REPORT

Diflufenican Tech: Toxicity to *Ankistrodesmus falcatus* in a 72-Hour Algal Growth Inhibition Test Supplemented with Testing for Recovery of Growth

Test Guideline(s): OECD No. 201 (2011)
Method C.3 of Commission Regulation (EU) No. 2016/266

Sponsor(s): Rotam Agrochem International Co. Ltd.
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Sponsor's Representative: [redacted]

Test Facility: [redacted]

Study Director: [redacted]

Study Identification: 20160072

Study Completion Date: September 26, 2016

Version: Final

GLP STATEMENT OF COMPLIANCE

IES Ltd Study Number: 20160072

Test Item: Diflufenican Tech

Study Director: [redacted]

Study Title: Diflufenican Tech: Toxicity to *Ankistrodesmus falcatus* in a 72-hour algal growth inhibition test supplemented with testing for recovery of growth

With the exception noted below the study was conducted in compliance with the Swiss Ordinance relating to Good Laboratory Practice, adopted May 18th, 2005 [SR 813.112.1]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C(97)186/Final].

These principles are compatible with GLP regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA), and Japan (MHLW, MAFF and METI).

Exclusions:
- Pre-experiments as mentioned in the report

There were no circumstances that may have affected the quality or integrity of the study.

Study Director: [redacted]

[redacted]

Date: 26 September, 2016

GLP QUALITY ASSURANCE STATEMENT

IES Ltd Study Number: 20160072

Test Item: Diflufenican Tech

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The general facilities and activities were inspected periodically and the results were reported to the person responsible and Test Facility Management.

Study procedures were periodically inspected. The Study Plan and this Final Report were audited by the Quality Assurance. The dates are given below. This Statement also confirms that this Final Report reflects the Raw Data.

Dates and Types of QA Inspections		**Date Reported to Study Director and Test Facility Management**
June 16, 2016	Study Plan Verification	June 16, 2016
July 05, 2016	Process-Based (test system)	July 05, 2016
August 18+19, 2016	Report Audit	August 19, 2016

Quality Assurance: [redacted]

Date: 26 September, 2016

GENERAL INFORMATION

General

Study Title:	Diflufenican Tech: Toxicity to *Ankistrodesmus falcatus* in a 72-hour algal growth inhibition test supplemented with testing for recovery of growth
Sponsors:	Rotam Agrochem International Co. Ltd. 26/F, E-Trade Plaza Chai Wan Hong Kong China UPL Europe Ltd 1st Floor, The Centre Birchwood Park Warrington WA3 6YN United Kingdom
Sponsor's Representative:	[redacted]
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Responsibilities

Study Director:	[redacted]
Deputy Study Director:	[redacted]
Analytical Chemistry:	[redacted]

Schedule

Study Initiation Date:	June 16, 2016
Experimental Starting Date:	June 17, 2016
Experimental Completion Date:	July 18 , 2016
Study Completion Date:	September 26, 2016

Archiving

[redacted]

TEST GUIDELINE(S)

This study followed the procedures indicated by the following internationally accepted Guidelines, Guidance and/or recommendations:

- OECD Guidelines for the Testing of Chemicals, No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, adopted 2006, corrected 2011.
- Commission Regulation (EU) No 2016/266 C.3: Algal Inhibition Test, 2016.

STUDY PLAN AMENDMENT(S) AND DEVIATION(S)

There were no Amendments to the Study Plan.

First Deviation:

Concerning	Alteration	Reason
4.5.2 Page 11	At the highest concentration only two replicates were prepared for the recovery periods, due to insufficient volume of the algal suspension after the filtration of the six replicates at the end of the exposure period.	The algal cell density after the exposure period was too low in this concentration.

Second Deviation:

Concerning	Alteration	Reason
4.5.2 Page 10	At the separation of the algae from the aged test medium after the first recovery period the algal cells were filtrated separately per replicate in the two highest concentrations instead of pooling the algae from all replicates per treatment.	The algal cell density was too low in these concentrations to use a pooled inoculum for the start of the second recovery period.

The Deviations had no impact on the integrity and quality of the study.

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1 SUMMARY

The impact of the test item Diflufenican Tech on the growth of the freshwater green algal species *Ankistrodesmus falcatus* was investigated in a 72-hour static test supplemented with testing for recovery of growth. The test performance was based on the OECD Guideline 201 (2011) and the Commission Regulation (EU) No 2016/266, C.3. The recovery of algal growth after the exposure period was recorded for two recovery periods of three days each resulting in six days recovery in total.

The nominal test item concentrations tested were 0.022, 0.046, 0.10, 0.22, 0.46 and 1.0 µg/L. Additionally, a control and a solvent control group were tested in parallel.

The test method is based on the OECD series on testing and assessment No. 23 on Aquatic Toxicity Testing of Difficult Substances and Mixtures, 2000.

The measured concentrations of the test item Diflufenican Tech in the test media of the nominal test concentrations of 0.022 to 1.0 µg/L were between 91 and 110% of the nominal values at the start of the exposure period. At the end of the exposure period (72 hours), 91 to 125% of the nominal values were found in these samples. At the start of both recovery periods all values were below the limit of quantification (LOQ = 0.0196 and 0.0208 µg/L at the first and the second day of analyses, respectively) except in the highest test concentration of 1.0 µg/L. Here the measured concentration of the test item was 0.022 µg/L at the start of the first recovery period. At the start of the second recovery period, the value of this concentration was below LOQ.

The reported biological results were based on the nominal concentrations of the test item since the correct dosage and the stability of the test item were confirmed. The slightly enhanced value of 125 % at the lowest tested concentration (0.022 µg/L) at the end of the exposure period was not taken into account, since 105% of nominal was measured at this concentration at test start. At this very low concentration minor analytical inaccuracies cannot be avoided.

For the exposure period, the biological results can be summarized as follows (based on nominal concentrations of the test item Diflufenican Tech):

	EC Values (0-72 h) in [µg/L]	
	Growth rate	**Yield**
EC_{10}	0.029	0.025
95% confidence interval	0.026 – 0.033	0.023 – 0.027
EC_{20}	0.040	0.029
95% confidence interval	0.036 – 0.043	0.027 – 0.031
EC_{50}	0.071	0.039
95% confidence interval	0.067 – 0.075	0.037 – 0.040
NOEC	0.022	0.022
LOEC	0.046	0.046

Over the test period, the biological results based on the average specific growth rates can be summarized as follows:

Nominal test item concentration [µg/L]	Inhibition of average specific growth rate µ during the test [%]		
	After exposure period (3 days)	After 3 days recovery	After 6 days recovery
0.022	-2.3	--	--
0.046	27*	-6.4	--
0.10	70*	-7.2	--
0.22	92*	19**	-4.1
0.46	98*	39**	-7.8
1.0	107*	92**	-13
NOEC/NOAEC [µg/L]	0.022	0.10	1.0

--: Not transferred, since full recovery in the previous test period
*: Mean value significantly lower than in the solvent control for growth rate and yield
**: Mean value significantly lower than in the pooled control for growth rate and yield

Note: Percentage inhibition values in excess of 100% are obtained when the biomass at the end of the interval is lower than at the start of the interval.

In conclusion, complete recovery of growth of the algae was demonstrated for all test concentrations at the latest after 6 days in test water free of test item. Thus, based on the complete recovery of algal growth within 6 days post exposure, the $NOAEC_{A.falcatus}$ (No Observed Ecologically Adverse Effect Concentration) for the growth of *Ankistrodesmus falcatus* after a 3-day exposure to Diflufenican Tech is 1.0 µg/L.

2 DEFINITIONS AND ABBREVIATIONS

Biomass:	Measurement variable (fluorescence or algal cell density) as surrogate measure for algal biomass.
Growth:	The increase of biomass over the test period.
Growth Rate (Average Specific Growth Rate):	The logarithmic increase in biomass during the exposure period.
Yield:	The value of the measurement variable at the end of the exposure period minus the measurement variable's value at the start of the exposure period, calculated to express biomass increase during the test.
EC_x:	The calculated concentration of test item that results in an x% reduction of the respective growth parameter relative to the controls within a stated exposure period.
LOEC (Lowest Observed Effect Concentration) :	The lowest test concentration at which a statistically significant inhibition of growth is determined relative to the controls.
NOEC (No Observed Effect Concentration):	The highest test concentration at which no statistically significant inhibition of growth is determined relative to the controls at the exposure phase.
NOAEC (No Observed Ecologically Adverse Effect Concentration):	The highest test concentration at which no statistically significant inhibition of growth is determined relative to the controls at the recovery phases.
Treatment:	Comprises test item treatments, control (test water only) and solvent control.

3 PURPOSE

The purpose of this study was to assess the toxicity of the test item Diflufenican Tech on the freshwater green algal species *Ankistrodesmus falcatus*. Exponentially growing cultures of this algal species were exposed to the test item over a period of 72 hours and the inhibition of algal growth in relation to control cultures were assessed over several generations. Thereafter, an additional experimental part was supplemented in which the recovery of algal growth was monitored.

4 TEST ITEM AND ANALYTICAL STANDARD

Information as provided by the Sponsor.

4.1 Test Item

Test Item Name:	Diflufenican Tech
Batch Number:	DFF-101/12
Chemical Name (IUPAC):	2',4'-difluoro-2-(a,a,a-trifluoro-*m*-tolyloxy)nicotinanilide
CAS-Number:	83164-33-4
Purity:	100% m/m, estimated error: ±0.6% (95% probability; n=5)
Molecular Formula:	$C_{19}H_{11}F_5N_2O_2$
Appearance:	White powder
Expiration Date:	16 October 2016
Storage Conditions (as provided by the Sponsor):	Room temperature
Storage Conditions (as handled at IES Ltd):	At room temperature at approximately 20 °C
Safety Precautions:	Routine hygienic procedures are sufficient to ensure personnel health and safety.
IES Code:	10469

4.2 Analytical Standard

The test item was used as analytical standard, since the purity of the test item was 100% ± 0.6%.

5 MATERIALS AND METHODS

5.1 Test System

The test organism used for the study was *Ankistrodesmus falcatus,* Strain No. CCAP 202/15A, supplied by the [redacted]. The algae were cultivated at [redacted] under standardized conditions according to the test guidelines.

An inoculum culture was set up four days before the start of the exposure. The algae were cultivated under the test conditions. The inoculum culture was diluted threefold one day before the start of the test to ensure that the algae were in the exponential growth phase when used to inoculate the test solutions.

The test method is recommended by the test guidelines.

