC_{50} of the reference compound 3,5-Dichlorophenol was in the range 5 – 30 mg/L

D. Test with toxic reference substance

A reference test with 3,5-Dichlorophenol showed an EC_{50} of 8.7 mg/L (95% CI: 5.4 – 13.9 mg/L).

E. Biological Endpoints Derived

From the results presented above the following biological endpoints can be derived:

EC_{50}: **>1000 mg a.i./L**
EC_{10}: **472.5 mg/L** (95% CI: 259.9 – 963.9 mg/L)

CONCLUSION

BYI 02960 (tech.) showed 23.3% respiration inhibition of activated sludge at the highest test item concentration of 1000 mg/L. Thus, the EC_{50} is > 1000 mg a.i./L.

IIA 8.16 Other/special studies

IIA 8.16.1 Other/special studies - laboratory studies

Effects on honey bees

In this section all studies in honey bees, which do not have specific dossier point headings, are summarized, this includes 10 day feeding studies on BYI 02960 and plant and environmental metabolites as well as a larvae feeding study.

Report:	**KIIA 8.16.1/01; Kling, A. (2010)**
Title:	BYI02960 – Assessment of Chronic Effects to the Honey Bee, *Apis mellifera* L., in a 10 Days Laboratory Feeding Test
Report No:	S10-02924
Document No:	M-400539-01-2
Guidelines:	**No specific guideline available**
Deviations:	**Not applicable**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The chronic effects of the test item BYI 02960 (Batch code: BYI 02960-01-03; TOX 08508-00; Purity 96.2% w/w) on the honey bee, *Apis mellifera* L., in a 10 days continuous feeding test in the laboratory were assessed.

Over a period of 10 days, honey bees were exposed to 50% (w/v) sucrose solution, containing nominally 100, 300, 1000, 3000 and 10000 µg a.i./L of the test item BYI 02960 by continuous and *ad libitum* feeding. All test item feeding solutions contained additionally 1% acetone. The control group was exposed for the same period of time under identical exposure conditions to an untreated 50% (w/v) sucrose feeding solution, also containing 1% acetone. Mortality, sublethal effects and behavioural observations were assessed every day throughout the 10 days exposure period.

Samples and retain samples of all feeding solutions and the stock solution were taken for analysis.

The accumulated nominal intake of the test item BYI 02960 via BYI 02960-treated sucrose solution was 0.04, 0.13, 0.48, 1.49 and 4.64 µg a.i./bee, respectively, after 10 days of continuous exposure.

It can be concluded that the continuous feeding of honey bees in the laboratory over a period of 10 consecutive days with the test item BYI 02960 at treatment levels of 100, 300, 1000, 3000 and 10000 µg a.i./L caused no adverse effect regarding mortality, sublethal effects and behaviour. Since at all other dose levels, higher and lower than 300 µg a.i./L, the food consumption was not statistically significantly different when compared to the food consumption of the control group, the statistically significantly reduced food consumption at the second highest dose level of 300 µg a.i./L should not be overestimated.

The NOEC (No Observed Effect Concentration) was determined to be 10000 µg a.i./L at the end of the test period.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description	Beige powder
Specification No.:	102000022313
Origin. Batch No.:	2009-000239 (Batch code: BYI 02960-01-03)
Sample description:	TOX 08508-00 (Test item code: 2010-01178)
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
Purity:	96.2% w/w (analytical)
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Stability:	Expiry date: 16.01.2011, when stored at +25 ± 5°C
Treatment	50 % (w/v) sucrose solution, containing the test item BYI 02960 at the nominal concentration levels of 100, 300, 1000, 3000 and 10000 µg a.i./L as well as 1% acetone
Control	50% (w/v) aqueous sucrose solution containing 1% acetone

2. Test organisms

Species:	*Apis mellifera* L.
Common name:	Honey bee
Age or developmental stage at test start:	Adult worker bees
Source:	Bred by Eurofins

B. Study design and methods

1. In life dates July 21 to 31, 2010

2. Experimental treatments

A study was conducted to determine the chronic effects of the test item BYI 02960 on the honey bee, *Apis mellifera* L., in a 10 days continuous feeding test in the laboratory.

The honey bees were continuously and *ad libitum* fed with a 50% (w/v) sucrose solution, containing the test item BYI 02960 at the nominal concentration levels of 100, 300, 1000, 3000 and 10000 µg a.i./L as well as 1% acetone, respectively. In the control group the honey bees received an untreated 50% (w/v) aqueous sucrose solution containing 1% acetone, *ad libitum*.

The feeding solutions were offered ad libitum to each cage of 10 bees in plastic syringes (Omnifix®, 5 mL, B. Braun, Melsungen, Germany). The tip of each syringe was removed so that the bees had access to the feeding solution. Every morning the syringes of all test cages (i.e. test item and control) were replaced by new syringes, filled with freshly prepared feeding solution. The weight of the syringes was determined before and after feeding on the next day in order to determine the mean food consumption of the bees per test cage.

During the entire test period, the bees were kept in cages made of stainless steel (base: 8.2 cm x 4 cm; height: 6 cm). The front side of the cages were equipped with a transparent pane so that the bees could be observed. The bottom of the cages consisted of a perforated board, which guaranteed sufficient air supply for the test animals. The test cages are lined with filter paper.

The feeding solutions were offered to the bees in syringes fitted into the test cages.

The study was carried out with the following treatments:

- five doses of the test item BYI 02960
- the control

At each test item treatment level, 10 replicates of 10 honey bees each were tested (i.e. in total 100 honey bees per test item concentration). The control group comprised 30 replicates of 10 honey bees each (i.e. in total 300 honey bees). A toxic standard was not included in this test, since a toxic reference substance had not been defined or validated for this type of study.

3. Observation and measurements

Mortality:

The number of dead bees in the individual test cages was recorded every day at about the same time of day during the 10 days test period.

Abnormal behavioural effects and behavioural differences were assessed according to the following categories and recorded in the raw data:

a = affected (bees still upright and attempting to walk but showing signs of reduced coordination)

ap = apathy (bees show only low or delayed reactions to stimulation [i.e. light, air blow] bees are either sitting motionless in the cage or walk but not correctly)

m = moribund (bees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation, bees may recover but usually die)

The mortality [%] in the test item treatment was calculated from the number of dead bees and the total number of introduced bees in the test item treatment group. The mortality of the test item treatment was corrected for corresponding control mortality, according to the formula of ABBOTT (1925), modified by SCHNEIDER-ORELLI (1947):

Corrected mortality $$M = \frac{t - c}{100 - c} \times 100$$

M = Corrected mortality (%)
t = Mortality in the test item group (%)
c = Mortality in the control group (%)

Food consumption:

The consumption of feeding solution per bee/day was calculated by dividing the total daily consumption per cage by the mean number of living bees at the corresponding time interval. The mean value per treatment group as well as the daily mean food consumption per bee was calculated.

The mean intake of test item per bee was calculated as follows:

$$\text{Mean intake of test item [µg a.i./bee]} = \frac{\text{Mean consumption of feeding solution [mg/bee]}}{\text{Density of the sucrose solution [g/cm}^3\text{]}} \text{ X } \text{Concentration of feeding solution [µg a.i./µL]}$$

Statistical evaluations:

Fisher's Exact Test (Bonferroni-Holms corrected, one-sided, $p \leq 0.05$) was used to evaluate whether there are significant differences between the mortality data of the test item treatment group and the control group.

For the statistical comparison of the food consumption, non-rounded values were taken. Data of food consumption were tested for normality using Shapiro-Wilks' test. If normality was observed, the t-Test (one-sided, $p \leq 0.05$) was applied. If the data deviated from the normal distribution, statistical analysis was performed by using the Mann Whitney – U test. Statistical calculations were made by using the statistical program SAS release Version 9.2.

Residue analysis:

The residue analysis of the feeding solutions was performed in an independent study by Bayer CropScience AG, Monheim, the analytical attached as an integral part of the study report.

Storage and shipment of samples:

Samples taken of the feeding solutions and the stock solution were stored deep frozen (≤ –18°C) in the Eurofins Agroscience Services EcoChem GmbH laboratory within 30 minutes. The samples were shipped on dry ice from the Eurofins Agroscience Services EcoChem GmbH - laboratory directly to the residue analysis laboratory of the sponsor (Bayer CropScience AG).

RESULTS AND DISCUSSION

A. Environmental Parameters

Measurements of climatic parameters during the test are summarized as follows:

Test temperature:	24 - 25°C
Relative air humidity:	60 - 68 %
Light intensity:	Darkness except during assessments

B. Verification of the Application Volume

The residue analysis of the feeding solutions was performed in an independent study (analytical report is part of the study report, see Appendix 3).

C. Biological Findings

The results of the 10 days feeding test with a control group fed with untreated sucrose solution containing 1% acetone and the test item treatments with 100, 300, 1000, 3000 and 10000 µg a.i. /L of BYI 02960, first dissolved in acetone and diluted in 50% sucrose solution are presented in the table below.

Table Mean consumption of feeding solution, mean intake of test item accumulated over all test days and cumulative mortality at the final assessment on day 10

Treatment Level [1]	Control	Test Item				
		100	300	1000	3000	10000
		[µg a.i./L]				
Mean consumption of feeding solution [mg/bee] [2]	57.4	52.4	50.8**	56.4	58.8	54.9
Mean intake accumulated over test days [µg a.i./bee]	-	0.04	0.13	0.48	1.49	4.64
Cumulative mortality [%]	18.0	9.0	14.0	11.0	24.0	10.0*
Corrected cumulative mortality [%]	-	-11.0	-4.9	-8.5	7.3	-9.8

[1] The control group was fed with untreated 50% (w/v) sucrose solution mixed with 1 % acetone; all test item treatment groups were fed with BYI 02960-treated 50% (w/v) sucrose solution mixed with 1% acetone

[2] The mean values per cage over the test period were used as basis for the calculation of the mean consumption of feeding solution per treatment over the test period

* Determined to be the NOEC (Fisher's Exact Test (Bonferroni-Holms corrected, one-sided, $p \leq 0.05$))

** Significantly reduced compared to the control group (t-test with Bonferroni-Holms correction, one-sided, $p \leq 0.05$)

D. Validity Criteria

Not applicable. No guideline is available for this type of study.

E. Biological Endpoints Derived

After 10 days of continuous exposure, mortality at all test item treatment levels was not significantly increased compared to the control group.

After 10 days of exposure, the cumulative control mortality accounted for 18.0%, as determined at the final assessment.

Furthermore, on all test item treatment levels, no remarkable sublethal effects or behavioural abnormalities were observed throughout the entire test period. The highest test item treatment level of 10000 µg a.i./L was determined to be the NOEC (No Observed Effect Concentration; Fisher's Exact Test; Bonferroni-Holms corrected, one-sided, $p \leq 0.05$).

At the 300 µg a.i./L test item treatment level, the mean food consumption per bee was slightly, although statistically significantly reduced when compared to the food consumption of the control group (t-Test with Bonferroni-Holms correction, one-sided, $p \leq 0.05$). However, at all other dose levels, higher and lower than 300 µg a.i./L (i.e. 100, 1000, 3000 and 10000 µg a.i./L), the food consumption was not statistically significantly different when compared to the food consumption of the control group.

The accumulated nominal intake of the test item BYI 02960 via BYI 02960-treated sucrose solution was 0.04, 0.13, 0.48, 1.49 and 4.64 µg a.i./bee after 10 days of continuous exposure.

From the results presented above the following biological endpoints can be derived:

NOEC 10000 µg a.i./L

CONCLUSION

It can be concluded that the continuous feeding of honey bees in the laboratory over a period of 10 consecutive days with the test item BYI 02960 at treatment levels of 100, 300, 1000, 3000 and 10000 µg a.i./L caused no adverse effect regarding mortality, sublethal effects and behaviour. Since at all other dose levels, higher and lower than 300 µg a.i./L, the food consumption was not statistically significantly different when compared to the food consumption of the control group, the statistically significantly reduced food consumption at the second highest dose level of 300 µg a.i./L should not be overestimated.

The NOEC (No Observed Effect Concentration) was determined to be 10000 µg a.i./L at the end of the test period.

Report:	**KIIA 8.16.1/02; Kling, A. (2012)**
Title:	BYI 02960-difluoroethyl-amino-furanone (BYI 02960-DFEAF) – Assessment of Chronic Effects to the Honey Bee, *Apis mellifera* L., in a 10 Days Continuous Laboratory Feeding Limit Test
Report No:	S11-01959
Document No:	M-425174-01-2
Guidelines:	**No specific guideline available**
Deviations:	**Not applicable**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The chronic effects of the test item BYI 02960-DFEAF (Origin Batch No: NLL 8671-12-1; Batch code: BCS-CC98193-01-03, TOX 09255-00; Purity 98.9% w/w) on the honey bee, *Apis mellifera* L., in a 10 days continuous feeding limit test in the laboratory were assessed.

Over a period of 10 days, honey bees were exposed to 50% (w/v) aqueous sucrose feeding solution, containing nominally 10000 µg a.i./L of the test item BYI 02960-DFEAF by continuous and *ad libitum* feeding. Because the test item was first dissolved in acetone and then diluted with aqueous sucrose solution, the final test item feeding solution contained 1% acetone. The control group was exposed for the same period of time under identical exposure conditions to untreated 50% (w/v) aqueous sucrose feeding solution, also containing 1% acetone. Mortality, sublethal effects and behavioural observations were assessed every day throughout the 10 days exposure period.

The accumulated nominal intake of the test item BYI 02960-DFEAF via BYI 02960-DFEAF - treated aqueous sucrose feeding solution was 4.35 µg a.i./bee after 10 days of continuous exposure.

Samples and retain samples of all feeding solutions and the stock solution were taken for chemical analysis.

It can be concluded that the continuous feeding of honey bees in the laboratory over a period of 10 consecutive days with the test item BYI 02960-DFEAF at the treatment level of 10000 µg a.i./L caused no adverse effect regarding mortality, sublethal effects and behaviour. As the overall mean daily food uptake (i.e. the average value over 10 days) in the test item treatment group was virtually identical to the untreated control group and because only on one single day during the 10 day continuous exposure period the mean food consumption per bee was significantly lower in the test item treatment group compared to the control group, it can be concluded that there was no repellent effect of the test item at the treatment level of 10000 µg a.i./L.

The NOEC (No Observed Effect Concentration) was determined to be 10000 µg a.i./L at the end of the test period.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960-difluoroethyl-amino-furanone (BYI 02960-DFEAF; BCS-CC98193)
Type of test material:	Pure metabolite
Chemical state and description	Powder, white
Origin Batch No.:	NLL 8671-12-1 (Batch code: BCS-CC98193-01-03)
Sample description:	TOX 09255-00 (Test item code: 2011-001522)
CAS#:	1134834-71-1
Purity:	98.9% w/w
Stability of test compound:	Expiry date: 16.08.2011, when stored at +10 to +30°C
Treatment	50% (w/v) aqueous sucrose feeding solution, containing nominally 10000 µg a.i./L of the test item BYI 02960-DFEAF as well as 1% acetone
Control	50% (w/v) aqueous sucrose feeding solution containing 1% acetone

2. Test organisms

Species:	*Apis mellifera* L.
Common name:	Honey bee
Age or developmental stage at test start:	Young adult worker bees
Source:	Bred by Eurofins

B. Study design and methods

1. In life dates June 7 to 17, 2011

2. Experimental treatments

A study was conducted to determine the chronic effects of the test item BYI 02960-DFEAF on the honey bee, *Apis mellifera* L., in a 10 days continuous feeding limit test in the laboratory. The NOEC value (No Observed Effect Concentration) for mortality was determined at the end of the test period.

The honey bees were fed continuously and *ad libitum* with a 50% (w/v) aqueous sucrose solution, containing the test item BYI 02960-DFEAF at the nominal concentration level of 10000 µg a.i./L as well as 1% acetone. For the preparation of the test item treatment level, the active substance content of the test item BCS-CC98193 (BYI 02960-DFEAF) was considered to be 100% and not corrected for the analysed content of 98.9% (w/w).

In the control group the honey bees received an untreated 50% (w/v) aqueous sucrose solution containing 1% acetone, *ad libitum*.

The feeding solutions were offered *ad libitum* to each cage of 10 bees in plastic syringes (Omnifix®, 5 mL, B. Braun, Melsungen, Germany). The tip of each syringe was removed so that the bees had access to the feeding solution. Every morning the syringes of all test cages (i.e. test item and control) were replaced by new syringes, filled with freshly prepared feeding solution. The weight of the syringes was determined before and after feeding on the next day in order to determine the mean food consumption of the bees per test cage.

During the entire test period, the bees were kept in cages made of stainless steel (base: 8.2 cm x 4 cm; height: 6 cm). The front side of the cages were equipped with a transparent pane so that the bees could be observed. The bottom of the cages consisted of a perforated board, which guaranteed sufficient air supply for the test animals. The test cages are lined with filter paper.