5.2 Test Water

Reconstituted test water (AAP Medium) prepared according to the test guidelines was used for algal cultivation and testing. Analytical grade salts were dissolved in sterile purified water to obtain the following concentrations:

Ingredients			Concentration
Macro-nutrients	$NaHCO_3$		15.0 mg/L
	K_2HPO_4		1.044 mg/L
	$MgSO_4$	× 7 H_2O	14.6 mg/L
	$MgCl_2$	× 6 H_2O	12.16 mg/L
	$CaCl_2$	× 2 H_2O	4.41 mg/L
	$NaNO_3$		25.5 mg/L
Trace elements	H_3BO_3		186.0 µg/L
	$MnCl_2$	× 4 H_2O	415.0 µg/L
	$ZnCl_2$		3.27 µg/L
	$CoCl_2$	× 6 H_2O	1.43 µg/L
	$CuCl_2$	× 2 H_2O	0.012 µg/L
	Na_2MoO_4	× 2 H_2O	7.26 µg/L
	$FeCl_3$	× 6 H_2O	160.0 µg/L
	Na_2EDTA	× 2 H_2O	300.0 µg/L

The water hardness (calculated) of the test water was 0.15 mmol/L (= 15 mg/L as $CaCO_3$). The pH was 7.5.

5.3 Material

125 mL Erlenmeyer flasks were used as incubation vessels. The volume of test solution in each test flask was 50 mL per replicate. Each test flask was covered with a glass lid. The test flasks were labeled with the study number and all necessary additional information to ensure unique identification.

5.4 Experimental Conditions

The test flasks were incubated in a temperature controlled orbital shaker (Multitron-Pro, Infors HT, Bottmingen/Switzerland) at a temperature of 23 °C. The test flasks were positioned randomly and repositioned daily. They were continuously illuminated by LED light installed above the test flasks. The light intensity was measured at the start of the exposure (Day 0) and each recovery period (Day 3 and 6, measurements at 9 places distributed over the experimental area at the surface of the test media). The light intensity over the test area did not exceed the range of ±15% of the mean value during the whole test. The mean measured light intensity at the level of the test solutions is summarized in Table 14.

5.5 Study Design

5.5.1 Exposure Period

The selection of the test concentrations was based on the results of a range-finding test (non GLP).

Nominal Concentration [µg test item/L]	Inhibition of yield after 72 hours [%]	Inhibition of average growth after 72 hours [%]
Pooled Controls	0.0	0.0
0.01	3.7	0.5
0.1	55	27
0.5	99	90
2.5	100	104
12.5	101	107

The following nominal concentrations of the test item were tested: 0.022, 0.046, 0.10, 0.22, 0.46 and 1.0 µg/L. Additionally, a control (test water without test item) and a solvent control (test water with solvent and without test item) group were tested in parallel.

The main test had to be performed twice since the first main test had to be repeated (the validity criteria were not fulfilled).

The test design included three replicates of the test concentrations and the control and six replicates of the solvent control during the exposure and the recovery periods. For the three highest concentrations additionally three replicates were prepared for the recovery period as described below to provide a sufficient number of algae for recovery in case the toxicity is high.

The exposure period was started using a nominal algal cell density of 10000 cells/mL. The algal cell density in the pre-culture was determined microscopically using a counting chamber (Neubauer chamber).

A static test design was applied. The duration of the exposure period was 72 hours.

5.5.2 Recovery Period

The Sponsor was informed which test concentrations were selected for the recovery periods prior to the start of each recovery period.

During the recovery, the algal cells were transferred every 72 hours to fresh test water to keep the concentrations of the nutrients in the test water sufficiently high. The number of algal cells was reduced to nominal 10000 cells/mL per replicate at the start of the recovery period and every 72 hours at the renewal of the test water, in order to allow exponential growth of the algae during the whole recovery period. At the concentrations with reduced growth of the algal cells due to high toxicity of the test item, the recovery phase had to be started with less than 10000 cells/mL or with two instead of three replicates (see Table 6).

5.5.2.1 First Recovery Period

Since no statistical difference in average specific growth rates and yield was observed between the test concentration of 0.022 µg/L and the solvent control after the exposure period, this treatment was excluded from recovery.

All higher concentration levels and the controls were used for the first recovery period. Therefore, the replicates per treatment were pooled and the algal cells were separated by filtration (Whatman type NC 45, 0.45µm). Rinsing of the algal cells with test water aimed to reduce potentially adsorbed test item. Subsequently, the algae were transferred into test water and the algal density of each pooled sample was determined by measuring the fluorescence intensity. The results [relative fluorescence units] were converted into biomass concentration [cells/mL] using a calibration line.

Three replicates per treatment were used for the first recovery period except the solvent control where six replicates were prepared. For the highest concentration of 1.0 µg/L only two replicates could be prepared, due to insufficient cell number after pooling and filtration of the six replicates at the end of the exposure period. The first recovery period was started using a nominal algal cell density of approximately 10000 cells/mL. The algal biomass of all replicates were determined by fluorescence measurement at the start of the first recovery phase, after 24, 48 and 72 hours. The duration of the first recovery period was 72 hours.

5.5.2.2 Second Recovery Period

Since no statistical difference in average specific growth rates was observed between the test concentrations of 0.046 and 0.10 µg/L and the pooled control after the first recovery period, these treatments were excluded from the second recovery period.

All concentration levels with statistically significant inhibition of growth rate and yield after the first recovery period (LOEC and above) were used for the second recovery period.

For the second recovery period, the procedure for the controls and the lowest concentration of 0.22 µg/L was the same as during the first recovery period. The replicates were pooled and the algal cells were separated by filtration (Whatman type NC 45, 0.45µm). After filtration the algal cells were rinsed with test water and re-suspended in test water without the test item.

At the two highest concentrations of 0.46 and 1.0 µg/L, the procedure was modified to ensure to have enough cells to start the second recovery period with the same number of replicates as for the first recovery period. The algal cells were filtered (Merck Nylon Type NC45) by replicate, rinsed with test water and re-suspended in 30 mL test water without the test item. The algal density of each sample was determined by measuring the fluorescence intensity. The results [relative fluorescence units] were converted into biomass concentration [cells/mL] using a calibration line.

Two replicates per treatment of the highest concentration of 1.0 µg/L, three replicates per the concentration of 0.22 and 0.46 µg/L and the control and six replicates per solvent control were set up with a nominal algal concentration of approximately 10000 cells/mL at the start of the second recovery period.

The algal biomass of all replicates and treatments were determined by fluorescence measurement at the start of the second recovery phase, after 24, 48 and 72 hours. The duration of the second recovery period was 72 hours.

5.6 Dosage

Due to the low water solubility of the test item, the organic solvent *N*,*N*-dimethylformamide (DMF) was used to dose the test item. The solvent was chosen based on its solubilizing properties and its relative non-toxicity to algae.

A stock solution with the test item concentration of 1.0 g/L was prepared by dissolving 20.01 mg of the test item Diflufenican Tech in 20 mL of DMF by using intense stirring for 10 minutes. This stock solution was diluted in a series of sequential dilutions with DMF to prepare the application solutions used for the dosage of the test concentrations.

For the preparation of the test media, 100 µL of each application solution was spiked into 1000 mL test water, using intense stirring for 10 minutes at room temperature. The solvent control was prepared by addition of 100 µL DMF per liter test water. For the control test water without addition of test item or solvent was used. The test media were prepared just before the start of the test.

The preparation of the test media was based on the OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures [3].

6 EVALUATIONS

6.1 Determination of Algal Biomass

A small volume (200-400 µL) of the algal suspension was withdrawn daily from each test flask for the measurement of the biomass, and was not replaced.

The cell numbers of the pre-culture were determined microscopically using a counting chamber (Neubauer chamber).

The algal biomass in the test samples was determined by fluorescence measurement (SpectraMax I3x, Molecular Devices Ltd, Wokingham Berkshire/UK). The measurements were performed at least in duplicate at an excitation of 440 nm and emission of 680 nm.

The cell number in the treatments at the end of the exposure period and the first recovery period after the filtration was determined by measuring the fluorescence intensity. The results [relative fluorescence units] were converted into biomass [cells/mL] using a calibration line. A dilution series of the pre-culture was used to determine the calibration line by comparison of cell numbers (counted using a microscope) and fluorescence intensity (measured using a fluorometer) under consideration of background fluorescence of the algal medium (see Figure 6).

At the end of the exposure and the recovery period(s), a sample was taken from several treatments to determine the potential influence of the test item on the shape and size of the algal cells. The algal cells were examined microscopically in the following samples:

	Solvent control	0.46 µg/L	1.0 µg/L
Exposure period	X		X
1st recovery period	X	X	X
2nd recovery period	X		X

6.2 Determination of Algal Growth Inhibition and EC Values

Inhibition of algal growth was determined from the following growth parameters:

a) the specific growth rate (μ)
b) the yield (Y)

using the following equations:

a) Specific growth rate (μ):

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i}$$

where: μ_{i-j}: average specific growth rate from time i to j
X_i: biomass at time i
X_j: biomass at time j

The average growth rate over the test duration and the section-by-section growth rates (daily growth rates between the sampling times) was calculated.

Inhibition of growth rate (I_r):

$$I_r = \frac{\mu_C - \mu_T}{\mu_C} \cdot 100\%$$

where: I_r: percent inhibition in average specific growth rate
μ_C: mean value for average specific growth rate in the pooled or solvent control
μ_T: average specific growth rate for the treatment replicate

b) Yield (Y):

$$Y = X_j - X_0$$

where: Y: yield
X_0: biomass (nominal value) at the start of the test
X_j: biomass at time j (at the end of the test)

Inhibition of yield (I_y):

$$I_y = \frac{(Y_C - Y_T)}{Y_C} \cdot 100\%$$

where: I_y: percent inhibition of yield
Y_C: mean value for yield in the pooled or solvent control
Y_T: value for yield for the treatment replicate

Growth rate and yield were calculated for each test flask. The mean values for both parameters were calculated for each treatment. The tabulated values represent rounded results obtained by calculation using the exact raw data.

At the end of the exposure period, the 72-hour EC_{10}, EC_{20} and EC_{50} values for the inhibition of average growth rate and yield and their 95% confidence intervals were calculated by Probit Analysis using linear maximum likelihood regression [4], [5]. For the determination of the LOEC and NOEC, the average growth rate and yield at the test concentrations were compared to the solvent control values by Williams t-test (one sided smaller, $\alpha = 0.05$) [6], [7] or Welch t-test with Bonferroni-Holm-adjustment (one sided smaller, $\alpha = 0.05$) [8], where appropriate. The normal distribution and the homogeneity of variances was checked by Shapiro-Wilk's Test and Levene's Test, respectively. If the homogeneity of variance check failed the Welch t-test for non-homogenous variances with Bonferroni-Holm-adjustment was used.

The assessment of recovery of growth of the exposed algae cultures was based on the average specific growth rates and the yield every 72 hours. The mean yield and growth rates of every treatment were determined and were compared with the pooled control by Williams or Welch t-tests, where appropriate. Based on these results, the $NOAEC_{A.falcatus}$ (No Observed Ecologically Adverse Effect Concentration) for the growth of *Ankistrodesmus falcatus* was determined.

Statistical analysis was performed using ToxRat Professional® [9].

6.3 Monitoring of Experimental Conditions

The pH was measured and recorded in each treatment at the start and end of each test period. The temperature in the incubator was monitored and recorded continuously. The appearance of the test media was also visually controlled and recorded daily during the exposure period.

6.4 Analysis of the Test Item Concentrations

For measurement of the actual concentrations of the test item, duplicate samples were taken from the test media of all test concentrations at the start of the exposure period (without algae) and daily during the exposure period (containing algae). At the same sampling times, duplicate samples were also taken from the solvent control.

For the daily sampling during the exposure period, additional flasks containing the test medium with algae were incubated for each treatment under the test conditions.

At the beginning of each recovery period, duplicate samples were taken to confirm that no or only a small amount of the test item was present in the test media during the recovery period. At the beginning of the first recovery period the samples of the concentrations 0.046 to 1.0 µg/L were taken. At the beginning of the second recovery period the samples of the concentrations 0.22 to 1.0 µg/L were taken. The samples of the lower concentrations were not taken, since these concentrations were below the NOEC, therefore they were excluded from the recovery period.

All samples were stored deep-frozen (at about -20 °C) immediately after sampling until analysis. In pre-experiments for investigation of the storage stability of the samples, the test item proved to be stable under these storage conditions[1].

The concentrations of the test item Diflufenican Tech were determined in one of the duplicate test medium samples from all treatments taken at the start and the end of the exposure period and at the start of the both recovery periods.

The analytical procedure and results are described in Appendix 1.

[1] These experiments were not performed according to the regulations of GLP. The raw data are archived under study number 20160075.

7 RESULTS AND DISCUSSION

7.1 Analytical Results

The measured concentrations of the test item Diflufenican Tech in the test media of the nominal test concentrations of 0.022 to 1.0 µg/L were between 91 and 110% of the nominal values at the start of the exposure period (see analytical Appendix 1). At the end of the exposure period, 91 to 125% of the nominal values were found in these samples. At the start of both recovery periods the values were below the limit of quantification (LOQ = 0.0196 and 0.0208 µg/L at the first and the second day of analyses, respectively), except in the highest test concentration of 1.0 µg/L. Here the measured concentration of the test item was 0.022 µg/L at the start of the first recovery period. At the start of the second recovery period the value of this concentration was below LOQ.

The reported biological results were based on the nominal concentrations of the test item since the correct dosage and the stability of the test item were confirmed. The slightly enhanced value of 125 % at the lowest tested concentration (0.022 µg/L) at the end of the exposure period was not taken into account since 105% of nominal was measured at this concentration at test start. At this very low concentration, minor analytical inaccuracies cannot be avoided.

7.2 Biological Results of the Exposure Period

The impact of the test item on the growth of the algae is shown in Table 1 to Table 4 and in Figure 1 to Figure 3.

The algal growth (biomass) in the solvent control was statistically significantly different from the control after 72 hours exposition phase (according to a Student-t test, $\alpha = 0.05$, two-sided). Therefore all treatments were compared with the solvent control.

The test item had a significant inhibitory effect on the growth rate µ and yield Y of the algae after the exposure period of 72 hours at the nominal test concentration of 0.046 µg/L and at all higher test concentrations (results of Williams and Welch t-tests, one-sided smaller, $\alpha = 0.05$, Table 2 and Table 3).

The 72-hour NOEC was determined to be 0.022 µg/L, since at this test concentration the growth rate µ and yield Y of the algae after 72 hours was not significantly lower than in the solvent control.

The NOEC and EC_X values for growth rate and yield during the exposure period are summarized in the conclusions.

The microscopic examination of the algal cells at the end of the exposure period revealed no difference between the shape and size of the algae at nominal concentration of 1.0 µg/L and the algae in the solvent control.

No remarkable observations were made concerning the appearance of the test media. All test media were clear solutions throughout the exposure period.

The pH increased in the control and the solvent control from 7.3 and 7.4 at the start of the exposure period to 8.8 at the end of the exposure period, respectively (Table 15) fulfilling the requirement of the OECD guideline that the pH of the control medium should not increase by more than 1.5 units during the test.

The pH of the test media with test item was in the range of 7.3 to 8.8 during the exposure period (Table 15). The water temperature during the test was maintained at 23 °C.

7.3 Recovery of Algal Growth

After the 3-day exposure period, the recovery of the algal growth was monitored during 72 hours for the test concentrations of 0.046 to 1.0 µg/L (Figure 4) and during a second period of 72 hours for the test concentrations of 0.22 to 1.0 µg/L (Figure 5).

The biomass of algae during the recovery periods is shown in Table 6 and Table 10.

7.3.1 Recovery of Algal Growth during the First Recovery Period

The algal growth (biomass) in the solvent control was not statistically significantly different from the control after the 72 hours of the first recovery period (according to a Student-t test, $\alpha = 0.05$, two-sided). Therefore all treatments were compared to the pooled control.

At the end of the first recovery period, the average growth rates and yield at the nominal test concentrations of 0.046 and 0.10 µg/L were not statistically significantly different from the pooled control and thus recovery in algal growth at these test concentrations could be demonstrated. At the nominal test concentrations from 0.22 to 1.0 µg/L, the average growth rates and the yield were statistically significantly different from the pooled control (results of Williams t-test, one-sided smaller, $\alpha = 0.05$, Table 7 and Table 8).

The microscopic examination of the algal cells at the end of the exposure period showed no difference between the algae growing at the nominal test concentration of 0.46 µg/L and the algal cells in the solvent control. The algal cells at the highest test concentration of 1.0 µg/L were brown colored compared to the algal cells in the solvent control.

7.3.2 Recovery of Algal Growth during the Second Recovery Period

The algal growth (biomass) in the solvent control was not statistically significantly different from the control after the 72 hours of the second recovery period (according to a Student-t test, $\alpha = 0.05$, two-sided). Therefore all treatments were compared with the pooled control.

Since growth inhibition (yield and biomass) was observed at the nominal test concentrations of 0.22, 0.46 and 1.0 µg/L after the first recovery period, the test was prolonged for 72 hours with these three test concentrations.

At the end of the second recovery period the average growth rate and yield at all tested concentrations were not significantly different from the pooled control (results of Williams and Welch t-tests, one-sided smaller, $\alpha = 0.05$, Table 11 and Table 12).

The microscopic examination of the algal cells at the end of the second recovery period revealed no difference between the shape and size of the algae at nominal 1.0 µg/L and the algae in the solvent control.

7.3.3 Water quality

At the start of the recovery periods, the pH values in the test media were between 7.3 and 7.6. At the end of the recovery periods, pH values between 7.2 and 9.2 were measured (Table 15). The water temperature during the recovery periods was maintained at 23 °C.

8 VALIDITY

The values for the validity criteria of the test were calculated by the statistic program ToxRat Professional® [9].

According to the test guidelines OECD 201 (2011) all validity criteria have been fulfilled after the 72 hours exposure period:

Parameter (in the controls)	Required for less frequently tested species	Present test after 72 hours exposure period	
		Control	Solvent Control
Biomass increase [factor of]	≥16	38	38
Daily growth rates CV [%]	≤35	8.5	15
Average specific growth rate CV [%]	≤10	5.0	6.4

CV: Coefficient of variation

9 CONCLUSION

For the exposure period, the biological results can be summarized as follows (based on nominal concentrations of the test item Diflufenican Tech):

	EC Values (0-72 h) in [µg/L]	
	Growth rate	**Yield**
EC_{10}	0.029	0.025
95% confidence interval	0.026 – 0.033	0.023 – 0.027
EC_{20}	0.040	0.029
95% confidence interval	0.036 – 0.043	0.027 – 0.031
EC_{50}	0.071	0.039
95% confidence interval	0.067 – 0.075	0.037 – 0.040
NOEC	0.022	0.022
LOEC	0.046	0.046

Over the test period the biological results based on the average specific growth rates can be summarized as follows:

Nominal test item concentration [µg/L]	**Inhibition of average specific growth rate µ during the test [%]**		
	After exposure period (3 days)	**After 3 days recovery**	**After 6 days recovery**
0.022	-2.3	--	--
0.046	27*	-6.4	--
0.10	70*	-7.2	--
0.22	92*	19**	-4.1
0.46	98*	39**	-7.8
1.0	107*	92**	-13
NOEC/NOAEC [µg/L]	0.022	0.10	1.0

--: Not transferred, since full recovery in the previous test period
*: Mean value significantly lower than in the solvent control for growth rate and yield
**: Mean value significantly lower than in the pooled control for growth rate and yield

Note: Percentage inhibition values in excess of 100% are obtained when the biomass at the end of the interval is lower than at the start of the interval.

In conclusion, complete recovery of growth of the algae was demonstrated for all test concentrations at the latest after 6 days in test water free of test item.

Thus, based on the complete recovery of algal growth within 6 days post exposure, the $NOAEC_{A.falcatus}$ (No Observed Ecologically Adverse Effect Concentration) for the growth of *Ankistrodesmus falcatus* after a 3-day exposure to Diflufenican Tech is 1.0 µg/L.

10 REFERENCES

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[10] European Commission, Directorate General Health and Consumer Protection (2000): Residues: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414. SANCO/3029/99 rev. 4, 11/07/2000.

TABLES

The tabulated values represent rounded results obtained by calculation using the exact raw data.

Table 1 Biomass of Algae during the 72-hour Exposure Period

Nominal test item concentration [µg/L]	Rep. no.	Biomass of algae*			
		0 hours	24 hours	48 hours	72 hours
Solvent Control	1	5.0	17.2	41.5	154.2
	2	4.0	13.0	46.1	142.6
	3	4.0	17.4	54.5	149.2
	4	3.2	14.2	54.0	157.4
	5	2.7	12.1	50.4	144.7
	6	4.5	13.2	48.4	140.8
	Mean	3.9	14.5	49.1	148.1
	SD	0.9	2.3	5.0	6.7
Control	1	5.4	14.3	47.7	176.9
	2	4.2	14.8	50.8	182.0
	3	3.8	15.1	47.4	178.9
	Mean	4.5	14.7	48.6	179.3
	SD	0.8	0.4	1.9	2.6
0.022	1	4.0	13.6	46.3	151.7
	2	3.0	12.3	35.7	127.7
	3	3.3	14.5	42.8	150.7
	Mean	3.4	13.4	41.6	143.4
	SD	0.5	1.1	5.4	13.6
0.046	1	3.1	13.2	20.1	63.3
	2	3.5	12.3	19.0	43.8
	3	3.2	10.5	15.1	36.0
	Mean	3.3	12.0	18.1	47.7
	SD	0.2	1.4	2.6	14.0
0.10	1	2.7	8.1	7.0	9.7
	2	4.0	11.0	7.1	11.1
	3	3.4	8.7	7.6	9.1
	Mean	3.4	9.2	7.2	10.0
	SD	0.6	1.5	0.3	1.0

Continued on next page

Table 1 Biomass of Algae during the 72-hour Exposure Period (continued)

Nominal test item concentration [µg/L]	Rep. no.	Biomass of algae*			
		0 hours	24 hours	48 hours	72 hours
0.22	1	4.2	8.4	4.8	6.0
	2	2.9	7.1	4.8	4.6
	3	3.3	6.3	5.5	3.6
	Mean	3.5	7.3	5.0	4.7
	SD	0.6	1.1	0.4	1.2
0.46	1	3.0	5.7	4.2	4.1
	2	2.9	5.9	3.8	3.5
	3	3.4	5.6	3.8	2.6
	Mean	3.1	5.7	3.9	3.4
	SD	0.3	0.2	0.2	0.8
1.0	1	3.8	5.1	2.9	2.9
	2	3.4	4.9	4.2	2.3
	3	2.8	4.7	3.4	2.4
	Mean	3.4	4.9	3.5	2.6
	SD	0.5	0.2	0.6	0.3

SD: Standard deviation
*: The biomass was determined by fluorescence measurement (at least duplicate measurements per replicate) and is given as relative fluorescence units (x 10^4). At the start of the test, the initial cell density was 10000 algal cells/mL. , 10000 algal cells/mL corresponding 4.7 x 10^4 (see Figure 6) relative fluorescence units.