The feeding solutions were offered to the bees in syringes fitted into the test cages.

The study was carried out with the following treatments:

- one concentration of the test item BYI 02960-DFEAF (difluoroethyl-amino-furanone)
- the control

The test item treatment was tested with 10 replicates (cages), each containing 10 bees (i.e. in total 100 honey bees). The control group comprised 30 replicates of 10 bees each (i.e. in total 300 honey bees).

A toxic standard was not included in this test, since a toxic reference substance had not been defined or validated for this type of study.

3. Observation and measurements

Mortality:

The number of dead bees in the individual test cages was recorded every day at about the same time of day during the 10 days test period.

Abnormal behavioural effects and behavioural differences were assessed according to the following categories and recorded in the raw data:

a = affected (bees still upright and attempting to walk but showing signs of reduced coordination)

ap = apathy (bees show only low or delayed reactions to stimulation [i.e. light, air blow] bees are either sitting motionless in the cage or walk but not correctly)

m = moribund (bees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation, bees may recover but usually die)

The mortality [%] in the test item treatment was calculated from the number of dead bees and the total number of introduced bees in the test item treatment group. The mortality of the test item treatment was corrected for corresponding control mortality, according to the formula of ABBOTT (1925), modified by SCHNEIDER-ORELLI (1947):

$$\text{Corrected mortality} \quad M = \frac{t - c}{100 - c} \times 100$$

M = Corrected mortality (%)
t = Mortality in the test item group (%)
c = Mortality in the control group (%)

Food consumption:

The consumption of feeding solution per bee/day was calculated by dividing the total daily consumption per cage by the mean number of living bees at the corresponding time interval. The mean value per treatment group as well as the daily mean food consumption per bee was calculated.

The mean intake of test item per bee was calculated as follows:

$$\text{Mean intake of test item [µg a.i./bee]} = \frac{\text{Mean consumption of feeding solution [mg/bee]}}{\text{Density of the sucrose solution [g/cm}^3\text{]}} \times \text{Concentration of feeding solution [µg a.i./µL]}$$

Statistical evaluations:

Fisher's Exact Test (Bonferroni-Holms corrected, one-sided, $p \leq 0.05$) was used to evaluate whether there are significant differences between the mortality data of the test item treatment group and the control group.

For the statistical comparison of the food consumption, non-rounded values were taken. Data of food consumption were tested for normality using Shapiro-Wilks' test. If normality was observed, the t-Test (one-sided, $p \leq 0.05$) was applied. If the data deviated from the normal distribution, statistical analysis was performed by using the Mann Whitney – U test. Statistical calculations were made by using the statistical program SAS release Version 9.2.

Residue analysis:

The residue analysis of the feeding solutions was performed in an independent study by Bayer CropScience AG, Monheim, the analytical attached as an integral part of the study report.

Storage and shipment of samples:

Samples taken of the feeding solutions and the stock solution were stored deep frozen (≤ –18°C) in the Eurofins Agroscience Services EcoChem GmbH laboratory within 30 minutes. The samples were shipped on dry ice from the Eurofins Agroscience Services EcoChem GmbH - laboratory directly to the residue analysis laboratory of the sponsor (Bayer CropScience AG).

RESULTS AND DISCUSSION

A. Environmental Parameters

Measurements of climatic parameters during the test are summarized as follows:

Test temperature:	25.1 – 25.8°C
Relative air humidity:	51.2 – 69.4 %
Light intensity:	Constant darkness except during observations

B. Verification of the Application Volume

The residue analysis of the feeding solutions was performed in an independent study (analytical report is part of the study report).

C. Biological Findings

The results of the 10 days feeding test with a control group fed with untreated sucrose solution containing 1% acetone and the test item treatment of 10000 µg a.i. /L of BYI 02960-DFEAF, first dissolved in acetone and diluted in 50% sucrose solution are presented in the table below.

Table **Mean consumption of feeding solution, mean intake of test item accumulated over all test days and cumulative mortality at the final assessment on day 10**

Treatment Level [1]	Control	Test Item
		10000 µg a.i./L
Overall mean daily consumption of aqueous sucrose feeding solution [mg/bee] [2]	51.4	51.5
Mean intake accumulated over test days [µg a.i./bee]	-	4.35
Cumulative mortality [%]	3.67	0.00*
Corrected cumulative mortality [%]	-	-3.81

[1] The control group was fed with untreated 50% (w/v) aqueous sucrose feeding solution mixed with 1% acetone; the test item treatment group was fed with 50% (w/v) aqueous sucrose feeding solution containing BYI 02960-DFEAF and 1% acetone
[2] The mean values per cage over the test period were used as basis for the calculation of the overall mean daily consumption of the aqueous sucrose feeding solution per treatment over the test period
* Determined to be the NOEC (not significantly different compared to the control, Fisher's Exact Test; Bonferroni-Holms corrected, one-sided, $p \leq 0.05$)

D. Validity Criteria

Not applicable. No guideline is available for this type of study.

E. Biological Endpoints Derived

After 10 days of continuous exposure, mortality in the test item treatment group was not significantly different compared to the control group. The cumulative control mortality accounted to 3.67%, as determined at the final assessment (day 10). In the test item treatment group at 10000 µg a.i./L, the cumulative mortality at the final assessment (day 10) accounted to 0.00% (corrected -3.81 %).

Furthermore, neither sublethal effects nor behavioural abnormalities were observed throughout the entire testing period in the test item treatment group. The test item treatment level of 10000 µg a.i./L was determined to be the NOEC (No Observed Effect Concentration, Fisher's Exact Test; (Bonferroni-Holms corrected, one-sided, $p \leq 0.05$).

The overall mean daily consumption of the aqueous sucrose feeding solution (i.e. the average value over 10 days) in the test item treatment group was virtually identical to the untreated control group (51.5 mg/bee in the test item treatment compared to 51.4 mg/bee in the control group).

The mean daily consumption of the aqueous sucrose feeding solution was statistically significantly reduced in the test item treatment group (42 mg/bee) compared to the control group (49 mg/bee) only on day 9, (t-test; one-sided, $p \leq 0.05$). On all other test days, the mean daily consumption of the aqueous sucrose feeding solution was almost identical in the test item treatment group compared to the control group (day-by-day comparison).

The accumulated nominal intake of the test item BYI 02960-DFEAF via BYI 02960-DFEAF - treated aqueous sucrose feeding solution was 4.35 µg a.i./bee after 10 days of continuous exposure.

From the results presented above the following biological endpoints can be derived:

NOEC 10000 µg a.i./L

CONCLUSION

It can be concluded that the continuous feeding of honey bees in the laboratory over a period of 10 consecutive days with the test item BYI 02960-DFEAF at the treatment level of 10000 µg a.i./L caused

no adverse effect regarding mortality, sublethal effects and behaviour. As the overall mean daily food uptake (i.e. the average value over 10 days) in the test item treatment group was virtually identical to the untreated control group and because only on one single day during the 10 day continuous exposure period the mean food consumption per bee was significantly lower in the test item treatment group compared to the control group, it can be concluded that there was no repellent effect of the test item at the treatment level of 10000 µg a.i./L.

The NOEC (No Observed Effect Concentration) was determined to be 10000 µg a.i./L at the end of the test period.

Report:	**KIIA 8.16.1/03; Kling, A. (2012)**
Title:	BYI 02960-hydroxy – Assessment of Chronic Effects to the Honey Bee, *Apis mellifera* L., in a 10 Days Continuous Laboratory Feeding Limit Test
Report No:	S11-01960
Document No:	M-425212-01-2
Guidelines:	**No specific guideline available**
Deviations:	**Not applicable**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The chronic effects of the test item BYI 02960-hydroxy (Origin Batch No: SES 11215-7-10; Batch code: BCS-CQ74364-PU-01; Purity 95.5% w/w) on the honey bee, *Apis mellifera* L., in a 10 days continuous feeding limit test in the laboratory were assessed.

Over a period of 10 days, honey bees were exposed to 50% (w/v) aqueous sucrose feeding solution, containing nominally 10000 µg a.i./L of the test item BYI 02960-hydroxy by continuous and *ad libitum* feeding. Because the test item was first dissolved in acetone and then diluted with aqueous sucrose solution, the final test item feeding solution contained 1% acetone. The control group was exposed for the same period of time under identical exposure conditions to untreated 50% (w/v) aqueous sucrose feeding solution, also containing 1% acetone. Mortality, sublethal effects and behavioural observations were assessed every day throughout the 10 days exposure period.

Samples and retain samples of all feeding solutions and the stock solution were taken for chemical analysis.

The accumulated nominal intake of the test item BYI 02960-hydroxy via BYI 02960-hydroxy - treated aqueous sucrose feeding solution was 4.20 µg a.i./bee after 10 days of continuous exposure.

It can be concluded that the continuous feeding of honey bees in the laboratory over a period of 10 consecutive days with the test item BYI 02960-hydroxy at the treatment level of 10000 µg a.i./L caused no adverse effect regarding mortality, sublethal effects and behaviour. As the overall mean daily food uptake (i.e. the average value over 10 days) in the test item treatment group was almost identical to the untreated control group and because on every single day during the 10 day continuous exposure period the mean food consumption per bee was not significantly lower in the test item treatment group compared to the control group, it can be concluded that there was no repellent effect of the test item at the treatment level of 10000 µg a.i./L.

The NOEC (No Observed Effect Concentration) was determined to be 10000 µg a.i./L at the end of the test period.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960-hydroxy (BCS-CQ74364)
Type of test material:	Pure metabolite
Chemical state and description	Solid, brown
Origin Batch No.:	SES 11215-7-10 (Batch code: BCS-CQ74364-PU-01)
Sample description:	Not available (Test item code: 2011-001604)
CAS#:	Not available
Purity:	95.5% w/w
Stability of test compound:	Expiry date: 15.12.2012, when stored at +5 ± 5°C
Treatment	50% (w/v) aqueous sucrose feeding solution, containing nominally 10000 µg a.i./L of the test item BYI 02960-hydroxy as well as 1% acetone
Control	50% (w/v) aqueous sucrose feeding solution containing 1% acetone

2. Test organisms

Species:	*Apis mellifera* L.
Common name:	Honey bee
Age or developmental stage at test start:	Young adult worker bees
Source:	Bred by Eurofins

B. Study design and methods

1. In life dates June 7 to 17, 2011

2. Experimental treatments

A study was conducted to determine the chronic effects of the test item BYI 02960-hydroxy on the honey bee, *Apis mellifera* L., in a 10 days continuous feeding limit test in the laboratory. The NOEC value (No Observed Effect Concentration) for mortality was determined at the end of the test period.

The honey bees were fed continuously and *ad libitum* with a 50% (w/v) aqueous sucrose solution, containing the test item BYI 02960-hydroxy at the nominal concentration level of 10000 µg a.i./L as well as 1% acetone. For the preparation of the test item treatment level, the active substance content of the test item BCS-CQ74364 (BYI 02960-hydroxy) was considered to be 100% and not corrected for the analysed content of 95.5% (w/w).

In the control group the honey bees received an untreated 50% (w/v) aqueous sucrose solution containing 1% acetone, *ad libitum*.

The feeding solutions were offered *ad libitum* to each cage of 10 bees in plastic syringes (Omnifix®, 5 mL, B. Braun, Melsungen, Germany). The tip of each syringe was removed so that the bees had access to the feeding solution. Every morning the syringes of all test cages (i.e. test item and control) were replaced by new syringes, filled with freshly prepared feeding solution. The weight of the syringes was determined before and after feeding on the next day in order to determine the mean food consumption of the bees per test cage.

During the entire test period, the bees were kept in cages made of stainless steel (base: 8.2 cm x 4 cm; height: 6 cm). The front side of the cages were equipped with a transparent pane so that the bees could

be observed. The bottom of the cages consisted of a perforated board, which guaranteed sufficient air supply for the test animals. The test cages are lined with filter paper.

The feeding solutions were offered to the bees in syringes fitted into the test cages.

The study was carried out with the following treatments:

- one concentration of the test item BYI 02960-hydroxy
- the control

The test item treatment was tested with 10 replicates (cages), each containing 10 bees (i.e. in total 100 honey bees). The control group comprised 30 replicates of 10 bees each (i.e. in total 300 honey bees).

A toxic standard was not included in this test, since a toxic reference substance had not been defined or validated for this type of study.

3. Observation and measurements

Mortality:

The number of dead bees in the individual test cages was recorded every day at about the same time of day during the 10 days test period.

Abnormal behavioural effects and behavioural differences were assessed according to the following categories and recorded in the raw data:

a = affected (bees still upright and attempting to walk but showing signs of reduced coordination)

ap = apathy (bees show only low or delayed reactions to stimulation [i.e. light, air blow] bees are either sitting motionless in the cage or walk but not correctly)

m = moribund (bees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation, bees may recover but usually die)

The mortality [%] in the test item treatment was calculated from the number of dead bees and the total number of introduced bees in the test item treatment group. The mortality of the test item treatment was corrected for corresponding control mortality, according to the formula of ABBOTT (1925), modified by SCHNEIDER-ORELLI (1947):

$$\text{Corrected mortality} \qquad M = \frac{t - c}{100 - c} \times 100$$

M = Corrected mortality (%)
t = Mortality in the test item group (%)
c = Mortality in the control group (%)

Food consumption:

The consumption of feeding solution per bee/day was calculated by dividing the total daily consumption per cage by the mean number of living bees at the corresponding time interval. The mean value per treatment group as well as the daily mean food consumption per bee was calculated.

The mean intake of test item per bee was calculated as follows:

$$\text{Mean intake of test item [µg a.i./bee]} = \text{Mean consumption of feeding solution [mg/bee]} \times \text{Concentration of feeding solution [µg a.i./µL]}$$

Density of the sucrose solution
[g/cm^3]

Statistical evaluations:

Fisher's Exact Test (Bonferroni-Holms corrected, one-sided, $p \leq 0.05$) was used to evaluate whether there are significant differences between the mortality data of the test item treatment group and the control group.

For the statistical comparison of the food consumption, non-rounded values were taken. Data of food consumption were tested for normality using Shapiro-Wilks' test. If normality was observed, the t-Test (one-sided, $p \leq 0.05$) was applied. If the data deviated from the normal distribution, statistical analysis was performed by using the Mann Whitney – U test. Statistical calculations were made by using the statistical program SAS release Version 9.2.

Residue analysis:

The residue analysis of the feeding solutions was performed in an independent study by Bayer CropScience AG, Monheim, the analytical attached as an integral part of the study report.

Storage and shipment of samples:

Samples taken of the feeding solutions and the stock solution were stored deep frozen (≤ –18°C) in the Eurofins Agroscience Services EcoChem GmbH laboratory within 30 minutes. The samples were shipped on dry ice from the Eurofins Agroscience Services EcoChem GmbH - laboratory directly to the residue analysis laboratory of the sponsor (Bayer CropScience AG).

RESULTS AND DISCUSSION

A. Environmental Parameters

Measurements of climatic parameters during the test are summarized as follows:

Test temperature:	25.1 – 25.8°C
Relative air humidity:	51.2 – 69.4 %
Light intensity:	Constant darkness except during observations

B. Verification of the Application Volume

The residue analysis of the feeding solutions was performed in an independent study (analytical report is part of the study report, see Appendix A3).

C. Biological Findings

The results of the 10 days feeding test with a control group fed with untreated sucrose solution containing 1% acetone and the test item treatment of 10000 µg a.i. /L of BYI 02960-hydroxy, first dissolved in acetone and diluted in 50% sucrose solution are presented in the table below.

Table **Mean consumption of feeding solution, mean intake of test item accumulated over all test days and cumulative mortality at the final assessment on day 10**

Treatment Level [1]	Control	Test Item
		10000 µg a.i./L
Overall mean daily consumption of aqueous sucrose feeding solution [mg/bee] [2]	51.4	49.6
Mean intake accumulated over test days [µg a.i./bee]	-	4.20
Cumulative mortality [%]	3.67	3.03*
Corrected cumulative mortality [%]	-	-0.66

[1] The control group was fed with untreated 50% (w/v) aqueous sucrose solution mixed with 1% acetone; the test item treatment group was fed with 50% (w/v) aqueous sucrose solution containing BYI 02960-hydroxy and 1% acetone

[2] The mean values per cage over the test period were used as basis for the calculation of the overall mean daily consumption of the aqueous sucrose feeding solution per treatment over the test period

* Determined to be the NOEC (not significantly different compared to the control, Fisher's Exact Test; Bonferroni-Holms corrected, one-sided, $p \leq 0.05$)

D. Validity Criteria

Not applicable. No guideline is available for this type of study.

E. Biological Endpoints Derived

After 10 days of continuous exposure, mortality in the test item treatment group was not significantly different compared to the control group. The cumulative control mortality accounted to 3.67%, as determined at the final assessment (day 10). In the test item treatment group at 10000 µg a.i./L, the cumulative mortality at the final assessment (day 10) accounted to 3.03% (corrected -0.66 %).