Table 2 Average Growth Rates (μ) during the Exposure Period

Nominal test item concentration	Average growth rate μ (day^{-1}) and inhibition of μ (I_r)					
	0-24 h		0-48 h		0-72 h	
[μg/L]	μ	I_r [%]	μ	I_r [%]	μ	I_r [%]
Solvent Control	1.326	0.0	1.275	0.0	1.219	0.0
0.022	1.374	-3.6	1.250	2.0	1.247	-2.3
0.046	1.302	1.8	0.854*	33.0	0.886*	27.3
0.10	1.009*	23.9	0.386*	69.7	0.364*	70.2
0.22	0.741*	44.1	0.188*	85.2	0.101*	91.7
0.46	0.618*	53.4	0.121*	90.5	0.024*	98.0
1.0	0.387*	70.8	0.022*	98.3	-0.091*	107.4

*: Mean value significantly lower than in the control
(according to Williams t-test, one-sided smaller, $\alpha = 0.05$)

Table 3 Yield (Y) during the Exposure Period

Nominal test item concentration	Yield Y (x 10^4) and inhibition of Y (I_y)					
	0-24 h		0-48 h		0-72 h	
[μg/L]	Y	I_y [%]	Y	I_y [%]	Y	I_y [%]
Solvent Control	10.6	0.0	45.2	0.0	144.2	0.0
0.022	10.0	5.7	38.2	15.7	140.0	3.0
0.046	8.8	17.6	14.8#	67.2	44.5#	69.2
0.10	5.9#	44.8	3.9#	91.5	6.6#	95.4
0.22	3.8#	64.4	1.5#	96.6	1.3#	99.1
0.46	2.6#	75.2	0.8#	98.1	0.3#	99.8
1.0	1.6#	85.4	0.2#	99.7	-0.8#	100.6

#: Mean value significantly lower than in the control
(according to Welch t-test, one-sided smaller, $\alpha = 0.05$)
Note: Percentage inhibition values in excess of 100% are obtained when the biomass at the end of the interval is lower than at the start of the interval.

Table 4 Average Section-by-Section Growth Rates during the Exposure Period

Nominal test item concentration	Section-by-section growth rates (day^{-1}) and inhibition of the growth rates (I_r)					
	0-24 h		24-48 h		48-72 h	
[µg/L]	µ	I_r [%]	µ	I_r [%]	µ	I_r [%]
Solvent Control	1.326	0.0	1.224	0.0	1.107	0.0
0.022	1.374	-3.6	1.126	8.0	1.241	-12.1
0.046	1.302	1.8	0.406	66.9	0.950	14.2
0.10	1.009	23.9	-0.238	119.4	0.320	71.1
0.22	0.741	44.1	-0.364	129.7	-0.075	106.8
0.46	0.618	53.4	-0.375	130.6	-0.170	115.3
1.0	0.387	70.8	-0.343	128.0	-0.316	128.5

Table 5 Sectional Growth Rate for Control and Solvent Control

		Section-by-section growth rates (day^{-1})		
		0-24 h	24-48 h	48-72 h
	Replicate	µ	µ	µ
Control	1	0.980	1.201	1.311
	2	1.265	1.236	1.276
	3	1.371	1.143	1.329
Solvent Control	1	1.229	0.877	1.314
	2	1.168	1.269	1.129
	3	1.479	1.141	1.007
	4	1.491	1.334	1.070
	5	1.507	1.426	1.055
	6	1.083	1.299	1.068

Table 6 Biomass of Algae during the First Recovery Period

Nominal test item concentration [µg/L]	Rep. no.	Biomass of algae*			
		0 hours¥	24 hours	48 hours	72 hours
Solvent Control	1	5.7	22.2	91.0	263.3
	2	6.3	20.4	91.2	271.8
	3	4.5	20.3	90.8	242.1
	4	4.4	20.6	90.8	244.4
	5	4.9	23.5	82.3	231.2
	6	4.9	21.4	95.2	258.2
	Mean	5.1	21.4	90.2	251.8
	SD	0.8	1.3	4.2	15.1
Control	1	4.9	24.9	92.2	252.0
	2	5.4	25.2	97.1	252.8
	3	5.0	20.9	93.2	236.3
	Mean	5.1	23.7	94.2	247.1
	SD	0.3	2.4	2.6	9.3
0.046	1	3.5	25.5	101.6	254.9
	2	4.7	22.8	116.1	273.7
	3	4.6	26.5	115.7	277.2
	Mean	4.3	24.9	111.1	268.6
	SD	0.6	1.9	8.3	12.0
0.10	1	4.1	20.1	89.2	245.3
	2	3.8	18.6	90.3	269.0
	3	3.8	20.0	77.1	248.1
	Mean	3.9	19.6	85.5	254.1
	SD	0.2	0.9	7.3	13.0
0.22	1	5.5	13.0	35.2	113.5
	2	4.6	10.5	34.9	118.1
	3	4.4	7.9	31.8	102.7
	Mean	4.8	10.5	34.0	111.4
	SD	0.6	2.5	1.9	7.9

Continued on next page

Table 6 Biomass of Algae during the First Recovery Period (continued)

Nominal test item concentration [µg/L]	Rep. no.	Biomass of algae*			
		0 hours¥	24 hours	48 hours	72 hours
0.46	1	3.1	5.4	12.9	38.0
	2	3.1	5.0	10.1	35.8
	3	3.3	5.0	11.7	31.3
	Mean	3.2	5.1	11.6	35.0
	SD	0.1	0.3	1.4	3.4
1.0	1	4.0	4.5	3.4	5.9
	2	3.5	3.9	3.2	4.5
	3	°	°	°	°
	Mean	3.7	4.2	3.3	5.2
	SD	0.4	0.5	0.1	1.0

SD: Standard deviation
*: The biomass was determined by fluorescence measurement (at least duplicate measurements per replicate) and is given as relative fluorescence units (x 10^4).
¥: The values of the biomass were determined in the fresh test solutions after filtration of the algal cells, see section 5.5.2.
°: Only two replicates could be prepared, due to insufficient cell number after pooling and filtration of the six replicates at the end of the exposure period.

Table 7 Average Growth Rates (μ) during the First Recovery Period

Nominal test item concentration	Average growth rate μ (day^{-1}) and inhibition of μ (I_r)					
	0-24 h		0-48 h		0-72 h	
[μg/L]	μ	I_r [%]	μ	I_r [%]	μ	I_r [%]
Pooled Control	1.471	0.0	1.446	0.0	1.299	0.0
0.046	1.768	-20.2	1.631	-12.8	1.382	-6.4
0.10	1.613	-9.7	1.543	-6.7	1.392	-7.2
0.22	0.758*	48.5	0.978*	32.4	1.048*	19.3
0.46	0.473*	67.8	0.641*	55.6	0.798*	38.5
1.0	0.111*	92.4	-0.062*	104.3	0.107*	91.8

*: Mean value significantly lower than in the control
(according to Williams t-test, one-sided smaller, $\alpha = 0.05$)
Note: Percentage inhibition values in excess of 100% are obtained when the biomass at the end of the interval is lower than at the start of the interval.

Table 8 Yield (Y) during the First Recovery Period

Nominal test item concentration	Yield Y (x 10^4) and inhibition of Y (I_y)					
	0-24 h		0-48 h		0-72 h	
[μg/L]	Y	I_y [%]	Y	I_y [%]	Y	I_y [%]
Pooled Control	17.1	0.0	86.4	0.0	245.1	0.0
0.046	20.7	-21.1	106.8	-23.6	264.3	-7.8
0.10	15.7	8.2	81.6	5.6	250.2	-2.1
0.22	5.6*	67.0	29.1*	66.3	106.6*	56.5
0.46	1.9*	88.7	8.4*	90.3	31.9*	87.0
1.0	0.4*	97.4	-0.4*	100.5	1.4*	99.4

*: Mean value significantly lower than in the control
(according to Williams t-test, one-sided smaller, $\alpha = 0.05$)
Note: Percentage inhibition values in excess of 100% are obtained when the biomass at the end of the interval is lower than at the start of the interval.

Table 9 Average Section-by-Section Growth Rates during the First Recovery Period

Nominal test item concentration	Section-by-section growth rates (day^{-1}) and inhibition of the growth rates (I_r)					
	0-24 h		24-48 h		48-72 h	
[µg/L]	µ	I_r [%]	µ	I_r [%]	µ	I_r [%]
Pooled Control	1.471	0.0	1.421	0.0	1.005	0.0
0.046	1.768	-20.2	1.494	-5.2	0.884	12.1
0.10	1.613	-9.7	1.473	-3.7	1.091	-8.5
0.22	0.758	48.5	1.197	15.7	1.188	-18.1
0.46	0.473	67.8	0.810	43.0	1.112	-10.6
1.0	0.111	92.4	-0.236	116.6	0.446	55.6

Note: Percentage inhibition values in excess of 100% are obtained when the biomass at the end of the interval is lower than at the start of the interval.

Table 10 Biomass of Algae during the Second Recovery Period

Nominal test item concentration [µg/L]	Rep. no.	Biomass of algae*			
		0 hours¥	24 hours	48 hours	72 hours
Solvent Control	1	5.0	7.5	36.0	104.0
	2	4.1	10.5	44.0	114.8
	3	3.9	11.3	45.7	120.9
	4	4.5	11.6	48.8	122.7
	5	3.2	13.3	42.9	117.5
	6	3.7	13.3	45.9	115.8
	Mean	4.1	11.3	43.9	115.9
	SD	0.6	2.2	4.4	6.6
Control	1	5.0	6.7	48.5	116.0
	2	4.4	14.8	42.7	129.6
	3	3.4	14.3	44.3	113.8
	Mean	4.3	11.9	45.2	119.8
	SD	0.8	4.6	3.0	8.6
0.22	1	4.7	13.5	56.2	144.9
	2	4.2	9.4	55.7	149.1
	3	4.6	12.9	58.9	147.7
	Mean	4.5	11.9	56.9	147.2
	SD	0.3	2.2	1.7	2.1
0.46	1	5.0	7.8	48.2	141.7
	2	3.8	17.2	68.1	200.5
	3	4.0	12.1	48.0	141.7
	Mean	4.3	12.4	54.8	161.3
	SD	0.6	4.7	11.6	33.9
1.0	1	5.3	21.0	71.5	202.9
	2	3.2	16.6	47.8	171.3
	Mean	4.3	18.8	59.6	187.1
	SD	1.5	3.1	16.7	22.4

SD: Standard deviation

*: The biomass was determined by fluorescence measurement (at least duplicate measurements per replicate) and is given as relative fluorescence units (x 10^4).