Furthermore, neither sublethal effects nor behavioural abnormalities were observed throughout the entire testing period in the test item treatment group. The test item treatment level of 10000 µg a.i./L was determined to be the NOEC (No Observed Effect Concentration, Fisher's Exact Test (Bonferroni-Holms corrected, one-sided, $p \leq 0.05$)).

The overall mean daily consumption of the aqueous sucrose feeding solution (i.e. the average value over 10 days) in the test item treatment group was almost identical to the untreated control group (49.6 mg/bee in the test item treatment compared to 51.4 mg/bee in the control group).

The mean daily consumption of the aqueous sucrose feeding solution was not significantly different between the control group and the test item treatment group throughout the entire testing period (day-by-day comparison).

The accumulated nominal intake of the test item BYI 02960-hydroxy via BYI 02960-hydroxy - treated aqueous sucrose feeding solution was 4.20 µg a.i./bee after 10 days of continuous exposure.

From the results presented above the following biological endpoints can be derived:

NOEC 10000 µg a.i./L

CONCLUSION

It can be concluded that the continuous feeding of honey bees in the laboratory over a period of 10 consecutive days with the test item BYI 02960-hydroxy at the treatment level of 10000 µg a.i./L caused no adverse effect regarding mortality, sublethal effects and behaviour. As the overall mean daily food uptake (i.e. the average value over 10 days) in the test item treatment group was almost identical to the untreated control group and because on every single day during the 10 day continuous exposure period the mean food consumption per bee was not significantly lower in the test item treatment group

compared to the control group, it can be concluded that there was no repellent effect of the test item at the treatment level of 10000 µg a.i./L.

The NOEC (No Observed Effect Concentration) was determined to be 10000 µg a.i./L at the end of the test period.

Report:	**KIIA 8.16.1/04; Kling, A. (2012)**
Title:	Difluoroacetic acid – Assessment of Chronic Effects to the Honey Bee, *Apis mellifera* L., in a 10 Days Continuous Laboratory Feeding Limit Test
Report No:	S11-01939
Document No:	M-425105-01-1
Guidelines:	**No specific guideline available**
Deviations:	**Not applicable**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The chronic effects of the test item difluoroacetic acid (Origin Batch No.: BCOO 5984-1-1; Batch code: BCS-AA56716-01-01; TOX 09400-00; Purity 95.8% w/w) on the honey bee, *Apis mellifera* L., in a 10 days continuous feeding limit test in the laboratory were assessed.

Over a period of 10 days, honey bees were exposed to 50% (w/v) aqueous sucrose feeding solution, containing nominally 10000 µg a.i./L of the test item difluoroacetic acid by continuous and *ad libitum* feeding. Because the test item was first dissolved in acetone and then diluted with aqueous sucrose solution, the final test item feeding solution contained 1% acetone. The control group was exposed for the same period of time under identical exposure conditions to untreated 50% (w/v) aqueous sucrose feeding solution, also containing 1% acetone. Mortality, sublethal effects and behavioural observations were assessed every day throughout the 10 days exposure period.

Samples and retain samples of all feeding solutions and the stock solution were taken for chemical analysis.

The accumulated nominal intake of the test item difluoroacetic acid via difluoroacetic acid - treated aqueous sucrose feeding solution was 3.79 µg a.i./bee after 10 days of continuous exposure.

It can be concluded that the continuous feeding of honey bees in the laboratory over a period of 10 consecutive days with the test item difluoroacetic acid at the treatment level of 10000 µg a.i./L caused no adverse effect regarding mortality, sublethal effects and behaviour. As the overall mean daily food uptake (i.e. the average value over 10 days) in the test item treatment group was almost identical to the untreated control group and because on every single day during the 10 day continuous exposure period the mean food consumption per bee was not significantly lower in the test item treatment group compared to the control group, it can be concluded that there was no repellent effect of the test item at the treatment level of 10000 µg a.i./L.

The NOEC (No Observed Effect Concentration) was determined to be 10000 µg a.i./L at the end of the test period

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	Difluoroacetic acid (BCS-AA56716)
Type of test material:	Pure metabolite (technical substance)
Chemical state and description	Liquid, light yellow
Origin Batch No.:	BCOO 5984-1-1 (Batch code:BCS-AA56716-01-01)
Sample No.:	TOX 09400-00 (Test item code: 2011-001527)
CAS#:	381-73-7
Purity:	95.8% w/w
Stability of test compound:	Expiry date: 19.10.2011, when stored at +10 to +30°C
Treatment	50% (w/v) aqueous sucrose feeding solution, containing nominally 10000 µg a.i./L of the test item difluoroacetic acid as well as 1% acetone
Control	50% (w/v) aqueous sucrose feeding solution containing 1% acetone

2. Test organisms

Species:	*Apis mellifera* L.
Common name:	Honey bee
Age or developmental stage at test start:	Young adult worker bees
Source:	Not stated

B. Study design and methods

1. In life dates 24 May to 03 June 2011

2. Experimental treatments

A study was conducted to determine the chronic effects of the test item difluoroacetic acid on the honey bee, *Apis mellifera* L., in a 10 days continuous feeding limit test in the laboratory. The NOEC value (No Observed Effect Concentration) for mortality was determined at the end of the test period.

The honey bees were fed continuously and *ad libitum* with a 50% (w/v) aqueous sucrose solution, containing the test item difluoroacetic acid at the nominal concentration level of 10000 µg a.i./L as well as 1% acetone. For the preparation of the test item treatment level, the active substance content of the test item BCS-AA56716 (difluoroacetic acid) was considered to be 100% and not corrected for the analysed content of 95.8% (w/w).

In the control group the honey bees received an untreated 50% (w/v) aqueous sucrose solution containing 1% acetone, *ad libitum*.

During the entire test period, the bees were kept in cages made of stainless steel (base: 8.2 cm x 4 cm; height: 6 cm). The front side of the cages were equipped with a transparent pane so that the bees could be observed. The bottom of the cages consisted of a perforated board, which guaranteed sufficient air supply for the test animals. The test cages are lined with filter paper.

The feeding solutions were offered to the bees in syringes fitted into the test cages.

The study was carried out with the following treatments:

- one concentration of the test item difluoroacetic acid
- the control

The test item treatment was tested with 10 replicates (cages), each containing 10 bees (i.e. in total 100 honey bees). The control group comprised 30 replicates of 10 bees each (i.e. in total 300 honey bees).

A toxic standard was not included in this test, since a toxic reference substance had not been defined or validated for this type of study.

3. Observation and measurements

Mortality:

The number of dead bees in the individual test cages was recorded every day at about the same time of day during the 10 days test period.

Abnormal behavioural effects and behavioural differences were assessed according to the following categories and recorded in the raw data:

a = affected (bees still upright and attempting to walk but showing signs of reduced coordination)

ap = apathy (bees show only low or delayed reactions to stimulation [i.e. light, air blow] bees are either sitting motionless in the cage or walk but not correctly)

m = moribund (bees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation, bees may recover but usually die)

The mortality [%] in the test item treatment was calculated from the number of dead bees and the total number of introduced bees in the test item treatment group. The mortality of the test item treatment was corrected for corresponding control mortality, according to the formula of ABBOTT (1925), modified by SCHNEIDER-ORELLI (1947):

$$\text{Corrected mortality} \qquad M = \frac{t - c}{100 - c} \times 100$$

M = Corrected mortality (%)
t = Mortality in the test item group (%)
c = Mortality in the control group (%)

Food consumption:

The consumption of feeding solution per bee/day was calculated by dividing the total daily consumption per cage by the mean number of living bees at the corresponding time interval. The mean value per treatment group as well as the daily mean food consumption per bee was calculated.

The mean intake of test item per bee was calculated as follows:

$$\text{Mean intake of test item [µg a.i./bee]} = \frac{\text{Mean consumption of feeding solution [mg/bee]}}{\text{Density of the sucrose solution [g/cm}^3\text{]}} \text{ X } \text{Concentration of feeding solution [µg a.i./µL]}$$

Statistical evaluations:

Fisher's Exact Test (Bonferroni-Holms corrected, one-sided, $p \leq 0.05$) was used to evaluate whether there are significant differences between the mortality data of the test item treatment group and the control group.

For the statistical comparison of the food consumption, non-rounded values were taken. Data of food consumption were tested for normality using Shapiro-Wilks' test. If normality was observed, the t-Test (one-sided, $p \leq 0.05$) was applied. If the data deviated from the normal distribution, statistical analysis was performed by using the Mann Whitney – U test. Statistical calculations were made by using the statistical program SAS release Version 9.2.

Residue analysis:

The residue analysis of the feeding solutions was performed in an independent study by Bayer CropScience AG, Monheim, the analytical attached as an integral part of the study report.

Storage and shipment of samples:

Samples taken of the feeding solutions and the stock solution were stored deep frozen (≤ –18°C) in the Eurofins Agroscience Services EcoChem GmbH laboratory within 30 minutes. The samples were shipped on dry ice from the Eurofins Agroscience Services EcoChem GmbH - laboratory directly to the residue analysis laboratory of the sponsor (Bayer CropScience AG).

RESULTS AND DISCUSSION

A. Environmental Parameters

Measurements of climatic parameters during the test are summarized as follows:

Test temperature:	25.1 – 25.4°C
Relative air humidity:	64.0 – 68.8 %
Light intensity:	Constant darkness except during observations

B. Verification of the Application Volume

The residue analysis of the feeding solutions was performed in an independent study (analytical report is part of the study report, see Appendix A3).

C. Biological Findings

The results of the 10 days feeding test with a control group fed with untreated sucrose solution containing 1% acetone and the test item treatment of 10000 µg a.i. /L of difluoroacetic acid, first dissolved in acetone and diluted in 50% sucrose solution are presented in the table below.

Table **Mean consumption of feeding solution, mean intake of test item accumulated over all test days and cumulative mortality at the final assessment on day 10**

Treatment Level [1]	Control	Test Item
		10000 µg a.i./L
Overall mean daily consumption of aqueous sucrose feeding solution [mg/bee] [2]	46.2	45.0
Mean intake accumulated over test days [µg a.i./bee]	-	3.79
Cumulative mortality [%]	2.33	1.00*
Corrected cumulative mortality [%]	-	-1.37

[1] The control group was fed with untreated 50% (w/v) aqueous sucrose solution mixed with 1% acetone; the test item treatment group was fed with 50% (w/v) aqueous sucrose solution containing difluoroacetic acid and 1% acetone

[2] The mean values per cage over the test period were used as basis for the calculation of the overall mean daily consumption of the aqueous sucrose feeding solution per treatment over the test period

* Determined to be the NOEC (not significantly different compared to the control, Fisher's Exact Test; Bonferroni-Holms corrected, one-sided, $p \leq 0.05$)

D. Validity Criteria

Not applicable. No guideline is available for this type of study.

E. Biological Endpoints Derived

After 10 days of continuous exposure, mortality in the test item treatment group was not significantly different compared to the control group. The cumulative control mortality accounted to 2.33%, as determined at the final assessment (day 10). In the test item treatment level of 10000 µg a.i./L, the cumulative mortality at the final assessment (day 10) accounted to 1.00% (corrected -1.37%).

Furthermore, neither sublethal effects nor behavioural abnormalities were observed throughout the entire testing period in the test item treatment group. The test item treatment level of 10000 µg a.i./L was determined to be the NOEC (No Observed Effect Concentration, Fisher's Exact Test (Bonferroni-Holms corrected, one-sided, $p \leq 0.05$)).

The overall mean daily consumption of the aqueous sucrose feeding solution (i.e. the average value over 10 days) in the test item treatment group was almost identical to the untreated control group (45.0 mg/bee in the test item treatment compared to 46.2 mg/bee in the control group).

The mean daily consumption of the aqueous sucrose feeding solution was not significantly different between the control group and the test item treatment group throughout the entire testing period (day-by-day comparison).

The accumulated nominal intake of the test item difluoroacetic acid via difluoroacetic acid - treated aqueous sucrose feeding solution was 3.79 µg a.i./bee after 10 days of continuous exposure.

From the results presented above the following biological endpoints can be derived:

NOEC 10000 µg a.i./L

CONCLUSION

It can be concluded that the continuous feeding of honey bees in the laboratory over a period of 10 consecutive days with the test item difluoroacetic acid at the treatment level of 10000 µg a.i./L caused no adverse effect regarding mortality, sublethal effects and behaviour. As the overall mean daily food uptake (i.e. the average value over 10 days) in the test item treatment group was almost identical to the untreated control group and because on every single day during the 10 day continuous exposure period the mean food consumption per bee was not significantly lower in the test item treatment group

compared to the control group, it can be concluded that there was no repellent effect of the test item at the treatment level of 10000 µg a.i./L.

The NOEC (No Observed Effect Concentration) was determined to be 10000 µg a.i./L at the end of the test period.

Report:	**KIIA 8.16.1/05; Kling, A. (2012)**
Title:	6-chloronicotinic acid – Assessment of Chronic Effects to the Honey Bee, *Apis mellifera* L., in a 10 Days Continuous Laboratory Feeding Limit Test
Report No:	S11-01957
Document No:	M-425155-01-2
Guidelines:	**No specific guideline available**
Deviations:	**Not applicable**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The chronic effects of the test item 6-chloronicotinic acid (Origin Batch No.: M12653; Batch code: AE F161089 00 1B99 0001; Purity 98.8% w/w) on the honey bee, *Apis mellifera* L., in a 10 days continuous feeding limit test in the laboratory were assessed.

Over a period of 10 days, honey bees were exposed to 50% (w/v) aqueous sucrose feeding solution, containing nominally 10000 µg a.i./L of the test item 6-chloronicotinic acid, by continuous and *ad libitum* feeding. Because the test item was first dissolved in acetone and then diluted with aqueous sucrose solution, the final test item feeding solution contained 1% acetone. The control group was exposed for the same period of time under identical exposure conditions to untreated 50% (w/v) aqueous sucrose feeding solution, also containing 1% acetone. Mortality, sublethal effects and behavioural observations were assessed every day throughout the 10 days exposure period.

Samples and retain samples of all feeding solutions and the stock solution were taken for chemical analysis.

The accumulated nominal intake of the test item 6-chloronicotinic acid via 6-chloronicotinic acid - treated aqueous sucrose solution was 4.18 µg a.i./bee after 10 days of continuous exposure.

It can be concluded that the continuous feeding of honey bees in the laboratory over a period of 10 consecutive days with the test item 6-chloronicotinic acid at the treatment level of 10000 µg a.i./L caused no adverse effect regarding mortality, sublethal effects and behaviour. As the overall mean daily food uptake (i.e. the average value over 10 days) in the test item treatment group was comparable to the untreated control group and because on every single day during the 10 day continuous exposure period the mean food consumption per bee was not significantly lower in the test item treatment group compared to the control group, it can be concluded that there was no repellent effect of the test item at the treatment level of 10000 µg a.i./L.

The NOEC (No Observed Effect Concentration) was determined to be 10000 µg a.i./L at the end of the test period.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	6-chloronicotinic acid (AE F161089)
Type of test material:	Pure metabolite
Chemical state and description	Beige powder
Origin Batch No.:	M12653 (Batch code: AE F161089 00 1B99 0001)
Sample description:	Not available (Test item code: 2011-001593)
CAS#:	5326-23-8
Purity:	98.8% w/w
Stability of test compound:	Expiry date: 09.07.2012, when stored at +5 ± 5°C
Treatment	50% (w/v) aqueous sucrose feeding solution, containing nominally 10000 µg a.i./L of the test item 6-chloronicotinic acid as well as 1% acetone
Control	50% (w/v) aqueous sucrose feeding solution containing 1% acetone

2. Test organisms

Species:	*Apis mellifera* L.
Common name:	Honey bee
Age or developmental stage at test start:	Young adult worker bees
Source:	Bred by Eurofins

B. Study design and methods

1. In life dates May 24 to June 3, 2011

2. Experimental treatments

A study was conducted to determine the chronic effects of the test item 6-chloronicotinic acid on the honey bee, *Apis mellifera* L., in a 10 days continuous feeding limit test in the laboratory. The NOEC value (No Observed Effect Concentration) for mortality was determined at the end of the test period.

The honey bees were fed continuously and *ad libitum* with a 50% (w/v) aqueous sucrose solution, containing the test item 6-chloronicotinic acid at the nominal concentration level of 10000 µg a.i./L as well as 1% acetone. For the preparation of the test item treatment level, the active substance content of the test item AE F161089 (6-chloronicotinic acid) was considered to be 100% and not corrected for the analysed content of 98.8% (w/w).

In the control group the honey bees received an untreated 50% (w/v) aqueous sucrose solution containing 1% acetone, *ad libitum*.