¥: The values of the biomass were determined in the fresh test solutions after filtration of the algal cells, see section 5.5.2.

Table 11 Average Growth Rates (μ) during the Second Recovery Period

Nominal test item concentration	Average growth rate μ (day^{-1}) and inhibition of μ (I_r)					
	0-24 h		0-48 h		0-72 h	
[μg/L]	μ	I_r [%]	μ	I_r [%]	μ	I_r [%]
Pooled Control	1.001	0.0	1.190	0.0	1.119	0.0
0.22	0.966	3.5	1.271	-6.8	1.164	-4.1
0.46	1.012	-1.2	1.269	-6.6	1.206	-7.8
1.0	1.503	-50.1	1.322	-11.0	1.268	-13.3

No statistically significant effect at all test concentrations compared to pooled control (according to Williams t-test and Welch t-test, one-sided smaller, $\alpha = 0.05$)

Table 12 Yield (Y) during the Second Recovery Period

Nominal test item concentration	Yield Y (x 10^4) and inhibition of Y (I_y)					
	0-24 h		0-48 h		0-72 h	
[μg/L]	Y	I_y [%]	Y	I_y [%]	Y	I_y [%]
Pooled Control	7.3	0.0	40.2	0.0	113.1	0.0
0.22	7.4	-1.2	52.5	-30.5	142.7	-26.2
0.46	8.1	-9.7	50.5	-25.6	157.0	-38.8
1.0	14.5	-97.6	55.3	-37.7	182.8	-61.7

No statistically significant effect at all test concentrations compared to pooled control (according to Williams t-test and Welch t-test, one-sided smaller, $\alpha = 0.05$)

Table 13 Average Section-by-Section Growth Rates during the Second Recovery Period

Nominal test item concentration	Section-by-section growth rates (day^{-1}) and inhibition of the growth rates (I_r)					
	0-24 h		24-48 h		48-72 h	
[μg/L]	μ	I_r [%]	μ	I_r [%]	μ	I_r [%]
Pooled Control	1.001	0.0	1.380	0.0	0.975	0.0
0.22	0.966	3.5	1.576	-14.2	0.950	2.5
0.46	1.012	-1.2	1.526	-10.5	1.080	-10.9
1.0	1.503	-50.1	1.141	17.4	1.160	-19.0

Table 14 Light Intensity during the Test Period

Light intensity [$\mu Es^{-1}m^{-2}$]	Day 0: Start of Exposure Period	Day 3: Start of First Recovery	Day 6: Start of Second Recovery
Minimum	65	65	63
Maximum	68	68	69
Mean	67	67	67

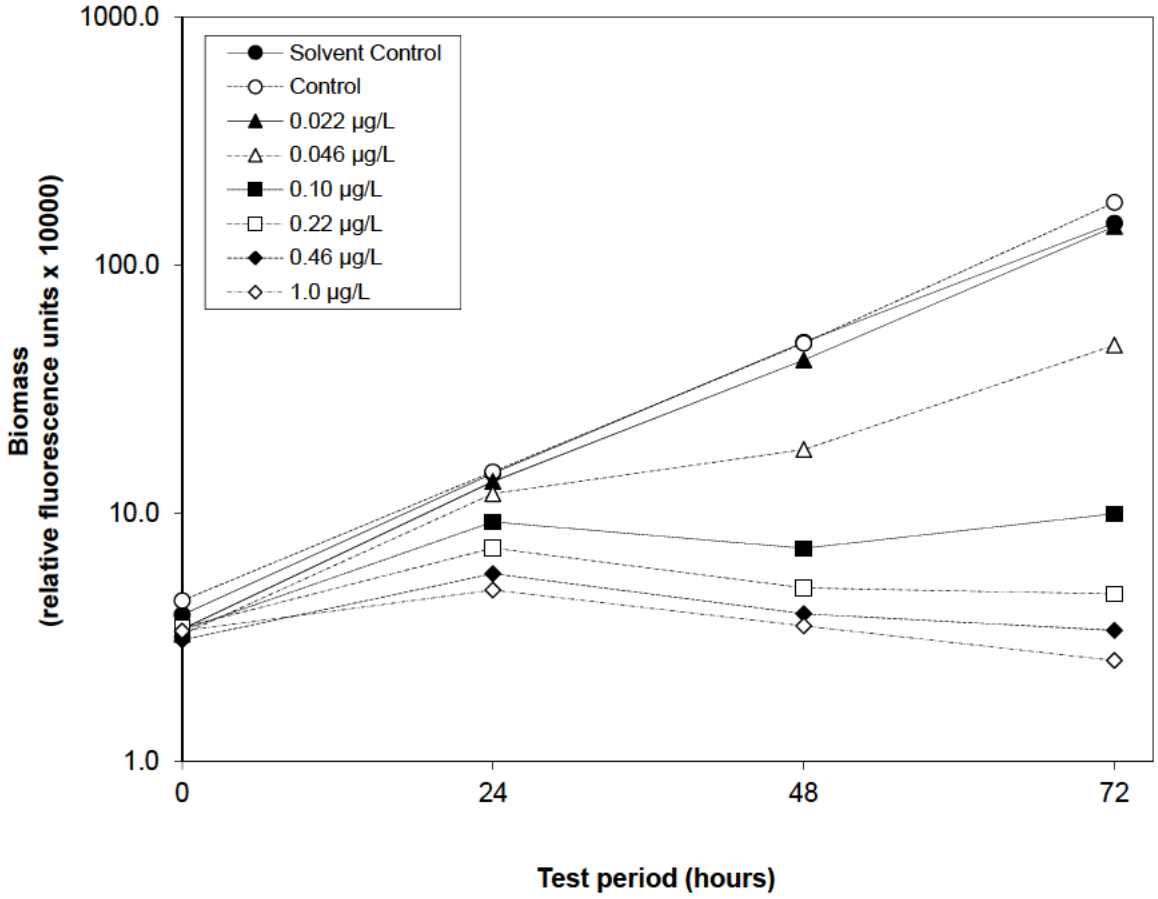
Table 15 pH Values in the Treatments

Nominal test item concentration	pH values Exposure Period		pH values First Recovery Period		pH values Second Recovery Period	
[mg/L]	Start	End	Start	End	Start	End
Control	7.3	8.8	7.6	8.9	7.3	8.7
Solvent Control	7.4	8.8	7.5	9.2	7.3	8.8
0.022	7.3	8.8	*	*	*	*
0.046	7.3	8.7	7.5	8.9	*	*
0.10	7.3	8.5	7.5	8.9	*	*
0.22	7.3	7.5	7.5	7.4	7.3	8.8
0.46	7.3	7.6	7.5	7.2	7.3	8.8
1.0	7.3	7.6	7.5	7.2	7.3	9.2

*: the pH was not measured since these test concentrations were not used for recovery.

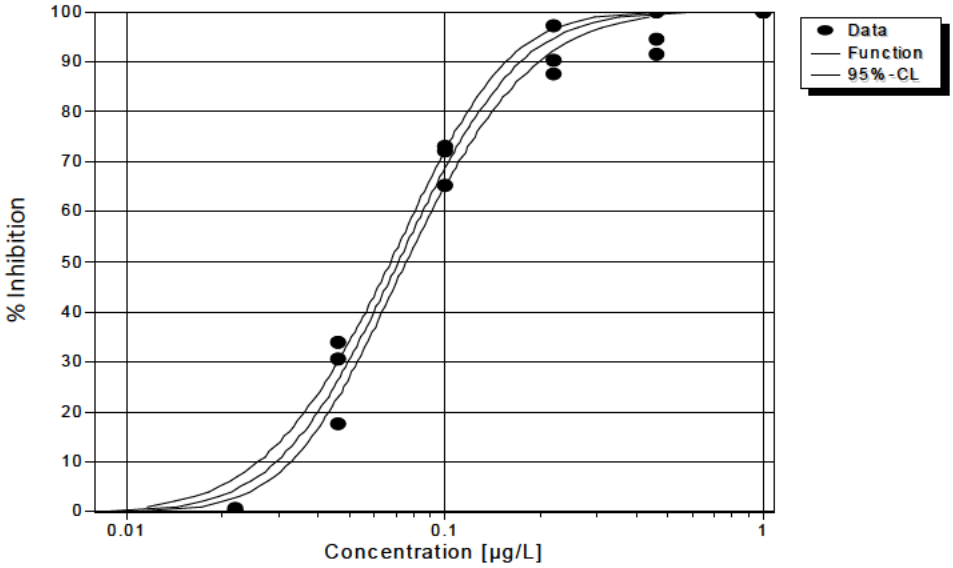
FIGURES

Figure 1 Growth Curves of the Algae during the 72-hour Exposure Period



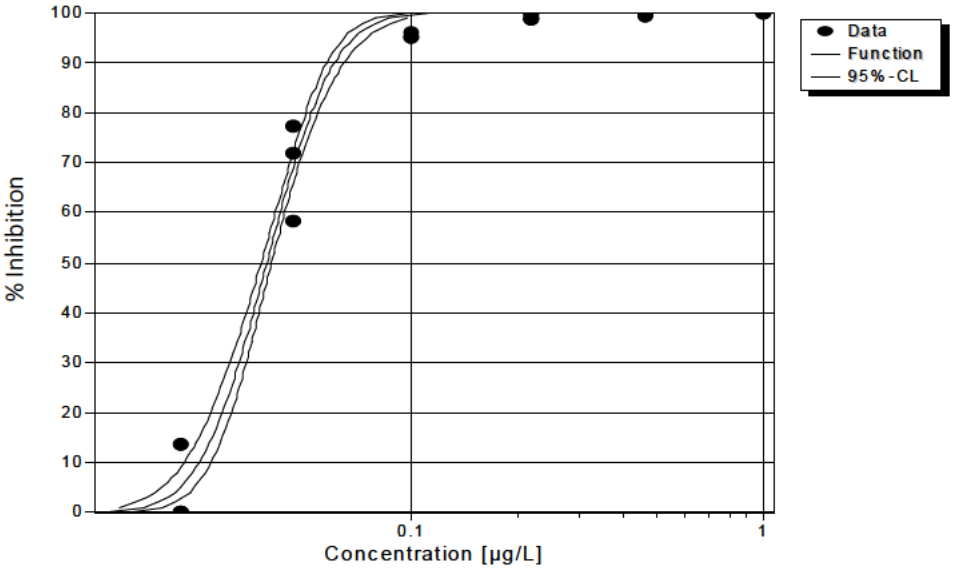
(Nominal concentrations of the test item Diflufenican Tech)

Figure 2 Concentration-Effect Relationship of Average Growth Rates after 72-hour Exposure Period