The feeding solutions were offered *ad libitum* to each cage of 10 bees in plastic syringes (Omnifix®, 5 mL, B. Braun, Melsungen, Germany). The tip of each syringe was removed so that the bees had access to the feeding solution. Every morning the syringes of all test cages (i.e. test item and control) were replaced by new syringes, filled with freshly prepared feeding solution. The weight of the syringes was determined before and after feeding on the next day in order to determine the mean food consumption of the bees per test cage.

During the entire test period, the bees were kept in cages made of stainless steel (base: 8.2 cm x 4 cm; height: 6 cm). The front side of the cages were equipped with a transparent pane so that the bees could be observed. The bottom of the cages consisted of a perforated board, which guaranteed sufficient air supply for the test animals. The test cages are lined with filter paper.

The feeding solutions were offered to the bees in syringes fitted into the test cages.

The study was carried out with the following treatments:

- one concentration of the test item 6-chloronicotinic acid
- the control

The test item treatment was tested with 10 replicates (cages), each containing 10 bees (i.e. in total 100 honey bees). The control group comprised 30 replicates of 10 bees each (i.e. in total 300 honey bees).

A toxic standard was not included in this test, since a toxic reference substance had not been defined or validated for this type of study.

3. Observation and measurements

Mortality

The number of dead bees in the individual test cages was recorded every day at about the same time of day during the 10 days test period.

Abnormal behavioural effects and behavioural differences were assessed according to the following categories and recorded in the raw data:

a = affected (bees still upright and attempting to walk but showing signs of reduced coordination)

ap = apathy (bees show only low or delayed reactions to stimulation [i.e. light, air blow] bees are either sitting motionless in the cage or walk but not correctly)

m = moribund (bees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation, bees may recover but usually die)

The mortality [%] in the test item treatment was calculated from the number of dead bees and the total number of introduced bees in the test item treatment group. The mortality of the test item treatment was corrected for corresponding control mortality, according to the formula of ABBOTT (1925), modified by SCHNEIDER-ORELLI (1947):

$$\text{Corrected mortality} \quad M = \frac{t - c}{100 - c} \times 100$$

M = Corrected mortality (%)
t = Mortality in the test item group (%)
c = Mortality in the control group (%)

Food consumption

The consumption of feeding solution per bee/day was calculated by dividing the total daily consumption per cage by the mean number of living bees at the corresponding time interval. The mean value per treatment group as well as the daily mean food consumption per bee was calculated.

The mean intake of test item per bee was calculated as follows:

$$\text{Mean intake of test item [µg a.i./bee]} = \frac{\text{Mean consumption of feeding solution [mg/bee]}}{\text{Density of the sucrose solution [g/cm}^3\text{]}} \text{ X } \text{Concentration of feeding solution [µg a.i./µL]}$$

Statistical evaluations:

Fisher's Exact Test (Bonferroni-Holms corrected, one-sided, $p \leq 0.05$) was used to evaluate whether there are significant differences between the mortality data of the test item treatment group and the control group.

For the statistical comparison of the food consumption, non-rounded values were taken. Data of food consumption were tested for normality using Shapiro-Wilks' test. If normality was observed, the t-Test (one-sided, $p \leq 0.05$) was applied. If the data deviated from the normal distribution, statistical analysis was performed by using the Mann Whitney – U test. Statistical calculations were made by using the statistical program SAS release Version 9.2.

Residue analysis:

The residue analysis of the feeding solutions was performed in an independent study by Bayer CropScience AG, Monheim, the analytical attached as an integral part of the study report.

Storage and shipment of samples:

Samples taken of the feeding solutions and the stock solution were stored deep frozen (≤ –18°C) in the Eurofins Agroscience Services EcoChem GmbH laboratory within 30 minutes. The samples were shipped on dry ice from the Eurofins Agroscience Services EcoChem GmbH - laboratory directly to the residue analysis laboratory of the sponsor (Bayer CropScience AG).

RESULTS AND DISCUSSION

A. Environmental Parameters

Measurements of climatic parameters during the test are summarized as follows:

Test temperature:	25.1 – 25.4°C
Relative air humidity:	64.0 – 68.8 %
Light intensity:	Constant darkness except during observations

B. Verification of the Application Volume

The residue analysis of the feeding solutions was performed in an independent study (analytical report is part of the study report, see Appendix A3).

C. Biological Findings

The results of the 10 days feeding test with a control group fed with untreated sucrose solution containing 1% acetone and the test item treatment of 10000 µg a.i. /L of 6-chloronicotinic acid first dissolved in acetone and diluted in 50% sucrose solution are presented in the table below.

Table **Mean consumption of feeding solution, mean intake of test item accumulated over all test days and cumulative mortality at the final assessment on day 10**

Treatment Level [1]	Control	Test Item
		10000 µg a.i./L
Overall mean daily consumption of aqueous sucrose feeding solution [mg/bee] [2]	46.2	49.7
Mean intake accumulated over test days [µg a.i./bee]	-	4.18
Cumulative mortality [%]	2.33	3.00*
Corrected cumulative mortality [%]	-	0.69

[1] The control group was fed with untreated 50% (w/v) aqueous sucrose feeding solution mixed with 1% acetone; the test item treatment group was fed with 50% (w/v) aqueous sucrose feeding solution containing 6-chloronicotinic acid and 1% acetone

[2] The mean values per cage over the test period were used as basis for the calculation of the overall mean daily consumption of the aqueous sucrose feeding solution per treatment over the test period

* Determined to be the NOEC (not significantly different compared to the control, Fisher's Exact Test; Bonferroni-Holms corrected, one-sided, $p \leq 0.05$)

D. Validity Criteria

Not applicable. No guideline is available for this type of study.

E. Biological Endpoints Derived

After 10 days of continuous exposure, mortality in the test item treatment group was not significantly different compared to the control group. The cumulative control mortality accounted to 2.33 %, as determined at the final assessment (day 10). In the test item treatment group at 10000 µg a.i./L, the cumulative mortality at the final assessment (day 10) accounted to 3.00 % (corrected 0.69 %).

Except for one moribund bee at the assessment after 4 days (d4) and one affected bee at the assessment after 5 days (d5), neither sublethal effects nor behavioural abnormalities were observed throughout the entire testing period in the test item treatment group. The test item treatment level of 10000 µg a.i./L was determined to be the NOEC (No Observed Effect Concentration, Fisher's Exact Test (Bonferroni-Holms corrected, one-sided, $p \leq 0.05$)).

The overall mean daily consumption of the aqueous sucrose feeding solution (i.e. the average value over 10 days) in the test item treatment group was comparable to the untreated control group (49.7 mg/bee in the test item treatment compared to 46.2 mg/bee in the control group).

The mean daily consumption of the aqueous sucrose feeding solution was not significantly different between the control group and the test item treatment group throughout the entire testing period (day-by-day comparison).

The accumulated nominal intake of the test item 6-chloronicotinic acid via 6-chloronicotinic acid - treated aqueous sucrose solution was 4.18 µg a.i./bee after 10 days of continuous exposure.

From the results presented above the following biological endpoints can be derived:

NOEC 10000 µg a.i./L

CONCLUSION

It can be concluded that the continuous feeding of honey bees in the laboratory over a period of 10 consecutive days with the test item 6-chloronicotinic acid at the treatment level of 10000 µg a.i./L caused no adverse effect regarding mortality, sublethal effects and behaviour. As the overall mean daily food uptake (i.e. the average value over 10 days) in the test item treatment group was comparable to the untreated control group and because on every single day during the 10 day continuous exposure period the mean food consumption per bee was not significantly lower in the test item treatment group

compared to the control group, it can be concluded that there was no repellent effect of the test item at the treatment level of 10000 µg a.i./L.

The NOEC (No Observed Effect Concentration) was determined to be 10000 µg a.i./L at the end of the test period.

Report:	**KIIA 8.16.1/06; Kling, A. (2012)**
Title:	6-chloropicolyl alcohol – Assessment of Chronic Effects to the Honey Bee, *Apis mellifera* L., in a 10 Days Continuous Laboratory Feeding Limit Test
Report No:	S11-01958
Document No:	M-425159-01-2
Guidelines:	**No specific guideline available**
Deviations:	**Not applicable**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

The chronic effects of the test item 6-chloropicolyl alcohol (Origin Batch No.: M06773; Batch code: AE F157983-PU-01; Purity 99.4% w/w) on the honey bee, *Apis mellifera* L., in a 10 days continuous feeding limit test in the laboratory were assessed.

Over a period of 10 days, honey bees were exposed to 50% (w/v) aqueous sucrose feeding solution, containing nominally 10000 µg a.i./L of the test item 6-chloropicolyl alcohol by continuous and *ad libitum* feeding. Because the test item was first dissolved in acetone and then diluted with aqueous sucrose solution, the final test item feeding solution contained 1% acetone. The control group was exposed for the same period of time under identical exposure conditions to untreated 50% (w/v) aqueous sucrose feeding solution, also containing 1% acetone. Mortality, sublethal effects and behavioural observations were assessed every day throughout the 10 days exposure period.

Samples and retain samples of all feeding solutions and the stock solution were taken for chemical analysis.

The accumulated nominal intake of the test item 6-chloropicolyl alcohol via 6-chloropicolyl alcohol - treated aqueous sucrose solution was 4.13 µg a.i./bee after 10 days of continuous exposure.

It can be concluded that the continuous feeding of honey bees in the laboratory over a period of 10 consecutive days with the test item 6-chloropicolyl alcohol at the treatment level of 10000 µg a.i./L caused no adverse effect regarding mortality, sublethal effects and behaviour. As the overall mean daily food uptake (i.e. the average value over 10 days) in the test item treatment group was comparable to the untreated control group and because only on one single day during the 10 day continuous exposure period the mean food consumption per bee was significantly lower in the test item treatment group compared to the control group, it can be concluded that there was no repellent effect of the test item at the treatment level of 10000 µg a.i./L.

The NOEC (No Observed Effect Concentration) was determined to be 10000 µg a.i./L at the end of the test period.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	6-chloropicolyl alcohol (AE F157983)
Type of test material:	Pure metabolite
Chemical state and description	Beige crystals
Origin Batch No.:	M06773 (Batch code: AE F157983-PU-01)
Sample description:	Not available (Test item code: 2011-001616)
CAS#:	21543-49-7
Purity:	99.4% w/w
Stability of test compound:	Expiry: 12.11.2017, when stored at +5 ± 5°C
Treatment	50% (w/v) aqueous sucrose feeding solution, containing nominally 10000 µg a.i./L of the test item 6-chloropicolyl alcohol as well as 1% acetone
Control	50% (w/v) aqueous sucrose feeding solution containing 1% acetone

2. Test organisms

Species:	*Apis mellifera* L.
Common name:	Honey bee
Age or developmental stage at test start:	Young adult worker bees
Source:	Bred by Eurofins

B. Study design and methods

1. In life dates May 24 to June 3, 2011

2. Experimental treatments

A study was conducted to determine the chronic effects of the test item 6-chloropicolyl alcohol on the honey bee, *Apis mellifera* L., in a 10 days continuous feeding limit test in the laboratory. The NOEC value (No Observed Effect Concentration) for mortality was determined at the end of the test period.

The honey bees were fed continuously and *ad libitum* with a 50% (w/v) aqueous sucrose solution, containing the test item 6-chloropicolyl alcohol at the nominal concentration level of 10000 µg a.i./L as well as 1% acetone. For the preparation of the test item treatment level, the active substance content of the test item AE F157983 (6-chloropicolyl alcohol) was considered to be 100 % and not corrected for the analysed content of 99.4% (w/w).

In the control group the honey bees received an untreated 50% (w/v) aqueous sucrose solution containing 1% acetone, *ad libitum*.

The feeding solutions were offered *ad libitum* to each cage of 10 bees in plastic syringes (Omnifix®, 5 mL, B. Braun, Melsungen, Germany). The tip of each syringe was removed so that the bees had access to the feeding solution. Every morning the syringes of all test cages (i.e. test item and control) were replaced by new syringes, filled with freshly prepared feeding solution. The weight of the syringes was determined before and after feeding on the next day in order to determine the mean food consumption of the bees per test cage.

During the entire test period, the bees were kept in cages made of stainless steel (base: 8.2 cm x 4 cm; height: 6 cm). The front side of the cages were equipped with a transparent pane so that the bees could be observed. The bottom of the cages consisted of a perforated board, which guaranteed sufficient air supply for the test animals. The test cages are lined with filter paper.

The feeding solutions were offered to the bees in syringes fitted into the test cages.

The study was carried out with the following treatments:

- one concentration of the test item 6-chloropicolyl alcohol
- the control

The test item treatment was tested with 10 replicates (cages), each containing 10 bees (i.e. in total 100 honey bees). The control group comprised 30 replicates of 10 bees each (i.e. in total 300 honey bees).

A toxic standard was not included in this test, since a toxic reference substance had not been defined or validated for this type of study.

3. Observation and measurements

Mortality

The number of dead bees in the individual test cages was recorded every day at about the same time of day during the 10 days test period.

Abnormal behavioural effects and behavioural differences were assessed according to the following categories and recorded in the raw data:

a = affected (bees still upright and attempting to walk but showing signs of reduced coordination)

ap = apathy (bees show only low or delayed reactions to stimulation [i.e. light, air blow] bees are either sitting motionless in the cage or walk but not correctly)

m = moribund (bees cannot walk and show only very feeble movements of legs and antennae, only weak response to stimulation, bees may recover but usually die)

The mortality [%] in the test item treatment was calculated from the number of dead bees and the total number of introduced bees in the test item treatment group. The mortality of the test item treatment was corrected for corresponding control mortality, according to the formula of ABBOTT (1925), modified by SCHNEIDER-ORELLI (1947):

$$\text{Corrected mortality} \qquad M = \frac{t - c}{100 - c} \times 100$$

M = Corrected mortality (%)
t = Mortality in the test item group (%)
c = Mortality in the control group (%)

Food consumption

The consumption of feeding solution per bee/day was calculated by dividing the total daily consumption per cage by the mean number of living bees at the corresponding time interval. The mean value per treatment group as well as the daily mean food consumption per bee was calculated.

The mean intake of test item per bee was calculated as follows:

$$\text{Mean intake of test item [µg a.i./bee]} = \frac{\text{Mean consumption of feeding solution [mg/bee]}}{\text{Density of the sucrose solution [g/cm}^3\text{]}} \text{ X } \text{Concentration of feeding solution [µg a.i./µL]}$$

Statistical evaluations:

Fisher's Exact Test (Bonferroni-Holms corrected, one-sided, $p \leq 0.05$) was used to evaluate whether there are significant differences between the mortality data of the test item treatment group and the control group.

For the statistical comparison of the food consumption, non-rounded values were taken. Data of food consumption were tested for normality using Shapiro-Wilks' test. If normality was observed, the t-Test (one-sided, $p \leq 0.05$) was applied. If the data deviated from the normal distribution, statistical analysis was performed by using the Mann Whitney – U test. Statistical calculations were made by using the statistical program SAS release Version 9.2.

Residue analysis:

The residue analysis of the feeding solutions was performed in an independent study by Bayer CropScience AG,.

Storage and shipment of samples:

Samples taken of the feeding solutions and the stock solution were stored deep frozen (≤ –18°C) in the Eurofins Agroscience Services EcoChem GmbH laboratory within 30 minutes. The samples were shipped on dry ice from the Eurofins Agroscience Services EcoChem GmbH - laboratory directly to the residue analysis laboratory of the sponsor (Bayer CropScience AG).

RESULTS AND DISCUSSION

A. Environmental Parameters

Measurements of climatic parameters during the test are summarized as follows:

Test temperature:	25.1 – 25.4°C
Relative air humidity:	64.0 – 68.8 %
Light intensity:	Constant darkness except during observations

B. Verification of the Application Volume

The residue analysis of the feeding solutions was performed in an independent study (analytical report is part of the study report, see Appendix A3).

C. Biological Findings

The results of the 10 days feeding test with a control group fed with untreated sucrose solution containing 1% acetone and the test item treatment of 10000 µg a.i. /L of 6-chloropicolyl alcohol, first dissolved in acetone and diluted in 50% sucrose solution are presented in the table below.

Table **Mean consumption of feeding solution, mean intake of test item accumulated over all test days and cumulative mortality at the final assessment on day 10**

Treatment Level [1]	Control	Test Item
		10000 µg a.i./L
Overall mean daily consumption of aqueous sucrose feeding solution [mg/bee] [2]	46.2	49.1
Mean intake accumulated over test days [µg a.i./bee]	-	4.13
Cumulative mortality [%]	2.33	5.00*
Corrected cumulative mortality [%]	-	2.73

[1] The control group was fed with untreated 50% (w/v) aqueous sucrose feeding solution mixed with 1% acetone; the test item treatment group was fed with 50% (w/v) aqueous sucrose feeding solution containing 6-chloropicolyl alcohol and 1% acetone

[2] The mean values per cage over the test period were used as basis for the calculation of the overall mean daily consumption of the aqueous sucrose feeding solution per treatment over the test period

* Determined to be the NOEC (not significantly different compared to the control, Fisher's Exact Test; Bonferroni-Holms corrected, one-sided, $p \leq 0.05$)

D. Validity Criteria

Not applicable. No guideline is available for this type of study.

E. Biological Endpoints Derived

After 10 days of continuous exposure, mortality in the test item treatment group was not significantly different compared to the control group. The cumulative control mortality accounted to 2.33%, as determined at the final assessment (day 10). In the test item treatment group at 10000 µg a.i./L, the cumulative mortality at the final assessment (day 10) accounted to 5.00 % (corrected 2.73%).

Furthermore, neither sublethal effects nor behavioural abnormalities were observed throughout the entire testing period in the test item treatment group. The test item treatment level of 10000 µg a.i./L was determined to be the NOEC (No Observed Effect Concentration, Fisher's Exact Test (Bonferroni-Holms corrected, one-sided, $p \leq 0.05$)).

The overall mean daily consumption of the aqueous sucrose feeding solution (i.e. the average value over 10 days) in the test item treatment group was comparable to the untreated control group (49.1 mg/bee in the test item treatment compared to 46.2 mg/bee in the control group).

The mean daily consumption of the aqueous sucrose feeding solution was statistically significantly reduced in the test item treatment group (46 mg/bee) compared to the control group (55 mg/bee) only on day 1 (t- test pooled; one-sided, $p \leq 0.05$). On all other test days, the mean daily consumption of the aqueous sucrose feeding solution was almost identical or even higher in the test item treatment group compared to the control group (day-by-day comparison).

The accumulated nominal intake of the test item 6-chloropicolyl alcohol via 6-chloropicolyl alcohol - treated aqueous sucrose solution was 4.13 µg a.i./bee after 10 days of continuous exposure.

From the results presented above the following biological endpoints can be derived:

NOEC 10000 µg a.i./L

CONCLUSION

It can be concluded that the continuous feeding of honey bees in the laboratory over a period of 10 consecutive days with the test item 6-chloropicolyl alcohol at the treatment level of 10000 µg a.i./L caused no adverse effect regarding mortality, sublethal effects and behaviour. As the overall mean daily

food uptake (i.e. the average value over 10 days) in the test item treatment group was comparable to the untreated control group and because only on one single day during the 10 day continuous exposure period the mean food consumption per bee was significantly lower in the test item treatment group compared to the control group, it can be concluded that there was no repellent effect of the test item at the treatment level of 10000 µg a.i./L.

The NOEC (No Observed Effect Concentration) was determined to be 10000 µg a.i./L at the end of the test period.

Report:	**KIIA 8.16.1/07; Nikolakis A., Theis M., Przygoda D.; (2010)**
Title:	BYI 02960 tech.: Effects of exposure to spiked diet on honey bee (*Apis mellifera carnica*) larvae in an *in vitro* laboratory testing design.
Report No:	E 318 3897-9
Document No:	M-406645-01-2
Guidelines:	**No validated guideline available. Study design according to the recommendations of the INRA (Institut National de la Recherche Agronomique) - method for testing pesticide toxicity to honeybee brood in laboratory conditions (January, 2008) and the recommendations of the honeybee larvae laboratory ring-test group, organized by ICPBR (Aupinel et al., 2009)**
Deviations:	**Not applicable**
GLP:	**Yes (certified laboratory)** The rearing of bee larvae in the bee hives was not part of GLP. The preparation of saturated solutions of K_2SO_4 and NaCl and the preparation of solutions for the disinfection of grafting cells as well as for the wetting of dental rolls were not part of GLP. The procedure of the disinfection of grafting cells and the preparation of the rearing plates, respectively test plates were not part of the GLP.

EXECUTIVE SUMMARY

The purpose of the biological part of this study was to assess the effects of BYI 02960 tech. (TOX 08508-00; Specification No.: 102000022313; Batch code: BYI 02960-01-03; content of a.i. (analysed): 96.2% w/w) on honey bee larvae, *Apis mellifera carnica*, after artificial feeding of spiked diet in an in vitro laboratory testing design. The purpose of the analytical part of this study was to quantify the concentration of BYI 02960 in spiked exposure diets, which were used to feed the larvae in the biological part of this study.

At day +1 (day 0 was the anticipated day of larval hatching), first instar bee larvae (*Apis mellifera carnica*) were transferred from their bee hive into an artificial *in vitro* testing system. The bee larvae were fed with standardised amounts of untreated artificial diet at day +1 and day +3. On day +4, +5 and +6, the bee larvae in the test item treatment groups were fed with standardized amounts of test item spiked artificial exposure diet. On day +4, the bee larvae in the reference item treatment group were fed with standardised amounts of reference item spiked artificial exposure diet. Concurrently, the bee larvae in the control group (on day +4, +5 and +6) and in the reference group (on day +5 and +6) received untreated artificial exposure diet, respectively. In the test item treatment groups, BYI 02960 (tech.) was incorporated into the artificial exposure diet at the nominal test concentrations of 150, 600, 2500 and 10000 µg a.i./kg diet, respectively. The actual concentration of BYI 02960 in the test item spiked exposure diet was determined according to analytical method 01206 by using High Performance Liquid Chromatography, coupled with tandem mass spectrometry.

During the development of the honeybee larvae, the larvae were incubated at about +35°C. From day +1 to +8, the relative humidity inside the incubator was on average about 95 ± 5% and from day +8 to +22 the mean relative humidity was about 80 ± 5%.

Mortality was determined on day +5, +6, +7, +8, +11, +13, +15 and +22. Dead test animals were discarded for sanitary reasons.

Five independent test runs were performed, from which 3 fulfilled both the INRA and the self-set validity criteria.

Overall, it can be concluded that the No Observed Effect Concentration (NOEC) as determined in this *in vitro* honeybee larvae study is ≥ 10000 µg BYI 02960 a.i./kg diet.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960
Type of test material:	Substance, technical
Chemical state and description:	Beige powder
Origin Batch No.:	2009-000239
Specification No.:	102000022313
Sample description:	TOX 08508-00
CAS name:	2(5H)-Furanone, 4-[[(6-chloro-3-pyridinyl)methyl](2,2-difuoroethyl)amino]
CAS#:	951659-40-8
Purity:	96.2% w/w (analytical)
IUPAC name:	4-[(6-chloropyridin-3-ylmethyl)(2,2-difluoroethyl)amino]furan-2(5H)-one
Stability of test compound:	Expiry date: 16.01.2011, when stored at +25 ± 5°C
Treatment	Artificial exposure diet containing the test item BYI 02960 at the nominal test concentrations of 150, 600, 2500 and 10000 µg a.i./kg diet
Reference Item	Dimethoate technical
Purity:	99.0%
Nominal test concentration:	3.0 µg a.i./larva
Basic larval diet (= negative control)	Artificial diet consisting of 50% royal jelly and 50% of an aqueous solution containing yeast extract, glucose and fructose in different proportions (dependent on the day of administration)

2. Test organisms

Species:	*Apis mellifera carnica*
Common name:	Honey bee
Age or developmental stage at test start:	First instar larvae, 1 day after hatching
Source:	Local beekeeper M. Flosbach, An der Gerichtslinde 12, 42929 Wermelskirchen, North-Rhine Westphalia, Germany

B. Study design and methods

1. In life dates June 14 to August 27, 2010

2. Experimental treatments

Principle of the testing procedure: At day +1[9], first instar bee larvae (*Apis mellifera carnica*) were transferred from their bee hive into an artificial *in vitro* testing system. The bee larvae were fed with standardised amounts of untreated artificial diet at day +1 and day +3. On day +4, +5 and +6, the bee larvae in the test item treatment groups were fed with standardised amounts of test item spiked artificial

[9] Day 0 was the anticipated day of larval hatching

exposure diet[10]. On day +4, the bee larvae in the reference item treatment group were fed with standardised amounts of reference item spiked artificial exposure diet. Concurrently, the bee larvae in the control group (on day +4, +5 and +6) and in the reference group (on day +5 and +6) received untreated artificial exposure diet, respectively. In the test item treatment groups, BYI 02960 (tech.) was incorporated into the artificial exposure diet at the nominal test concentrations of 150, 600, 2500 and 10000 µg a.i./kg diet. The actual concentration of BYI 02960 in the test item spiked exposure diet was determined according to analytical method 01206 by using High Performance Liquid Chromatography, coupled with tandem mass spectrometry.

During the development of the honeybee larvae, the larvae were incubated at about +35°C.

The feeding of the larvae took place once a day (except of day +2) according to the following schedule:

Table: Feeding regime of the honey bee larvae

Treatment group	**Day**	**+1**	**+2**	**+3**	**+4**	**+5**	**+6**
	Diet Type	**A**	**n.a.**	**B**	**C**		
Control	Volume of diet/larvae [µL]	20 (untreated)	n.a.	20 (untreated)	30 (untreated)	40 (untreated)	50 (untreated)
Test item	Volume of diet/larvae [µL]	20 (untreated)	n.a.	20 (untreated)	30 (treated)	40 (treated)	50 (treated)
Reference	Volume of diet/larvae [µL]	20 (untreated)	n.a.	20 (untreated)	30 (treated)	40 (untreated)	50 (untreated)

n.a.: not applicable

3. Observation and measurements

Time table of mortality assessments:

Larva: Before start of exposure (i.e. from day +1 until day +4), dead larvae were systematically removed for sanitary reasons. Exposure of the larvae to the treated diet started on day +4; during exposure (i.e. from day +4 until day +6) and after exposure, mortality was determined on day +5, +6, +7 and +8. Dead larvae were recorded and thereafter discarded.

Pupa: Not emerged bees were counted on day +22. During the pupal stage, obviously dead pupae were recorded and thereafter discarded on several dates for sanitary reasons.

Adult: Emerged adult bees were counted on day +22.

RESULTS AND DISCUSSION

A. Environmental Parameters

From day +1 to day +8, the larvae were incubated in a hermetic container at about +35°C, containing a dish filled with a saturated solution of K_2SO_4 in order to keep a water saturated atmosphere of on average 95 ± 5% relative humidity. For the control group a separate incubator with the same test conditions as described above was used from day +4 to day +8, to avoid any potential contamination of

[10] Remark: This study is designed to address chronic exposure of bee larvae to the test item. However, since a reliable design for a full chronic exposure (i.e. feeding with standardised amounts of treated artificial diet from day +1 to +6) has not yet been developed nor ring-tested or validated, and no appropriate validity criteria are so far defined for control mortality during day +1 to +3 (in this phase, very high control mortalities are regularly observed), the exposure of the bee larvae to the test item will take place in this study from day +4 onwards until day +6, where more stable conditions in terms of control mortality are to be expected.

the control larvae. From day +8 until day +22, the larvae were transferred into a hermetic container at about +35°C, containing a dish with a saturated solution of NaCl in order to keep a relative humidity of on average 80 ± 5%.

The desired test conditions were recorded with suitable and calibrated instruments and the obtained measurements are documented in the raw data.

The experimental unit was the 48-well culture plate made of polystyrene. For the test, the following test groups were used:

- Control group: 1 plate
- Test item treatment group: Four test concentrations,1 plate per test concentration, respectively
- Reference group: Dimethoate, 1 plate

The nominal test concentrations and the calculated, nominal feeding rates of the test groups used in this study are presented in following table:

Table: Feeding rates of honey bee larvae during the exposure period

Treatment groups	**Treatment on:**	**day +4**	**day +5**	**day +6**
	Volume of exposure diet C:	**30 µL (≈ 33 mg)**	**40 µL (≈ 44 mg)**	**50 µL (≈ 55 mg)**
	Treatment level [µg a.i./kg diet]	**Feeding rate [µg a.i./larva]**		
Control	n.a.	n.a.	n.a.	n.a.
Test item	150	0.00495	0.0066	0.00825
	600	0.0198	0.0264	0.033
	2500	0.0825	0.11	0.1375
	10000	0.33	0.44	0.55
Reference	90909	3.0	n.a.	n.a.

n.a.: not applicable

B. Verification of the Application Volume

For the analytical determination of the content of BYI 02960 in the test item spiked exposure diets, samples of the remaining spiked diets were immediately deep-frozen, after feeding of the honeybee larvae on day +4, +5 and +6 was completed. The samples were labelled with the study number and all necessary additional information to assure unmistakable identification. The actual concentration of BYI 02960 in the test item spiked exposure diet was determined according to analytical method 01206, by using High Performance Liquid Chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS) and electrospray ionization (ESI).

The analytical determination of the BYI 02960 concentration in the spiked exposure diets of the test item treatment groups revealed for all five test runs [test runs 2, 4 and 5] the following results:

150 µg a.i./kg diet - treatment level: On average 95 - 118% [test runs 2, 4 and 5; 95 - 109%] of nominal
600 µg a.i./kg diet - treatment level: On average 100 - 140% [test runs 2, 4 and 5; 100 - 110%] of nominal
2500 µg a.i./kg diet - treatment level: On average 87 - 106% [test runs 2, 4 and 5; 87 - 104%] of nominal
10000 µg a.i./kg diet - treatment level: On average 88 - 108% [test runs 2, 4 and 5; 88 - 108%] of nominal

C. Biological Findings

Table: Control and test item performance and associated statistical evaluation:

Test object	**Honeybee larvae *(Apis mellifera carnica)***					
	Control (untreated exposure diet)	**Test item (BYI 02960 - spiked exposure diet)**				**Reference item (dimethoate - spiked exposure diet)**
Test concentration (µg a.i./kg diet)	**---**	**150**	**600**	**2500**	**10000**	**3.0 (µg a.i./larva)**
Test run No. 1 [a]						
Mortality until day +22 [%]	31.3 [a]	39.6	29.2	41.7	45.8	89.6
Abbott-corrected mortality until day +22 [%]	0.0	12.1	-3.0	15.2	21.2	84.8
Test run No. 2						
Mortality until day +22 [%]	16.7	31.0	38.1	21.4	16.7	100
Abbott-corrected mortality until day +22 [%]	0.0	17.1	25.7	5.7	0.0	100
Statistical comparison to the control [b]	---	n.s.	n.s.	n.s.	n.s.	---
NOEC [b]	≥ 10000 µg a.i./kg diet					---
LOEC [b]	> 10000 µg a.i./kg diet					---
Test run No. 3 [a]						
Mortality until day +22 [%]	32.4 [a]	40.5	35.1	40.5	48.6	91.9
Abbott-corrected mortality until day +22 [%]	0.0	12.0	4.0	12.0	24.0	88.0
Test run No. 4						
Mortality until day +22 [%]	18.8	16.7	10.4	16.7	10.4	95.8
Abbott-corrected mortality until day +22 [%]	0.0	-2.6	-10.3	-2.6	-10.3	94.9
Statistical comparison to the control [b]	---	n.s.	n.s.	n.s.	n.s.	---
NOEC [b]	≥10000 µg a.i./kg diet					---
LOEC [b]	>10000 µg a.i./kg diet					---
Test run No. 5						
Mortality until day +22 [%]	28.6	31.0	26.2	31.0	40.5	100
Abbott-corrected mortality until day +22 [%]	0.0	3.3	-3.3	3.3	16.7	100
Statistical comparison to the control [b]	---	n.s.	n.s.	n.s.	n.s.	---
NOEC [b]	≥10000 µg a.i./kg diet					---
LOEC [b]	>10000 µg a.i./kg diet					---

[a]Although control performance met the validity criteria as stated in the INRA - method for testing pesticide toxicity to honeybee brood in laboratory conditions (January 2008), the self-set validity criterion for control performance at the end of the test (i.e. ≤ 30%) was not met; no distinct differences in larval mortality can be observed at concentrations of up to and including 10000 µg BYI 02960 a.i./kg diet (as the self-set validity criterion was not met, no detailed statistical evaluation is presented; however, when subjecting the data to statistical analysis, there is no statistical significance up to and including 10000 µg a.i./kg diet; Chi^2 Test [Bonferroni-Holms corrected, one-sided, $\alpha = 0.05$])

[b] Chi^2 Test, (Bonferroni-Holms corrected, one-sided, $\alpha = 0.05$)
n.s.: mean value not statistically significantly different compared to the control

D. Validity Criteria

In total, five independent test runs were conducted. In all test runs, the validity criteria as stated in the INRA - method for testing pesticide toxicity to honeybee brood in laboratory conditions (January 2008) and proposed by the recommendations of the honeybee larvae laboratory ring-test group (AUPINEL *et al.*, 2009) were met (i.e. mortality in the control group ≤ 15% and in the reference group ≥ 50% until day +7). In addition to the validity criteria as proposed by the ring-test group, an additional self-set validity criterion was employed (i.e. mortality in the control group ≤ 30% until day +22). This self-set validity criterion was applied in order to exclude test runs from which it is difficult to derive biologically meaningful information due to elevated mortality levels.