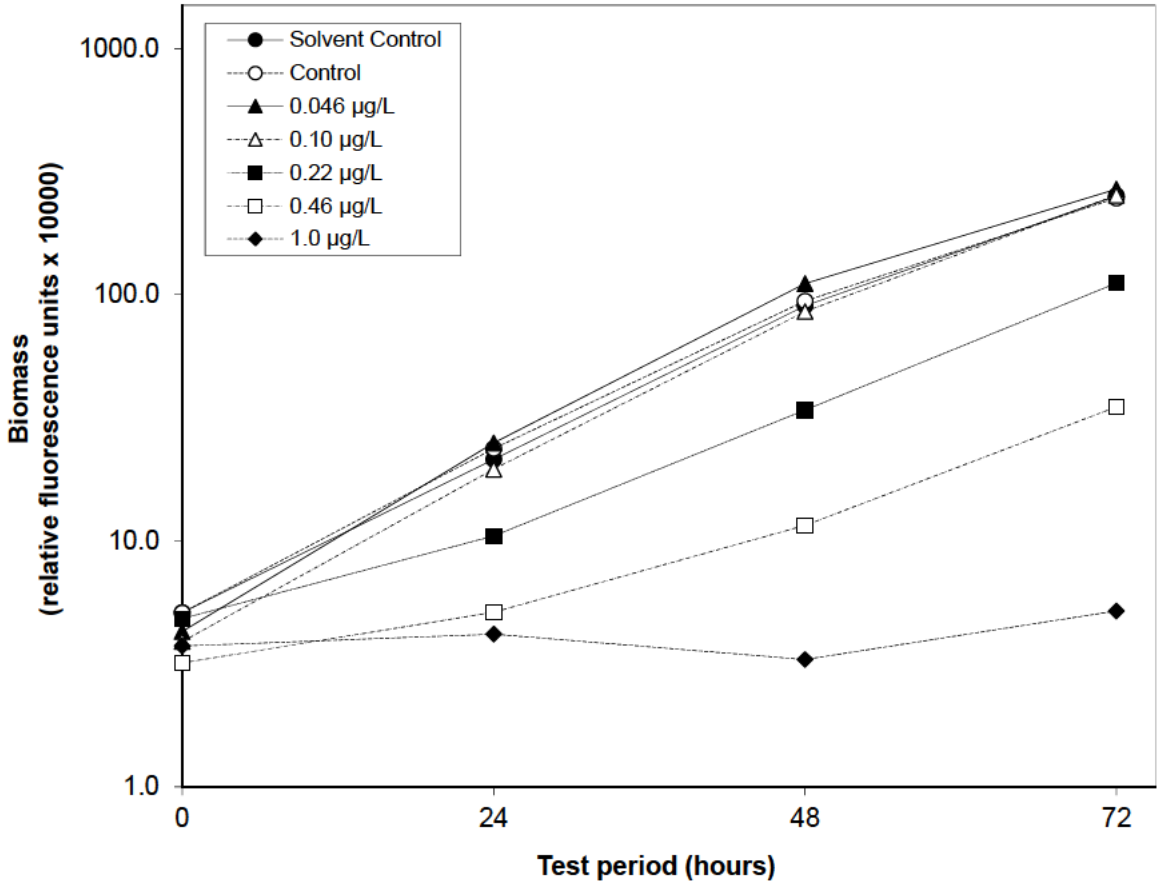
Nominal concentrations of the test item Diflufenican Tech

Figure 3 Concentration-Effect Relationship of Yield after 72-hour Exposure Period



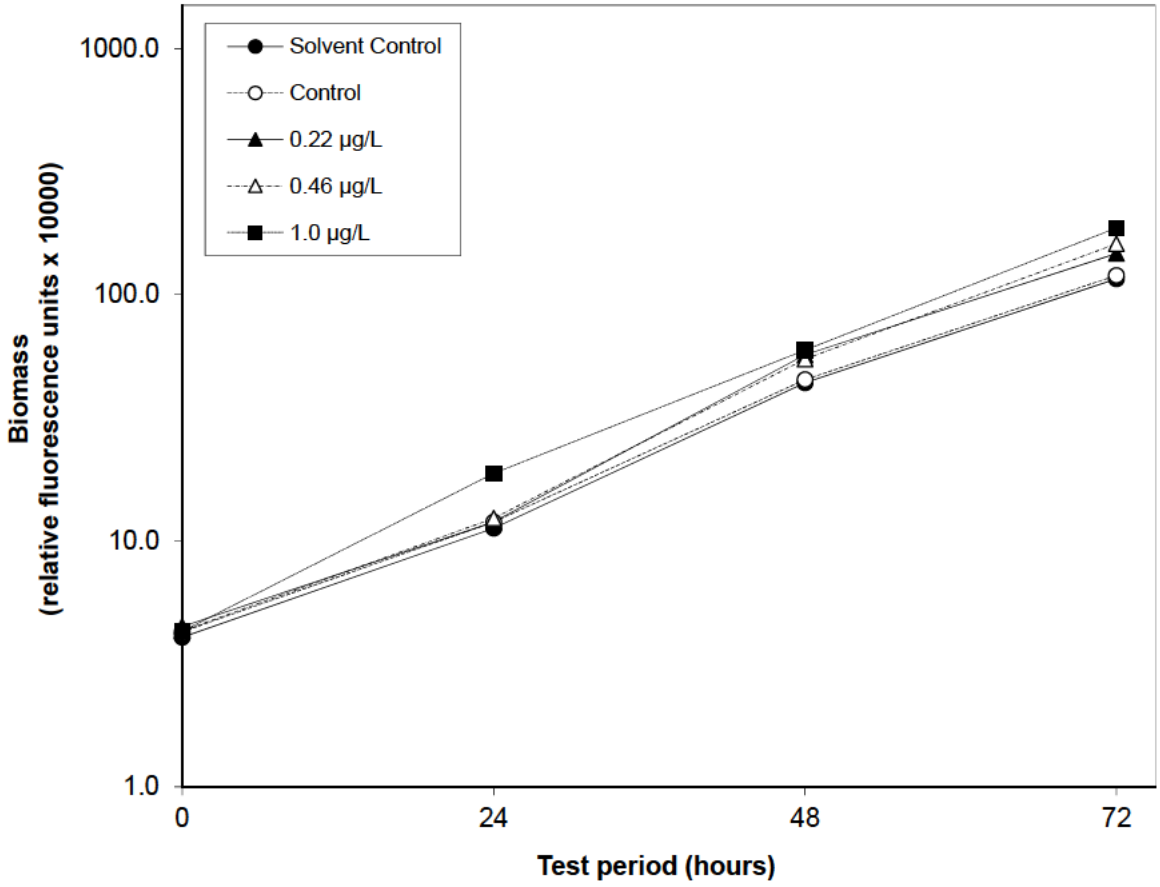
Nominal concentrations of the test item Diflufenican Tech

Figure 4 Growth Curves of the Algae during the First Recovery Period



(Nominal concentrations of the test item Diflufenican Tech during the 72-hour exposure period)

Figure 5 Growth Curves of the Algae during the Second Recovery Period



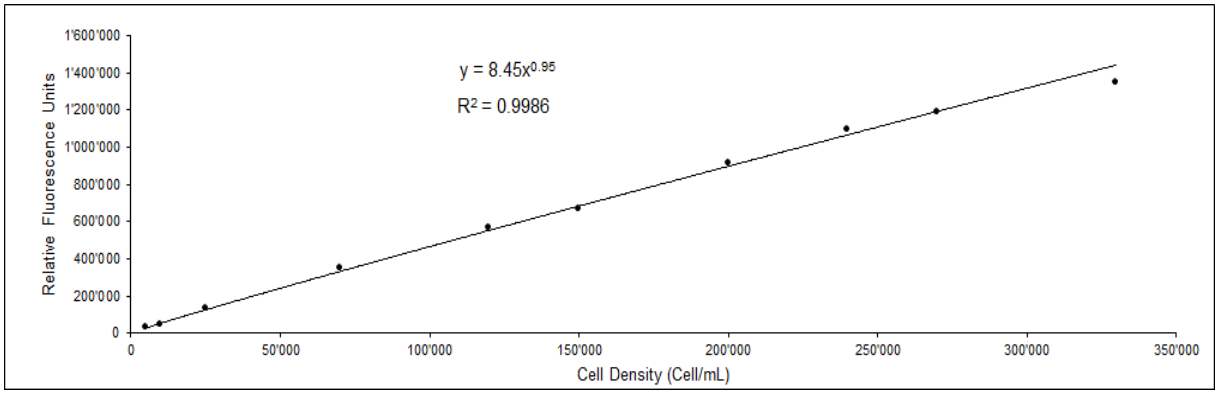
(Nominal concentrations of the test item Diflufenican Tech during the 72-hour exposure period)

Figure 6 Calibration of Cell Density by Fluorescence Measurement

Calibration Data of Cell Density

Cell Density [Cell/mL]	Relative Fluorescence Units	Deviation of Calculated from Effective Value [%]
5000	28063	3.5
10000	47178	-10.5
25000	130993	5.1
70000	351429	6.3
120000	565787	2.4
150000	669341	-2.2
200000	910343	1.5
240000	1096649	2.9
270000	1187256	-0.5
330000	1344240	-7.2

Calibration Plot



APPENDICES

Appendix 1 Analytical Investigations

ANALYTICAL PART TO REPORT

Diflufenican Tech: Toxicity to *Ankistrodesmus falcatus* in a 72-Hour Algal Growth Inhibition Test Supplemented with Testing for Recovery of Growth

Sponsors:

Rotam Agrochem International Co. Ltd.
26/F, E-Trade Plaza
Chai Wan
Hong Kong
China

UPL Europe Ltd
1st Floor, The Centre
Birchwood Park
Warrington WA3 6YN
United Kingdom

Test Facility: [redacted]

Analytical Chemistry: [redacted]

Study Identification: 20160072

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1 MATERIALS AND METHODS

1.1 Introduction

The test item concentrations in the test samples were determined by high performance liquid chromatography-mass spectrometry (HPLC-MS/MS) using external calibration. The test item gave a chromatographic profile consisting of a single peak. The analytical method was validated according to SANCO/3029/99 rev. 4 [10].

The analytical method was developed by [redacted] These experiments were not performed according to the regulations of GLP [redacted]

1.2 Test Item

The test item is described in the biological part of this study.

1.3 Analytical Standard

The test item described in the biological part of this study was also used as the analytical standard.

1.4 Analytical Procedure

1.4.1 Storage

The samples from day 0 were stored frozen until analysis was performed (at about -20°C). The other samples were analysed at the day of sampling.
The storage period between sampling and actual analysis for the samples from day 0 was 3 days. The stability during storage was confirmed by analysis of samples from the range finder test. These experiments were not confirmed according to the regulations of GLP ([redacted]).