Table: Control performance in the individual test runs and associated validity criteria

Validity criteria	Origin of validity criteria	Validity threshold	Obtained results				
			Test run	Test run	Test run	Test run	Test run
			No. 1	No. 2	No. 3	No. 4	No. 5
Mortality in the control group until day +7	INRA - method for testing pesticide toxicity to honeybee brood in laboratory conditions (January 2008)	≤ 15%	14.6%	9.5%	5.4%	2.1%	11.9%
Mortality in the reference group until day +7 (Abbott)		≥ 50%	73.2%	81.6%	88.6%	83.3%	78.4%
Mortality in the control group until day +22	Self-set	≤ 30%	31.3% ‡	16.7%	32.4% ‡	18.8%	28.6%

‡ **Actual control performance at the end of the test has not met the self-set validity criterion**

E. Biological Endpoints Derived

Overall, three test runs (2, 4 and 5) fulfilled both, the validity criteria as proposed by the INRA-method (January, 2008) for testing pesticide toxicity to honeybee brood in laboratory conditions and the self set validity-criterion of ≤ 30% mortality in the control group until day +22. The statistical processing of the data as obtained in the test runs 2, 4 and 5 consistently revealed no statistically significant effects on mortality of exposed honeybee larvae until day +22 (end of the test, emergence) at concentrations of up to and including 10000 µg BYI 02960 a.i./kg diet (Chi^2 Test, Bonferroni-Holms corrected, one-sided, $\alpha = 0.05$). This conclusion is supported by the findings of the test runs 1 and 3.

CONCLUSION

All five independent test runs, as performed during the course of this *in vitro* honeybee larvae study, comply with the validity criteria as proposed by the INRA-method (January, 2008) for testing pesticide toxicity to honeybee brood in laboratory conditions (i.e. until day +7, ≤ 15% mortality in the control group and ≥ 50% mortality in the reference group), three independent test runs (test runs 2, 4 and 5) fulfilled both, the validity criteria as proposed by the INRA-method (January 2008) and the self-set validity criterion(i.e. ≤ 30% mortality in the control group until day +22). The analytical determination of BYI 02960 in the exposure diets of the test item treatment group revealed that the actual concentrations were well in line with the nominal concentrations. The statistical processing of the data as obtained in the test runs 2, 4 and 5 consistently revealed no statistically significant effects on mortality of exposed honeybee larvae until day +22 (end of the test, emergence) at concentrations of up to and including 10000 µg BYI 02960 a.i./kg diet (Chi^2 Test, Bonferroni-Holms corrected, one-sided, $\alpha = 0.05$). The outcome of this statistical evaluation is further supported by the findings of the test runs 1 and 3.

Overall, it can be concluded that the No Observed Effect Concentration (NOEC) as determined in this *in-vitro* honeybee larvae study is ≥ 10000 µg BYI 02960 a.i./kg diet.

IIA 8.16.2 Other/special studies - field studies

Effect of BYI 02960 on soil litter bag degradation

In this section the effect of BYI 02960 on soil litter bag degradation is described, this study is a registration requirement in Europe, however there is no specific OECD data heading for the study.

Report:	**KIIA 8.16.2/01; Leicher, T. (2011)**
Title:	BYI 02960: Effects on soil litter degradation after spray application
Report No:	LRT-SLD-45/11
Document No:	M-413408-01-2
Guidelines:	**Guidance Document on the Breakdown of Organic Matter in Litter Bags (OECD Series on Testing and Assessment, Number 56, 2006)**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

This study was designed to evaluate the effect of BYI 02960 on soil litter degradation. The test item was applied as formulated product BYI 02960 SL 200 G (Specification No.: 102000021884-01, Batch-ID.: 2010-001067, Sample description: TOX08907-00, analysed content: 201.0 g/L (17.1 % w/w), density: 1.175 g/mL).

The test item was applied twice by spraying, 1st to represent the plateau concentration and 2nd to represent the yearly application rate on six plots on a field in Germany (Bayer Experimental Farm Höfchen, Burscheid).

On June 14, 2010 BYI 02960 SL 200 G was applied at a rate of 150 g a.i./ha to the treatment plots and by careful harrowing, the test item was incorporated into the upper 10 cm soil layer to achieve a plateau concentration of 100 µg a.i./kg soil dry weight in 0 - 10 cm soil depth. Two days later, untreated summer wheat, variety “Chamsin”, was sown onto all plots. The seed rate was 230 kg/ha. Forty litterbags per plot were buried. On the same day BYI 02960 SL 200 G was applied at a single rate of 300 g a.i./ha to the treatment plots simulating the cumulative annual use of BYI 02960. This application rate corresponds to 1490 mL test item/ha or 1754 g test item/ha.

The degradation of the straw was determined for the time periods of 0 – 29 days, 0 – 92 days and 0 - 217 days by recording the weight of undegraded straw (after grinding by a ball mill and incineration).

217 days after burying of litter bags in soil treated with BYI 02960 (150 g BYI 02960/ha for plateau concentration and 300 g BYI 02960/ha for the annual rate) a difference in straw degradation of 3% was observed, which is below the trigger value of 10%.

From the results of this study it can be concluded that soil residues of BYI 02960 after long term use (including plateau concentration equivalent to a rate of 150 g a.i./ha combined with an annual rate of 300 g a.i./ha as spray application) have no influence on organic matter breakdown 217 days after application.

MATERIAL AND METHODS

A. Materials

1. Test material

Test item:	BYI 02960 SL 200 G
Type:	Formulated product (soluble (liquid) concentrate)
Chemical state and description:	Liquid, clear brown
Specification No.:	102000021884-01
Batch No.:	2010-001067
Nominal content of active substance:	BYI 02960: 200 g/L
Analytical content of active substance:	BYI 02960: 17.0% w/w, 201.0 g/L according to certificate of analysis
Density:	1.175 g/mL at 20°C
Stability of test compound:	Expiry date:14.06.2012, when stored at 25 ± 5°C (+2°C to +30°C are also acceptable)

2. Test soil

Soil nomenclature	Stark toniger Schluff (according to DIN 19682)
Study site	Am Hohenseh 4051 of Bayer Experimental Farm Höfchen, Burscheid, Germany
Particle size distribution (%) according to DIN:	
clay (< 0.002 mm):	19.4
silt (0.002 - 0.063 mm):	77.9
sand (0.063 - 2.0 mm):	2.7
Soil properties:	
pH (1 N KCl)	6.47
Microbial C (mg/kg dws)	477
C_{org} (%)	1.09
C_{anorg} (%)	0.06
N (%)	0.129
P (mg/kg dws)	731.62
$CaCO_3$ (%)	0.3
Water holding capacity (g H_2O/100g dws)	48.8
Cation exchange capacity (meq/100 g dry weight soil):	14.5
History of soil:	
Plant protection products not used since:	2009
Fertilisers not used since:	2009
Crops:	summer wheat

B. Study design and methods

1. In life dates June 10, 2010 to May 20, 2011

2. Design of biological test

Six plots in the field Am Hohenseh 4051 of Bayer Experimental Farm Höfchen, Burscheid, Germany were treated with BYI 02960. Six plots served as untreated control plots. The field Am Hohenseh 4051 was not treated with BYI 02960 in any formulation for at least the last three years.

A concentration of 100 µg a.i./kg soil dry weight was selected for the plateau concentration in the upper 10 cm soil layer. This corresponds to an application rate of 150 g a.i./ha equivalent to 750 mL test item/ha or 877 g test item/ha.

On June 14, 2010 BYI 02960 SL 200 G was applied at a rate of 150 g a.i./ha in a water volume of 300 L/ha to the treatment plots and by careful harrowing, the test item was incorporated into the upper 10 cm soil layer to achieve a plateau concentration of 100 µg a.s./kg soil dry weight in 0 - 10 cm soil

depth. On June 16, 2010, untreated summer wheat, variety "Chamsin", was sown with a seed rate of 230 kg/ha onto all plots and 40 litterbags per plot were buried. At the same day BYI 02960 SL 200 G was applied at a single rate of 300 g a.i./ha in a water volume of 300 L/ha to the treatment plots simulating the cumulative annual use of BYI 02960. This application rate corresponds to 1490 mL test item/ha or 1754 g test item/ha.

Litterbags (Polyester, 22 x 12 cm, mesh size 8 mm) filled with 4 ± 0.1 g dry wheat straw, were purchased from EcoTech Company, Nikolausstraße 7, 53129 Bonn, Germany. The bags were closed with a cable tie. A coloured label was fixed on each bag which served as a mark above the soil to recover the bags after they had been buried.

On June 18, 2010 each treatment plot and control plot was irrigated with about 6 mm water. Up to June 20, 2010, 3.5 mL precipitation was observed.

3. Observation and measurements

Soil samples were taken on June 14, 2010, directly after application and incorporation of the plateau concentration and on June 21, 2010, 5 days after application of the annual rate. The degradation of the straw was determined for the time periods of 0 – 29 days, 0 – 92 days and 0 - 217 days by recording the weight of undegraded straw (after grinding by a ball mill and incineration). Day 0 was set on June 16, 2010 when litterbags had been buried. Calculating the difference of the weight of straw at the start of the experiment and the remaining weight at sampling time allowed determination of the degree of degradation.

4. Statistical analysis

In order to determine whether the results reveal statistically significant differences, the weight (in gram) of degraded-straw of the two variants (untreated control and treatments with BYI 02960 SL 200 G) were analysed with the program ToxRatPro, Version 2.09. Cochran's test was conducted to test for homogeneity of variances and Kolmogorov-Smirnow Test was conducted to test for normal distribution. If the data were normally distributed and homogeneity of variance was given, a Student-t test (two sided, $\alpha = 0.05$) for homogeneous variances was performed. If data were not normally distributed, or if homogeneity of variance was not given the test was repeated with transformed data. If the transformed data too were not normally distributed or homogeneity of variance was not given the non-parametric Mann &Whitney Pair-wise U-test (Wilcoxon rank sum test, two sided, $\alpha = 0.05$) was chosen.

RESULTS AND DISCUSSION

A. Weather conditions

Rainfall and temperature during the study are depicted in the following table:

Table: Monthly Precipitation and average temperature during test period

Month (2010)	Monthly precipitation (mm)	Monthly average temperature (°C) 2 m above ground
June	12.3	18.01
July	88.0	21.15
August	203.0	17.05
September	109.0	13.57
October	60.6	10.01
November	129.0	6.20
December	67.5	- 1.63
January 2011	113.9	4.5
Sum June 2010 to January 2011	783.2	

B. Analytical results

Findings are well in agreement with analytical limits as specified by the EPFES guideline (50% to 150% of the nominal concentration should be reached) thereby confirming the application of the test substance. The limit of quantification (LOQ) was 5 µg/kg dry weight soil.

Table: Nominal and analytically verified amounts of BYI 02960

	Nominal application rate	
	[µg a.i./kg dry soil]	[g a.i./ha] *
Plateau concentration	100	150
Cumulative annual application	200	300
∑	300	450
	Analysed concentrations	
	[µg a.i./kg dry soil]	% of nominal amount
Plateau concentration [soil samples taken after incorporation]	89	89
Plateau concentration + Cumulative annual application [soil samples taken six days after application]	237	79

* Conversions from µg a.i./kg soil to g a.i./ha and vice versa assume a soil depth of 10 cm and a soil density of 1.5 g/mL

C. Biological Findings

The results of this study showed no statistically significant difference in proportion of straw degradation between untreated control plots and the plots treated with BYI 02960 SL 200 G for of litter-bags 29 and 217 days after introduction into the soil.

Table Effects of BYI 02960 SL 200 G on organic matter degradation

Means of 4 plots	Control	BYI 02960 SL 200 G	% of Control [1)]	% Effect [2)]
0 – 29 d*			92	8
g straw degraded	0.93	0.86		
% straw degraded	23.37	21.56		
0 – 92 d*			118	-18
g straw degraded	2.12	2.51**		
% straw degraded	52.95	62.73		
0 – 217 d*			97	3
g straw degraded	3.06	2.96		
% straw degraded	76.38	74.06		

* day 0 was set on June 16, 2010, when the litterbags had been buried
** statistically significant difference to the control (student-t test, α = 0.05, two sided)
1) corresponding to degraded straw in g, formula: (mass loss treatment*100)/mass loss control
2) corresponding to degraded straw in g, formula: ((mass loss control-mass loss treatment)/mass loss control)*100

D. Validity Criteria

A degradation of ≥ 60 % straw in untreated control was reached after 217 days after introduction of litterbags into soil. A coefficient of variation of ≤ 40 % for soil litter degradation for the data generated within 217 days in the control plots of the study was achieved (20.72 %, 8.25 % and 7.24 % after 29, 92 and 217 days, respectively).
Thus the two validity criteria were fulfilled as recommended by the OECD guideline. The study was terminated since degradation of straw was ≥ 60 % in the untreated control after 217 days.

E. Biological endpoint

217 days after burying of litter bags in soil treated with BYI 02960 (150 g BYI 02960/ha for plateau concentration and 300 g BYI 02960/ha for the annual rate) a difference in straw degradation of 3 % was observed, which is below the trigger value of 10 %.

CONCLUSION

From the results of this study it can be concluded that soil residues of BYI 02960 after long term use (including plateau concentration equivalent to a rate of 150 g a.i./ha combined with an annual rate of 300 g a.i./ha) have no influence on organic matter breakdown 217 days after application.

Report:	**KIIA 8.16.2/02; Leicher, T. (2011)**
Title:	BYI 02960: Effects on soil litter degradation if applied as seed treatment
Report No:	LRT-SLD-46/11
Document No:	M-413416-01-2
Guidelines:	**OECD No. 56, 2006 (OECD Series on Testing and Assessment)**
Deviations:	**None**
GLP:	**Yes (certified laboratory)**

EXECUTIVE SUMMARY

This study was designed to evaluate the effect of BYI 02960 on soil litter degradation. The test item was applied as formulated product BYI 02960 SL 200 G (Specification No.: 102000021884-01, Batch-ID.: 2010-001067, Sample description: TOX 08907-00, analysed content: 201.0 g/L (17.1% w/w), density: 1.175 g/mL) and BYI 02960 FS 480 G (Specification No.: 102000022677-01, Batch-ID.: 2010-001101, sample description: TOX 08940-00, analysed content: 481.4 g/L (40.4% w/w), density: 1.191 g/mL.

The test item was applied twice. First by spraying, represent the plateau concentration (as would occur after multi-year use) and second, as seed treatment to represent the annual application rate. The study was performed on six plots on a field in Germany (Bayer Experimental Farm Höfchen, Burscheid).

On June 14, 2010 BYI 02960 SL 200 G was applied at a rate of 150 g a.i./ha to the treatment plots and by careful harrowing, the test item was incorporated into the upper 10 cm soil layer to achieve a plateau concentration of 100 µg a.i./kg soil dry weight in 0 - 10 cm soil depth. Two days later, summer wheat, variety “Chamsin”, treated with BYI 02960 FS 480 G was sown onto treatment plots. The seed rate of 230 kg/ha leaded to an application rate of 265 g BYI 02960/ha (nominal). A degree of loading of 109.7 % was reached with the analysed content of 115.37 g a.i./100 kg seeds.

Litter bags were buried in six treatment plots and six untreated plots, respectively, and the degradation of the straw was observed for a period of 217 days. Results obtained from the untreated plots were compared with those of the treatment plots.

The degradation of the straw was determined for the time periods of 0 – 29 days, 0 – 92 days and 0 - 217 days by recording the weight of undegraded straw (after grinding by a ball mill and incineration). 217 days after burying of litter bags in soil treated with BYI 02960 SL 200 G (150 g BYI 02960/ha for plateau concentration) and the annual rate in form of treated summer wheat seed (nominally 265 g BYI 02960/ha), a difference in straw degradation of -5% compared to the untreated control was observed, which is below the trigger value of 10%. Therefore, it can be concluded that soil residues of BYI 02960 after long term use (including plateau concentration equivalent to a rate of 150 g a.i./ha combined with an annual rate of 265 g a.i./ha as seed treatment) have no influence on organic matter breakdown 217 days after application.

MATERIAL AND METHODS

A. Materials

1. Test materials

Test item 1	
(spray for plateau concentration):	BYI 02960 SL 200 G
Type:	Formulated product (soluble (liquid) concentrate)
Chemical state and description:	Liquid, clear brown
Specification No.:	102000021884-01
Batch No.:	2010-001067
Sample description:	TOX 08907-00
Nominal content of active substance:	BYI 02960: 200 g/L
Analytical content of active substance:	BYI 02960: 17.0% w/w, 201.0 g/L according to certificate of analysis
Density:	1.175 g/mL at 20°C
Stability of test compound:	Expiry date: 14.06.2012, when stored at 25 ± 5°C (+2°C to +30°C are also acceptable)

Test item 2	
(used as seed treatment):	BYI 02960 FS 480 G
Type:	Formulation (Flowable concentrate for seed treatment)
Chemical state and description:	Beige suspension
Specification No.:	102000022677-01
Batch No.:	2010-001101
Sample description:	TOX 08940-00
Nominal content of active substance:	BYI 02960: 480 g/L
Analytical content of active substance:	BYI 02960: 481.4 g/L
Density:	1.191 g/L
Stability of test compound:	Expiry date: 12.03.2012, when stored at 25 ± 5°C (+2°C to +30°C are also acceptable)

Seed dressing:

Cereal:	Summer wheat 'Chamsin'
Dosage:	219 mL BYI 02960 FS 480/dt
Nominal content of active substance:	105.12 g a.i./100 kg seeds
Analytical content of active substance:	115.37 g a.i./100 kg seeds
Degree of loading:	109.7 %
Stability of test compound:	Approved until 04.11.2010, when stored at 25 ± 5°C (+2°C to +30°C are also acceptable)

2. Test soil

Soil nomenclature:	Stark toniger Schluff (according to DIN 19682)
Study site:	Field: "Am Hohenseh 4051", Bayer Experimental Farm Höfchen, Burscheid, Germany
Particle size distribution (%) according to DIN:	
Clay (< 0.002 mm):	19.4
Silt (0.002 - 0.063 mm):	77.9
Sand (0.063 - 2.0 mm):	2.7
Soil properties:	
pH (1 N KCl)	6.47
Microbial C (mg/kg dws)	477
C_{org} (%)	1.09
C_{anorg} (%)	0.06
N (%)	0.126
P (mg/kg dws)	731.62
$CaCO_3$ (%)	0.3
Water holding capacity (g H_2O/100g dws):	48.8
Cation exchange capacity (meq/100 g dry weight soil):	14.5
History of soil:	
Plant protection products not used since:	2009
Fertiliser not used since:	2009
Crops:	Summer wheat

B. Study design and methods

1. In life dates June 10, 2010 to May 20, 2011

2. Design of biological test

Six plots in the field Am Hohenseh 4051 of Bayer Experimental Farm Höfchen, Burscheid, Germany were treated with BYI 02960 formulated as BYI 02960 applied as SL 200 G (Specification No.: 102000021884-01, Batch-ID.: 2010-001067, sample description: TOX 08907-00, analysed content: 201 g/L (17.1% w/w), density: 1.175 g/mL, approved until June 14, 2012) and BYI 02960 FS 480 G (Specification No.: 102000022677-01, Batch-ID.: 2010-001101, sample description: TOX 08940-00, analysed content: 481.4 g/L (40.4% w/w), density: 1.191 g/mL, approved until March 12, 2012).

A concentration of 100 µg a.i./kg soil dry weight was selected for the plateau concentration in the upper 10 cm soil layer. This corresponds to an application rate of 150 g a.i./ha equivalent to 750 mL test item/ha or 877 g test item/ha. Six plots served as untreated control plots.

On June 14, 2010 BYI 02960 SL 200 G was applied at a rate of 150 g a.i./ha in a water volume of 300 L/ha to the treatment plots. On June 16, 2010, untreated summer wheat, variety "Chamsin", was sown with a seed rate of 230 kg/ha onto the control plots. Also on June 16, 2010, dressed summer wheat, variety "Chamsin" treated with BYI 02960 FS 480 G was sown onto the treatment plots (TRE 1-6). After sowing 40 litterbags per plot were buried. Litterbags (Polyester, 22 x 12 cm, mesh size 8 mm)

filled with 4 ± 0.1 g dry wheat straw, had been purchased from EcoTech Company, Nikolausstraße 7, 53129 Bonn, Germany. The bags were closed with a cable tie. A coloured label was fixed on each bag which served as a mark above the soil to recover the bags after they had been buried.

3. Observation and measurements

Soil samples were taken on June 14, 2010, directly after application and incorporation of the plateau concentration and on June 21, 2010, 5 days after application of the annual rate. The degradation of the straw was determined for the time periods of 0 – 29 days, 0 – 92 days and 0 - 217 days by recording the weight of undegraded straw (after grinding by a ball mill and incineration). Day 0 was set on June 16, 2010 when litterbags had been buried. Calculating the difference of the weight of straw at the start of the experiment and the remaining weight at sampling time allowed determination of the degree of degradation.

4. Statistical analysis

In order to determine whether the results reveal statistically significant differences, the weight (in gram) of degraded-straw of the two variants (untreated control and treatments with BYI 02960) were analysed with the program ToxRatPro, Version 2.09 from ToxRat Solutions GmbH, Naheweg 15, 52477 Alsdorf, Germany.

Cochran's test was conducted to test for homogeneity of variances and Kolmogorov-Smirnow Test was conducted to test for normal distribution. If the data were normally distributed and homogeneity of variance was given, a Student-t test (two sided, $\alpha = 0.05$) for homogeneous variances was performed. If data were not normally distributed, or if homogeneity of variance was not given the test was repeated with transformed data. If the transformed data too were not normally distributed or homogeneity of variance was not given the non-parametric Mann & Whitney Pair-wise U-test (Wilcoxon rank sum test, two sided, $\alpha = 0.05$) was chosen.

RESULTS AND DISCUSSION

A. Weather conditions

Rainfall and temperature during the study are depicted in the following table:

Table: Monthly Precipitation and average temperature during test period

Month (2010)	Monthly precipitation (mm)	Monthly average temperature (°C) 2 m above ground
June	12.3	18.01
July	88.0	21.15
August	203.0	17.05
September	109.0	13.57
October	60.6	10.01
November	129.0	6.20
December	67.5	- 1.63
January 2011	113.9	4.5
Sum June 2010 to January 2011	783.2	-

B. Analytical findings

Findings are well in agreement with analytical limits as specified by the EPFES guideline (50% to 150% of the nominal concentration should be reached) thereby confirming the application of the test substance. The limit of quantification (LOQ) was 5 µg/kg dry weight soil.

Table: Nominal and analytically verified amounts of BYI 02960

	Nominal application rate	
	[µg a.i./kg dry soil]	**[g a.i./ha] ***
Plateau concentration	100	150
Cumulative annual application	177	265
∑	277	415
	Analysed concentrations	
	[µg a.i./kg dry soil]	% of nominal amount
Plateau concentration [soil samples taken after incorporation]	94	94
Plateau concentration + Cumulative annual application [soil samples taken six days after application]	243	88

* All conversions from µg a.i./kg soil to g a.i./ha and vice versa assume a soil depth of 10 cm and a soil density of 1.5 g/mL

C. Biological findings

Soil litter degradation is presented in the following table.

Table: Effects of BYI 02960 SL 200 G and BYI 02960 FS 480 G on organic matter degradation

Means of 4 plots	**Control**	**BYI 02960**	**% of Control 1)**	**% Effect 2)**
0 – 29 d*			100	0
g straw degraded	0.93	0.93		
% straw degraded	23.37	23.37		
0 – 92 d*			99	1
g straw degraded	2.12	2.09		
% straw degraded	52.95	52.35		
0 – 217 d*			105	-5
g straw degraded	3.06	3.20		
% straw degraded	76.38	80.08		

* day 0 was set on June 16, 2010, when the litterbags had been buried
1) Corresponding to degraded straw in g, formula: (mass loss treatment*100)/mass loss control
2) Corresponding to degraded straw in g, formula: ((mass loss control-mass loss treatment)/mass loss control)*100

No statistically significant difference in proportion of straw degradation was observed between untreated control plots and the plots treated with BYI 02960.

D. Validity Criteria

A degradation of ≥ 60 % straw in untreated control was reached after 217 days after introduction of litterbags into soil. A coefficient of variation of ≤ 40 % for soil litter degradation for the data generated within the 217 days in the control plots of the study was achieved (20.72%, 8.25% and 7.24% after 29, 92 and 217 days, respectively).
Thus the two validity criteria were fulfilled as recommended by the OECD guideline. The study was terminated since degradation of straw was ≥ 60% in the untreated control after 217 days.

E. Biological endpoint

217 days after burying of litter bags in soil treated with BYI 02960 (150 g BYI 02960/ha for plateau concentration and the annual rate of 265 g BYI 02960/ha as seed treatment a difference in straw degradation of -5% was observed, which is below the trigger value of 10 %.

CONCLUSION

It can be concluded, that soil residues of BYI 02960 after long term use (including plateau concentration equivalent to a rate of 150 g a.i./ha combined with an annual rate of 265 g a.i./ha as seed treatment) have no influence on organic matter breakdown 217 days after application.

IIA 8.17 Summary and evaluation of points IIA 7 and IIA 8.1 to 8.16

Summary of Point IIA 7, Environmental Fate

The route of degradation of BYI 02960 in aerobic soil has been determined in European and American soils with four different label positions under standard laboratory conditions at 20ºC for 120 days. Under aerobic conditions two major metabolites were observed, DFA (maximum 33.9%) and 6-CNA (maximum 17.1%). In all label positions there was significant mineralization to $^{14}CO_2$ (maximum ca. 59%) with relatively low formation of non-extractable residues (max. ca. 34%). The results indicate that BYI 02960 is degraded in aerobic soil by microbial activity with an overall mean DT_{50} (trigger value) of 73 days.

Under anaerobic soil conditions BYI 02960 was stable and it was concluded that photolysis on the soil surface would not be a significant route of degradation.

For BYI 02960 in standard batch equilibrium studies on 6 soils the adsorption K_{oc} ranged from 74.9 to 132.2 mL/g , desorption K_{doc} were higher indicating significant stronger sorption. In time dependent sorption studies the sorption of BYI 02960 was shown to increase over time with an ageing factor of 2.4 to 4.4. The K_{oc} of the major metabolite 6-CNA was determined in four soils (excluding one soil with very low organic carbon content and the sediment) ranged from 70 to 129 indicating medium mobility. The $K_{oc\ ads}$ for the metabolite DFA determined in five soils ranged from 1.7 to 9.5 indicating high mobility in soil.

The hydrolysis study of BYI 02960 in sterile buffer solutions of pH 4, 7 and 9 showed that the active substance is hydrolytically stable under environmental conditions. Photolytically BYI 02960 degraded very rapidly in sterile buffer and natural water studies, based BYI 02960 should degrade with a DT_{50} of less than a week, if exposed to sunlight. The major degradates were identified as BYI 02960-succinamide (found at max. 39.6% of applied) and BYI 02960-azabicyclosuccinamide (found at max. 25.9% of applied).

BYI 02960 is regarded as stable under anaerobic aquatic condition, and no major metabolites were formed.

The aerobic biotransformation of BYI 02960 was studied in two water-sediment systems, for a maximum of 120 days in the darkness at 20°C. The test item was applied with three radiolabels per test system. Dissipation of BYI 02960 from the water phase was mainly characterized by rapid partitioning into the sediment where it is slowly degraded and mineralized. DFA (difluoroacetic acid) was observed as a degradation product. In the water phases DFA accounted for up to 6.0%, in the sediment extracts for max. 0.9% of the applied radioactivity. No further significant degradation products were observed in the studies except mineralization to carbon dioxide (max. 8.5% of applied) and formation of NER (max. 26.6% of applied). The DT_{50} value for BYI 02960 in the entire water/sediment systems was in the range of 193 to 285 days.

In a supportive study the fate of BYI 02960 was investigated in pond water and sediment in outdoor microcosms as an aquatic model ecosystem for lentic aquatic freshwater systems with different trophic levels. The dissipation of BYI 02960 from the supernatant water phase with a mean of 81 days was caused by translocation into the sediment and by degradation. The overall degradation (mean of 95 days) was faster under the prevailing outdoor conditions compared to the standardized laboratory water sediment studies considering the rapid degradation due to photolysis this may due to the enhanced degradation due to sunlight under the outdoor test conditions.

In a further water-sediment study the degradation behavior of DFA applied as test item was investigated. Mineralization to carbon dioxide (max. 25.1% of applied) and formation of NER (max. 15.8% of applied) was measured during the study period.

Summary of IIA point 8

In the following, the endpoints of the active ingredient resulting from the ecotoxicological studies are summarised.

Endpoints for Birds

Test species	Test design	Ecotoxicological endpoint	Reference
BYI 02960			
Colinus virginianus	acute, oral	LD_{50} 232 mg a.i./kg bw	[redacted] & [redacted] (2010) M-386036-01-1, KIIA 8.1.1/01
Serinus canaria	acute, oral	LD_{50} 330 mg a.i./kg bw	[redacted] & [redacted] (2011) M-408514-01-1, KIIA 8.1.1/02
Gallus gallus domesticus	acute, oral	LD_{50} >2000 mg a.i./kg bw	Barfknecht & Wilkens (2011) M-420519-01-2, KIIA 8.1.1/03
Anas platyrhynchos	5-day-feeding	LC_{50} >4741 mg a.i./kg diet ≡ >825 mg a.i./kg bw/d NOEL 2238 mg a.i./kg diet ≡ 459 mg a.i./kg bw/d	[redacted], Lam & [redacted] (2010) M-388718-01-1 KIIA 8.1.2/01
Colinus virginianus	5-day-feeding	LC_{50} >4876 mg a.i./kg diet ≡ >470 mg a.i./kg bw/d NOEL 1133 mg a.i./kg diet ≡ 170 mg a.i./kg bw/d	[redacted] & Lam (2010) M-394535-01-1 KIIA 8.1.2/02
Anas platyrhynchos	20-week feeding chronic, reproduction	NOAEL ≥845 mg a.i./kg diet ≡ ≥81 mg a.i./kg bw/d	[redacted], [redacted] & Lam (2011), M-412917-02-1,KIIA 8.1.4/01
Colinus virginianus	23-week feeding chronic, reproduction	NOAEL 302 mg a.i./kg diet ≡ 40 mg a.i./kg bw/d	[redacted], [redacted] & Lam (2012), M-424704-01-1, KIIA 8.1.4/02

Endpoints for Aquatic organisms: Freshwater Fish

Test species	Test design	Ecotoxicological endpoint [mg a.i./L]	Reference
Oncorhynchus mykiss	acute, 96 h	LC_{50} > 74.2 (mm)[2] NOEC ≥ 74.2 (mm)	[redacted] & Lam (2010) M-390611-01-1, KIIA 8.2.1.1/01
Pimephales promelas	acute, 96 h	LC_{50} > 70.5 (mm) NOEC ≥ 70.5 (mm)	[redacted]& Lam (2010) M-392560-01-1, KIIA 8.2.1.2/01
Cyprinus carpio	acute, 96 h	LC_{50} > 100 (mm) NOEC ≥ 100 (mm)	Bruns (2011) M-420407-01-2, KIIA 8.2.1.2/02
Pimephales promelas	early life stage (ELS), 35d	NOEC 4.41 (mm) LOEC 8.41 (mm)	[redacted] & Lam (2011) M-409339-01-1, KIIA 8.2.4/01

mm = mean measured

Endpoints for Aquatic organisms: Invertebrates

Test species	Test design	Ecotoxicological endpoint [mg a.i./L]	Reference
BYI 02960			
Daphnia magna	acute, 48 h	EC_{50} > 77.6 (mm) NOEC ≥ 77.6 (mm)	Banman & Lam (2009) M-357476-01-1, KIIA 8.3.1.1/01
Daphnia magna	chronic, static renewal	NOEC 3.2 (nom) LOEC 6.4 (nom)	Riebschlaeger (2011) M-414066-01-2, KIIA 8.3.2.1/01
Chironomus riparius	acute, 48 h	EC_{50} 0.062 (nom) NOEC 0.025 (nom)	Bruns (2011) M-414739-01-1, KIIA 8.3.1.2/01
Chironomus riparius	chronic, spiked water, 28 d	NOEC 0.0105 (mi) LOEC 0.0213 (mi) EC_{50} 0.0353 (mi) EC_{15} 0.0219 (mi)	Bruns (2011) M-401792-01-2 KIIA 8.3.2.2/01
BYI 02960-succinamide			
Daphnia magna	chronic, 21 d	NOEC 43.3 (nom) LOEC 100 (nom)	Riebschlaeger (2012) M-424700-01-2, KIIA 8.3.2.1/02
Chironomus riparius	acute, 48 h	EC_{50} > 100 (mi) NOEC 71 (mi)	Bruns (2011) M-417386-01-3, KIIA 8.3.1.2/02
BYI 02960-azabicyclosuccinamide			
Chironomus riparius	acute, 48 h	EC_{50} > 100 (mi) NOEC 71 (mi)	Bruns (2011) M-424404-01-1, KIIA 8.3.1.2/03
DFA (tested as Sodium difluoro acetate)			
Daphnia magna	acute, 48 h	EC_{50} > 10 (nom) NOEC 10 (nom)	Bruns (2011) M-409326-01-2, KIIA 8.3.1.1/02
Chironomus riparius	chronic, spiked water, 28 d	NOEC ≥ 100 (nom)	Bruns (2011) M-415913-01-2, KIIA 8.3.2.2/02
6-chloronicotinic acid (6-CNA)			
Daphnia magna	acute, , 48 h	EC_{50} > 95.1 (mm) NOEC 95.1 (mm)	McElligott (1997) M-196569-01-1, KIIA 8.3.1.1/03
Chironomus tentans	acute, 96 h	LC_{50} >1 (mi) NOEC 1 (mi)	Bowers & Lam (1998) M-048448-01-1, KIIA 8.3.1.2/04
Chironomus riparius	chronic, spiked water, 28 d	NOEC ≥ 100 (nom)	Bruns (2011) M-416604-02-2, KIIA 8.3.2.2/03

mm = mean measured concentration; nom = nominal concentration; mi = initial measured concentration