1.4.2 Reagents and Solvents

Water	prepared in-house using an ELGA water purification system
Test water	as described in the biological part of this study
Methanol	Sigma Aldrich, no. 34860

1.4.3 Preparation of Calibration Solutions

Day of analysis 1: Analysis of samples of sampling days 0, 3 and recovery phase 1

The test item (30.77 mg) was dissolved in methanol and made up to the mark in a 25 mL volumetric flask to prepare a stock solution with a concentration of 1231 mg/L.
A stock solution aliquot of 0.1 mL was diluted to 100 mL with methanol to obtain a diluted stock solution with a concentration of 1231 µg/L. An aliquot of 0.1 mL of the diluted stock

solution was diluted to 50 mL with a mixture of methanol / test water (1:1, v:v) to obtain a working solution with a concentration of 2.46 µg/L.
Defined volumes of this working solution were further diluted with a mixture of methanol / test water (1:1, v:v) to obtain calibration solutions of the test item in the range of 0.00981 to 0.757 µg/L. These solutions were used to calibrate the analytical system.

Day of analysis 2: Analysis of samples of recovery phase 2

The test item (32.77 mg) was dissolved in methanol and made up to the mark in a 25 mL volumetric flask to prepare a stock solution with a concentration of 1311 mg/L.
A stock solution aliquot of 0.1 mL was diluted to 100 mL with methanol to obtain a diluted stock solution with a concentration of 1311 µg/L. An aliquot of 0.1 mL of the diluted stock solution was diluted to 50 mL with a mixture of methanol / test water (1:1, v:v) to obtain a working solution with a concentration of 2.62 µg/L.
Defined volumes of this working solution were further diluted with a mixture of methanol / test water (1:1, v:v) to obtain calibration solutions of the test item in the range of 0.00914 to 0.0260 µg/L. These solutions were used to calibrate the analytical system.

1.4.4 Preparation of Spiked Samples

To demonstrate the validity of the method, untreated test water was spiked with the test item.

Day of analysis 1:

The test item (31.13 mg) was dissolved in methanol and made up to the mark in a 25 mL volumetric flask to prepare a stock solution with a concentration of 1245 mg/L.
A stock solution aliquot of 0.2 mL was diluted to 20 mL with methanol to obtain a diluted stock solution with a concentration of 12.45 mg/L.
An aliquot of 0.21 mL of the diluted stock solution was further diluted to 20 mL with a mixture of methanol / test water (1:1, v:v) to obtain a fortification solution 1 with a concentration of 131 µg/L. An aliquot of 0.3 mL of the fortification solution 1 was diluted to 20 mL with a mixture of methanol / test water (1:1, v:v) to obtain a fortification solution 2 with a concentration of 1.96 µg/L.
Each 0.1 mL of fortification solution 1 and 2 were diluted to 10 mL with test water to obtain spiked samples with concentrations of 0.0196 µg/L and 1.31 µg/L. Five spiked recovery samples were freshly prepared per concentration level, subjected to the same treatment as a test sample but without storage and subsequently analyzed. In addition, test water without the test item was analyzed (analytical blank).

Day of analysis 2:

The test item (41.21 mg) was dissolved in methanol and made up to the mark in a 25 mL volumetric flask to prepare a stock solution with a concentration of 1648 mg/L.
A stock solution aliquot of 0.16 mL was diluted to 20 mL with methanol to obtain a diluted stock solution with a concentration of 13.19 mg/L.
An aliquot of 0.21 mL of the diluted stock solution was further diluted to 20 mL with a mixture of methanol / test water (1:1, v:v) to obtain a fortification solution 1 with a concentration of 138 µg/L. An aliquot of 0.3 mL of the fortification solution 1 was diluted to 20 mL with a

mixture of methanol / test water (1:1, v:v) to obtain a fortification solution 2 with a concentration of 2.08 µg/L.
0.1 mL of fortification solution 2 were diluted to 10 mL with test water to obtain spiked samples with concentrations of 0.0208 µg/L. Five spiked recovery samples were freshly prepared at this concentration level, subjected to the same treatment as a test sample and subsequently analyzed. In addition, test water without the test item was analyzed (analytical blank).

1.4.5 Analysis of Samples

Test samples and control samples of sampling day 0 were thawed at room temperature for about 1 hour and shaken manually to obtain homogeneous sample solutions. The other samples were worked-up at the day of sampling.
The samples (10 mL) were diluted to 20 mL with methanol resulting in a sample preparation factor of 2. The test and control samples from sampling day 3 were centrifuged (2465 g, 5 min) before HPLC/MS analysis due to the presence of algae. The centrifugation process was verified by subjecting one spike sample per concentration level to this treatment.

1.4.6 Instrumental Setup (HPLC/MS/MS conditions)

Autosampler: Agilent G7167B Multisampler
Pump: Agilent G7120A High Speed Pump
Detector: AB Sciex QTRAP 6500

PreColumn: Phenomenex Security Guard C18; 4 mm x 3 mm
Column: Water Acquity C18 UPLC; 50 mm x 2.1 mm; 1.7 µm
Columne temperature: 40°C in a thermostatic oven

Eluent A: Water with 0.1 % formic acid
Eluent B: Methanol with 0.1 % formic acid

Gradient:

Minutes	% Eluent A	% Eluent B
0.0	50	50
2.0	0	100
2.5	0	100
2.6	50	50
3.5	50	50

Flow Rate: 400 µL/minute
Injection Volume: 20 µL

Ionization Mode: ESI positive
Heater Gas Temperature: 500°C
Spray Voltage: 5500 V

Scan Mode: Multiple reaction monitoring (MRM)

MS/MS Conditions:

	Ion Polarity	Precursor Ion	Product Ion	Dwell Time [ms]	Collision Energy [V]
Quantifier *	positive	394.9	246.0	150	47.0
Qualifier	positive	394.9	266.0	150	33.0

** used for evaluation*

Retention Time: Approximately 2.1 minutes

1.4.7 Data Evaluation

Injected samples were quantified by peak areas with reference to the respective calibration curve. The latter was obtained by correlation of the peak area of the calibration solutions to their corresponding concentration in µg/L.
The correlation was performed using the potential function shown below. The results obtained are presented in Table 1. From the calibration curve, the concentration x of the test item in an injected sample was calculated by equation 1:

$$\ln y = \ln a + b \times \ln x \quad or \quad y = a \times x^{b} \quad or \quad x = \sqrt[b]{\frac{y}{a}} \qquad (1)$$

where: x: concentration of the test item in injected sample [µmol/L]
y: peak area of the test item in injected sample [counts]
ln a: y-axis intercept
b: slope

The concentration of the test item in a sample was calculated by equation 2:

$$c = x \cdot F \qquad (2)$$

where: c: concentration of the test item in the sample [µg/L]
x: concentration of the test item in injected sample [µg/L]
F: sample preparation factor (F = 2)

The concentration determined in a test sample as percentage of the nominal concentration (= recovery rate R) was calculated by equation 3:

$$R = \% \text{ nominal} = \frac{c}{c_{nom}} \cdot 100\% \qquad (3)$$

where: R: recovery rate or % nominal
c: determined concentration in the test sample [µg/L]
c_{nom}: nominal concentration in the test sample [µg/L]

2 RESULTS

2.1 Validation of Analytical Method According to SANCO/3029/99 rev. 4

Specificity

The analyzed analytical control (test water) and the biological control samples did not affect the chromatogram at the retention time of the test item with regard to the limit of detection (LOD): the signal observed in these samples was less than 30 % of the limit of quantification (LOQ) and thus below the LOD.
The calibration solutions contained a peak specific for the test item, which area changed accordingly with known concentration.
This shows the specificity of the analytical method.

Calibration

An example of the calibration data for the calibration solutions of the test item are given in Table 1. The correlation coefficient R^2 of the calibration curves used were 0.9996 (day of analysis 1) and 0.996 (day of analysis 2). This reflects the linearity of the analytical system within the calibration range of 0.00981 to 0.757 µg test item /L (day of analysis 1) and 0.00914 to 0.0260 µg test item /L (day of analysis 2), covering the measured and nominal range of the concentrations in the samples from the biological test. Typical chromatograms are shown in Figures 2 and 3.

Accuracy (Recovery) and Precision

Concurrent with the sample analysis, a set of recovery samples fortified at relevant concentrations of the test item (0.0196 and 1.31 µg/L) was prepared five-fold and analyzed at the day of analysis 1. A 2nd set of recovery samples fortified at the low-level was prepared five-fold and analyzed at the day of analysis 2. The results obtained for the concentrations of the test item in the recovery samples are presented in Table 2. A representative chromatogram is given in Figure 4.

For day of analysis 1, the average recoveries for the non-centrifuged samples were found to be 109 % and 91 % of the spiked values with relative standard deviations of 12.7 % and 0.7 % respectively. For day of analysis 2, the average recovery was found to be 84 % of the spiked values with a relative standard deviation of 3.3 %.

The recoveries for the centrifuged samples were found to be 98 % and 91 % of the spiked values.

The method was considered to be sufficiently accurate and precise for the purpose of this test. The test sample results were not corrected for recovery.

Limit of Quantification (LOQ)

The limit of quantification for the test item in the test samples was derived from the lowest concentration of spiked samples which was validated. The LOQ is 0.0196 µg test item/L for day of analysis 1 and 0.0208 µg test item/L for day of analysis 2.

Limit of Detection (LOD)

The limit of detection (LOD) was calculated to be 0.006 µg/L (one third of the LOQ).

2.2 Test Samples

The results obtained for the concentrations of the test samples are presented in Table 4.

The concentrations as % nominal found in the test samples ranged from 91 % to 110 % at test start and from 91 % to 125 % at test end, thereof all except of one value between 91 % and 100 %. The slightly enhanced value of 125 % in the low-level sample can be due to some variation of the analytical method at this very low concentration level.

The concentrations in the samples from the recovery phase were below the limit of quantification, except in the highest test concentration of 1.0 µg/L at the start of the first recovery phase which was found to be 0.022 µg/L.

Typical chromatograms for the test samples are shown in Figure 5 to Figure 8.

3 CONCLUSION

The analytical procedure for determination of the test item in test water showed acceptable recoveries and relative standard deviations. The method of analysis was validated according to SANCO/3029/99 rev. 4 [10] and proven to be suitable for use.

The concentrations of the test item in the test samples showed the correct dosage and stability during the performance of the test.

The results obtained for the samples from the algae recovery phase showed the correct preparation of these samples.