Endpoints for Aquatic organisms: Marine organisms

Test species	Test design	Ecotoxicological endpoint [mg a.i./L]	Reference
Cyprinodon variegatus	static acute, 96 h	LC_{50} > 83.9 (mm) NOEC 83.9 (mm)	[redacted] & Lam (2009) M-357479-01-1, KIIA 8.11.1/01
Crassostrea virginica	acute, flow-through, 96 h	EC_{50} > 29 (mm) NOEC ≥ 29 (mm)	Gallagher, Kendall & Krueger (2009), M-361668-01-1, KIIA 8.11.1/02
Americamysis bahia	static acute, 96 h	EC_{50} 0.26 (mm) NOEC 0.12 (mm)	Gallagher, Kendall & Krueger (2009), M-364620-01-1, KIIA 8.11.1/03
Americamysis bahia	life cycle, flow-through, 28 d	NOEC 0.0132 (mm) LOEC 0.0236 (mm)	Claude, Kendall & Krueger (2011), M-420783-01-1, KIIA 8.11.1/04

mm = mean measured

Endpoints for Aquatic organisms: Algae and Aquatic plants

Test species	Test design	Ecotoxicological endpoint [mg a.i./L]	Reference
BYI 02960			
Pseudokirchneriella subcapitata	growth inhibition, 96 h	E_rC_{50} > 80 (nom) NOE_rC ≥ 80 (nom)	[redacted] & Lam (2010) M-397552-01-1, KIIA 8.4/01
Lemna gibba	growth inhibition, 7 d	$E_bC_{50\ (frond\ no.)}$ > 67.7 (mm) $E_rC_{50(frond\ no)}$ > 67.7 (mm) $NOEC_{(frond\ no)}$ 34.2 (mm)	[redacted], [redacted] & Lam (2010), M-398376-01-1 KIIA 8.6/01
BYI 02960-succinamide			
Pseudokirchneriella subcapitata	growth inhibition test, 72 h	E_rC_{50} > 10 (nom) NOE_rC ≥ 10 (nom)	[redacted] (2011), M-414090-01-2 KIIA 8.4/03
DFA (tested as Sodium difluoro acetate)			
Pseudokirchneriella subcapitata	growth inhibition test, 72 h	E_rC_{50} > 10 (nom) NOE_rC 10 (nom)	Bruns (2011), M-409118-01-2 KIIA 8.4/02
6-chloronicotinic acid (6-CNA)			
Pseudokirchneriella subcapitata	growth inhibition test, 72 h	ErC_{50} > 100 [A] (nom)	Bruns (2012), M-424145-01-2 KIIA 8.4/04

[A] E_rC_{50} at a test concentration of 100 mg p.m./L, pH adjusted (pH 7.5 - 8.2)

Endpoints for Aquatic organisms: Amphibians

Test species	Test design	Ecotoxicological endpoint [mg a.i./L]	Reference
BYI 02960			
Xenopus laevis	acute, 48 h	LC_{50} > 73.8 (mm) NOEC ≥ 73.8 (mm)	[redacted] & Lam (2011) M-417822-01-1, KIIA 8.2.1.1/02

Endpoints for Honeybees: Acute contact and oral toxicity test

Test species	Test design	Ecotoxicological endpoint	Reference
BYI 02960			
Apis mellifera	Acute contact & oral	$LD_{50\ contact,\ 96\ h}$ 122.8 µg a.i./bee $LD_{50\ oral,\ 48\ h}$ 1.2 µg a.i./bee	[redacted] & [redacted] (2008) M-308904-02-1, KIIA 8.7.1/01
Difluoro-ethyl-amino-furanone (DFEAF)			
Apis mellifera	Acute contact & oral	$LD_{50\ contact,\ 48\ h}$ >100 µg a.i./bee $LD_{50\ oral,\ 48\ h}$ >81.5 µg a.i./bee	[redacted], 2010 M-398557-01-2, KIIA 8.7.1/02
BYI 02960-hydroxy			
Apis mellifera	Acute contact & oral limit test,	$LD_{50\ contact,\ 48\ h}$ >100 µg a.i./bee $LD_{50\ oral,\ 48\ h}$ >105.3 µg a.i./bee	[redacted], 2011 M-409606-01-2, KIIA 8.7.1/03
Difluoroacetic acid (DFA)			
Apis mellifera	Acute contact & oral limit test	$LD_{50\ contact,\ 48\ h}$ >100 µg a.i./bee $LD_{50\ oral,\ 48\ h}$ >107.9 µg a.i./bee	[redacted], 2010 M-367915-01-2, KIIA 8.7.1/04
6-chloronicotinic acid (6-CNA)			
Apis mellifera	Acute contact & oral limit test	$LD_{50\ contact,\ 48\ h}$ >100 µg a.i./bee $LD_{50\ oral,\ 48\ h}$ >107.1 µg a.i./bee	[redacted], 2010 M-395279-01-2, KIIA 8.7.1/05
6-chloropicolyl alcohol (6-CPA)			
Apis mellifera	Acute contact & oral limit test	$LD_{50\ contact,\ 48\ h}$ >100 µg a.i./bee $LD_{50\ oral,\ 48\ h}$ >106.7 µg a.i./bee	[redacted], 2010 M-361234-01-2, KIIA 8.7.1/06

Honeybees: Further testing

Test species	Test design	Ecotoxicological endpoint	Reference
BYI 02960			
Apis mellifera	Chronic effects: 10 d continuous feeding (laboratory), adult honeybees	No adverse effects (mortality & behavior); NOEC = 10000 µg a.i./L	Kling, 2010 M-400539-01-2 KIIA 8.16.1/01
Difluoro-ethyl-amino-furanone (DFEAF)			
Apis mellifera	Chronic effects: 10 d continuous feeding (laboratory), adult honeybees	No adverse effects (mortality & behavior); NOEC = 10000 µg a.i./L	Kling, 2012 M-425174-01-2 KIIA 8.16.1/02
BYI 02960-hydroxy			
Apis mellifera	Chronic effects: 10 d continuous feeding (laboratory), adult honeybees	No adverse effects (mortality & behavior); NOEC = 10000 µg a.i./L	Kling, 2012 M-425212-01-2 KIIA 8.16.1/03
Difluoroacetic acid (DFA)			
Apis mellifera	Chronic effects: 10 d continuous feeding (laboratory), adult honeybees	No adverse effects (mortality & behavior); NOEC = 10000 µg a.i./L	Kling, 2012 M-425105-01-1 KIIA 8.16.1/04
6-chloronicotinic acid (6-CNA)			
Apis mellifera	Chronic effects: 10 d continuous feeding (laboratory), adult honeybees	No adverse effects (mortality & behavior); NOEC = 10000 µg a.i./L	Kling, 2012 M-425155-01-2 KIIA 8.16.1/05
6-CHMP			
Apis mellifera	Chronic effects: 10 d continuous feeding (laboratory), adult honeybees	No adverse effects (mortality & behavior); NOEC = 10000 µg a.i./L	Kling, 2012 M-425159-01-2 KIIA 8.16.1/06

Endpoints for Non-target arthropods

Test species	Test design	Ecotoxicological endpoint	Reference
BYI 02960			
Aphidius rhopalosiphi	Laboratory, glass plates	LR_{50} < 0.5 g a.i./ha	Jans (2010) M-366965-01-2, KIIA 8.8.1.1/01
Typhlodromus pyri	Laboratory, glass plates	LR_{50} 17.3 g a.i./ha	Jans (2010) M-366957-01-2, KIIA 8.8.1.2/01

Endpoints for Soil organisms

Test species	Test design	Ecotoxicological endpoint	Reference
BYI 02960			
Eisenia fetida	acute, 14 d (10% peat in test soil)	LC_{50} 192.9 mg a.i./kg dws	Leicher (2010) M-363742-01-2, KIIA 8.9.1/01
BYI 02960 SL 200			
Eisenia fetida	reproduction, 56 d (10% peat in test soil)	NOEC 8.9 mg prod./kg dws	Leicher (2010) M-392964-01-2, KIIA 8.9.2/01
Folsomia candida	chronic, 28 d (5% peat in test soil)	NOEC 8.47 mg prod./kg dws	Frommholz (2009) M-359728-01-2, KIIA 8.14/01
Hypoaspis aculeifer	chronic, 14 d (5% peat in test soil)	NOEC ≥1000 mg prod./kg dws	Kratz (2010) M-358752-01-21,KIIA 8.14/04
Difluoroacetic acid (DFA)			
Eisenia fetida	acute, 14 d (10% peat in test soil)	LC_{50} >1000 mg p.m./kg dws	Leicher (2007) M-368835-01-2,KIIA 8.9.1/02
Eisenia fetida	reproduction, 56 d (10% peat in test soil)	NOEC 62.0 mg p.m./kg dws	Leicher (2010) M-398061-01-2, KIIA 8.9.2/02
Folsomia candida	chronic, 28 d (5% peat in test soil)	NOEC ≥100 mg p.m./kg dws	Frommholz (2010) M-368675-01-2,KIIA 8.14/02
Hypoaspis aculeifer	chronic, 14 d (5% peat in test soil)	NOEC ≥1000 mg p.m./kg dws	Kratz (2010) M-390091-01-2,KIIA 8.14/05
6-chloronicotinic acid (6-CNA)			
Eisenia fetida	acute, 14 d (10% peat in test soil)	LC_{50} ≥1000 mg p.m./kg dws	Wetton (1999) M-196591-01-1,KIIA 8.9.1/03
Eisenia fetida	reproduction, 56 d (10% peat in test soil)	NOEC 95 mg p.m./kg dws	Leicher (2011) M-413562-02-2, KIIA 8.9.2/03
Folsomia candida	chronic, 28 d (5% peat in test soil)	NOEC 90 mg p.m./kg dws	Frommholz (2010) M-407861-01-2, KIIA 8.14/03
Hypoaspis aculeifer	chronic, 14 d (5% peat in test soil)	NOEC ≥100 mg p.m./kg dws	Kratz, A. (2011) M-404434-01-2, KIIA 8.14/06

dws = dry weight soil

Endpoints for Organic matter breakdown:

Test species	Test design	Ecotoxicological endpoint	Reference
BYI 02960 SL 200G			
Soil litter degradation	217 d, spraying	soil treated with 150 g a.i./ha for plateau concentration + the annual rate of 300 g a.i./ha) → No influence	Leicher (2011) M-413408-01-2 KIIA 8.16.2/01
BYI 02960 SL 200 & FS 480			
Soil litter degradation	217 d, seed treatment	soil treated with 150 g a.i./ha for plateau concentration + the annual rate in form of treated summer wheat seed (265 g a.i./ha) → No influence	Leicher (2011) M-413416-01-2 KIIA 8.16.2/02

Endpoints for Soil micro-organisms

Test species	Test design	Ecotoxicological endpoint		Reference
BYI 02960				
N-cycle	28 d	no influence	0.3 kg a.i./ha 3 kg a.i./ha	Frommholz (2009) M-359803-01-1, KIIA 8.10.1/01
C-cycle	28 d	no influence	0.3 kg a.i./ha 3 kg a.i./ha	Schulz (2011) M-417194-01-1, KIIA 8.10.2/01
6-chloronicotinic acid				
N-cycle	28 d	no influence	1.0 kg p.m./ha	Frommholz (2011) M-408028-01-1, KIIA 8.10.1/02

dws = dry weight soil

Non-target terrestrial plants

Test species	Test design	Ecotoxicological endpoint	Reference
BYI 02960 SL 200			
11 plant species	Vegetative vigour test, application rate 410 g a.i./ha	No adverse effects >25% on survival, visual phytotoxicity, growth, shoot length and shoot dry weight	Gosch (2010) M-397734-01-1 KIIA 8.12/01
11 plant species	Seedling emergence test, application rate 410 g a.i./ha	No adverse effects >25% on emergence, survival, visual phytotoxicity, growth, shoot length and shoot dry weight	Gosch (2010) M-397727-01-1 KIIA 8.12/02

Efficacy of metabolite DFA compared to the parent compound

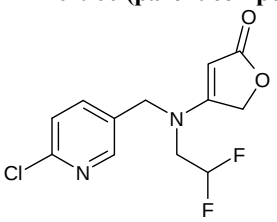
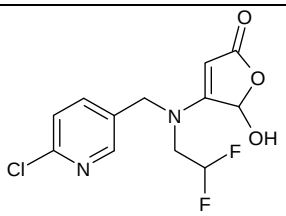
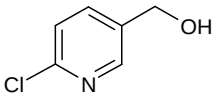
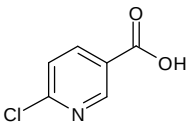
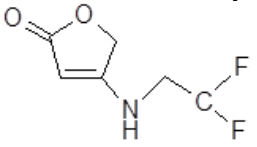
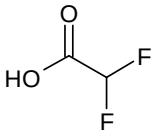
Test species	Test design	Ecotoxicological endpoint	Reference
Difluoroacetic acid			
Sucking and biting insects on bell pepper, cotton and cabbage	Microtiter plate test and whole plant screening (greenhouse)	In all test systems it could be demonstrated that the metabolite DFA does not exhibit any substantial insecticidal efficacy compared to the parent compound BYI 02960	Voerste & Malsam (2010) M-386333-01-1 KIIA 8.14.1/01

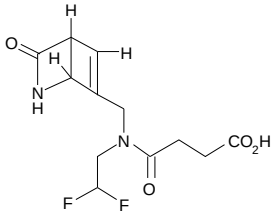
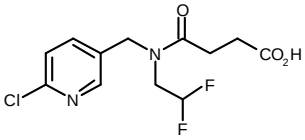
Sewage treatment

Test species	Test design	Ecotoxicological endpoint	Reference
BYI 02960			
Activated sludge	Respiration inhibition test	EC_{50} >1000 mg a.i./L EC_{10} 472.5 mg a.i./L	Caspers (2010) M-377311-01-1, KIIA 8.15/01

List of BYI 02960 metabolites included in this section

In the original study reports on BYI 02960 the metabolites are sometimes named by different synonyms, the metabolites referred to in this section are summarized below. Full details are provided in Document N.

	Name, Structure **IUPAC name** **CAS name, [CAS number]**	**Molecular formula** **molar mass** **Other names / codes**	**Occurrence**
a.s.	**BYF 02960 (parent compound)** 	C_{12} H_{11} Cl F_2 N_2 O_2 288.68 g/mol Flupyradifurone	all matrices
M03	BYI 02960-OH		
		C12 H11 Cl F2 N2 O3 304.68 g/mol BYI 02960-hydroxy BCS-CQ74364	Animal, Plant:
M21	BYI 02960-CHMP		
		C6 H6 Cl N O 143.57 g/mol 6-CPA (6-chloro-picolylacohol) BCS-AA52175	Plant:
M27	6-CNA 	C_6 H_4 Cl N O_2 157.56 g/mol 6-chloronicotinic acid IC-0 (in reports from Nippon Soda Co. Ltd.) BYI 02960-6-CNA BCS-AA35572	Animal, Plant: Environment Aerobic soil (major)
M34	BYI 02960-difluoroethyl-amino-furanone 	C_6 H_7 F_2 N O_2 163.12 g/mol DFEAF	Animal, Plant
M44	DFA 	C_2 H_2 F_2 O_2 96.03 g/mol difluroacetic acid BYI 02960-DFA BCS-AA56716 (In aquatic studies, tested as sodium difluoroacetate (Na-salt of difluoroacetic acid) (code: BCS-AB60481)	Animal, Plant: Environment Aerobic Soil (major) Aerobic water/Sediment (major)

	Name, Structure IUPAC name CAS name, [CAS number]	Molecular formula molar mass Other names / codes	Occurrence
M47	**BYI 02960-azabicyclosuccinamide**		
		$C_{12}H_{14}F_2N_2O_4$ 288.25 g/mol BCS-CS64875 (Tested as BYI 02960-azabicyclosuccinamide Na-Salt, BCS-CU93236)	Environment Water – aquatic photolysis (major)
M48	**BYI 02960-succinamide**		
		$C_{12}H_{13}ClF_2N_2O_3$ 306.69 g/mol BCS-CR74729	Environment Water – Aquatic photolysis (major)