TABLES AND FIGURES

Table 1 Calibration Data of Test Item (for Day of Analysis 1)

Concentration of Test Item [µg/L]	Peak Area [counts]	Deviation of Calculated from Effective Value [%]
0.00981	69170	4.2
0.0147	100614	3.1
0.0244	151119	-4.9
0.0483	290468	-4.8
0.0947	581439	0.4
0.1823	1082295	-0.1
0.3755	2178253	1.0
0.6330	3511781	-1.2
0.7574	4333368	2.9

Figure 1 Calibration Plot

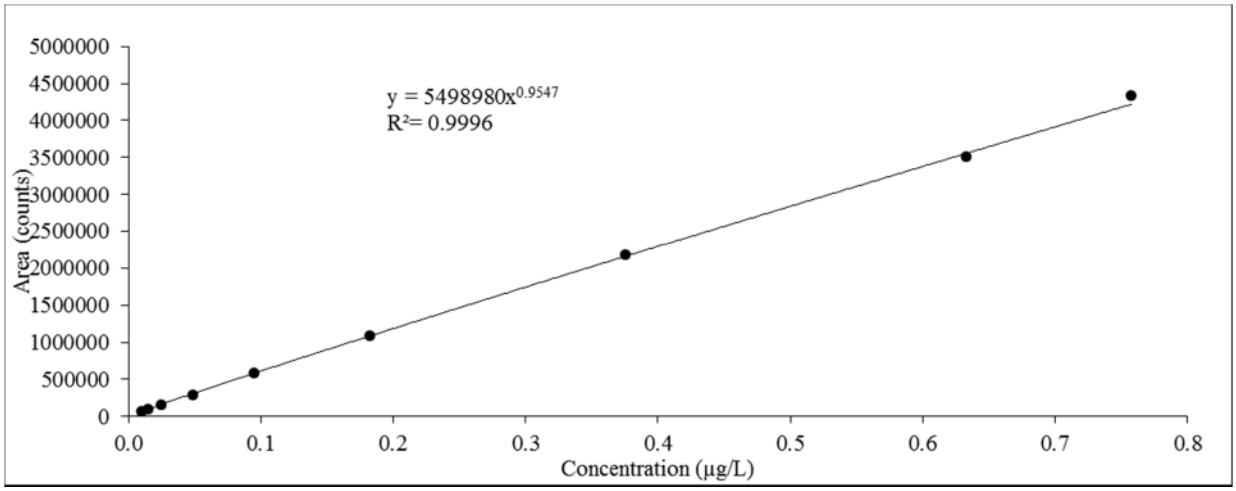


Table 2 Results for Non-Centrifuged Spiked Samples

Nominal Concentration of Test Item c_{nom} [µg/L]	Measured Concentration of Test Item in Spiked Sample x [µg/L]	Sample Preparation Factor F	Concentration of Test Item Determined in Spiked Sample c [µg/L]	Recovery R [%]	Accuracy (Average Recovery) [%]	Precision (Relative Standard Deviation of Recovery) [%]
Day of analysis 1						
0	n.d.	2	< LOQ	n.a.	n.a.	n.a.
0.0196	0.0099	2	0.0197	101		
0.0196	0.0121	2	0.0241	123		
0.0196	0.0119	2	0.0238	121	109	12.7
0.0196	0.0110	2	0.0219	112		
0.0196	0.0089	2	0.0177	90		
1.31	0.596	2	1.19	91		
1.31	0.595	2	1.19	91		
1.31	0.593	2	1.19	91	91	0.7
1.31	0.591	2	1.18	90		
1.31	0.602	2	1.20	92		
Day of analysis 2						
0	n.d.	2	< LOQ	n.a.	n.a.	n.a.
0.0208	0.0086	2	0.0172	83		
0.0208	0.0086	2	0.0171	83		
0.0208	0.0092	2	0.0184	88	84	3.3
0.0208	0.0086	2	0.0172	83		
0.0208	0.0085	2	0.0169	81		
				Acceptance target	70-110 %	≤ 20 %

n.d. = no test item detected

LOQ = 0.0196 µg test item /L (day of analysis 1) or 0.0208 µg test item /L (day of analysis 2)

n.a. = not applicable

The tabulated values of the samples represent rounded results obtained by calculation using the exact raw data.

Table 3 Results for Centrifuged Spiked Samples

Nominal Concentration of Test Item c_{nom} [µg/L]	Measured Concentration of Test Item in Spiked Sample x [µg/L]	Sample Preparation Factor F	Concentration of Test Item Determined in Spiked Sample c [µg/L]	Recovery R [%]
Day of analysis 1				
0	n.d.	2	< LOQ	n.a.
0.0196	0.0096	2	0.0192	98
1.31	0.595	2	1.19	91

n.d. = no test item detected
LOQ = 0.0196 µg test item /L (day of analysis 1)
n.a. = not applicable

The tabulated values of the samples represent rounded results obtained by calculation using the exact raw data.

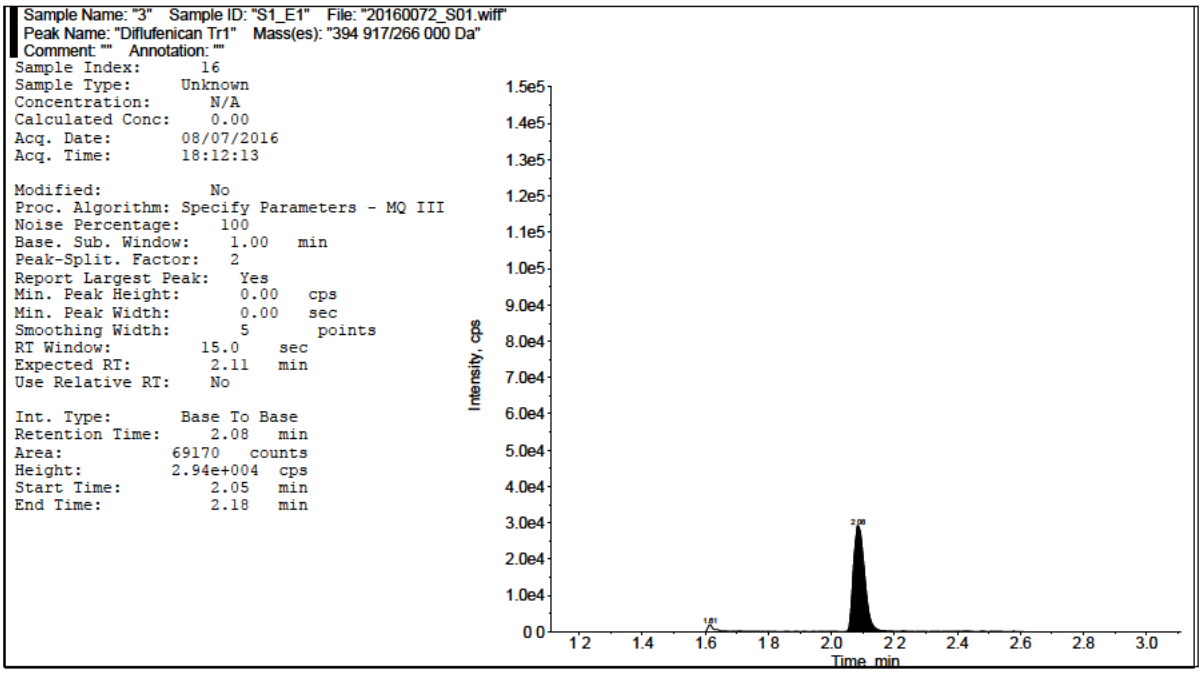
Table 4 Results for Test Samples

Sampling Day [d]	Nominal Concentration of Test Item c_{nom} [µg/L]	Measured Concentration of Test Item x [µg/L]	Day of Analysis	Sample Preparation Factor F	Determined Concentration of Test Item c [µg/L]	% of Nominal Concentration [%]
0	Solvent Control	< 0.0098	1	2	< LOQ	n.a.
(fresh)	0.022	0.0115	1	2	0.0231	105
	0.046	0.0241	1	2	0.0482	105
	0.10	0.0538	1	2	0.108	108
	0.22	0.116	1	2	0.232	105
	0.46	0.253	1	2	0.506	110
	1.0	0.456	1	2	0.913	91
3	Solvent Control	< 0.0098	1	2	< LOQ	n.a.
(aged)	0.022	0.0138	1	2	0.0275	125
	0.046	0.0230	1	2	0.0460	100
	0.10	0.0453	1	2	0.091	91
	0.22	0.107	1	2	0.214	97
	0.46	0.218	1	2	0.436	95
	1.0	0.468	1	2	0.937	94
Recovery	Solvent Control*	< 0.0098	1	2	< LOQ	n.a.
Phase 1	*0.046**	< 0.0098	1	2	< LOQ	n.a.
	*0.10**	< 0.0098	1	2	< LOQ	n.a.
	*0.22**	< 0.0098	1	2	< LOQ	n.a.
	*0.46**	< 0.0098	1	2	< LOQ	n.a.
	*1.0**	0.0112	1	2	0.022	n.a.
Recovery	Solvent Control*	< 0.0104	2	2	< LOQ	n.a.
Phase 2	*0.22**	< 0.0104	2	2	< LOQ	n.a.
	*0.46**	< 0.0104	2	2	< LOQ	n.a.
	*1.0**	< 0.0104	2	2	< LOQ	n.a.

LOQ = 0.0196 µg test item /L (day of analysis 1) or 0.0208 µg test item /L (day of analysis 2)
n.a. = not applicable
* = concentration during exposure phase

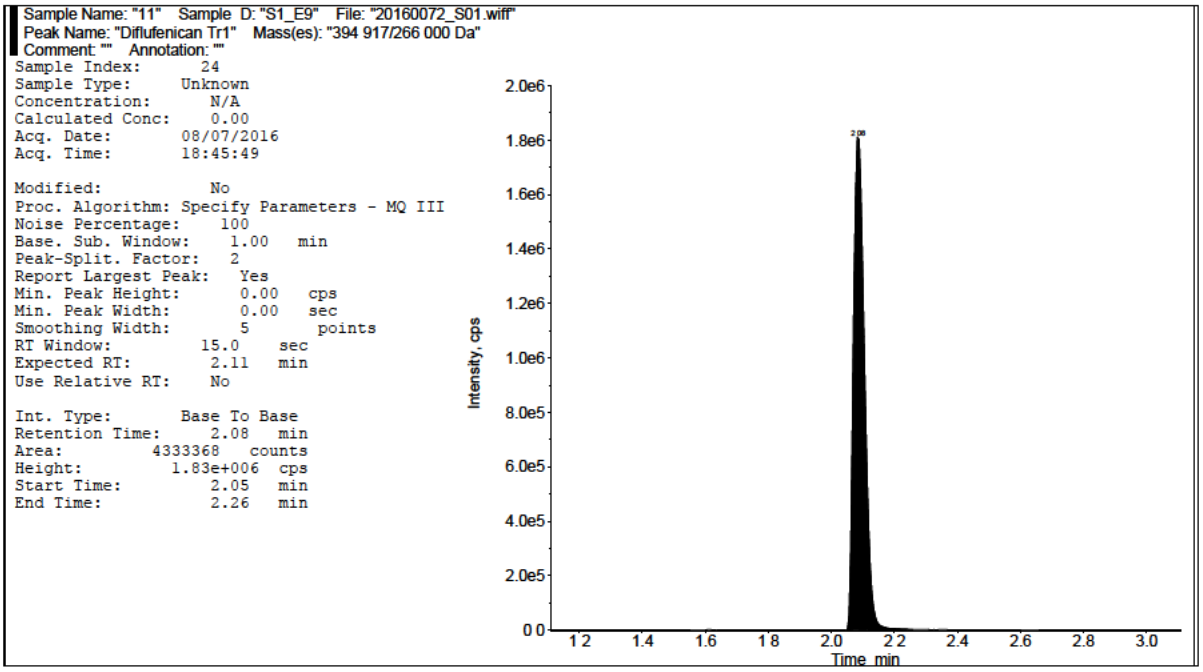
The tabulated values of the samples represent rounded results obtained by calculation using the exact raw data.

Figure 2 Chromatogram of Calibration Solution (Low-Level)



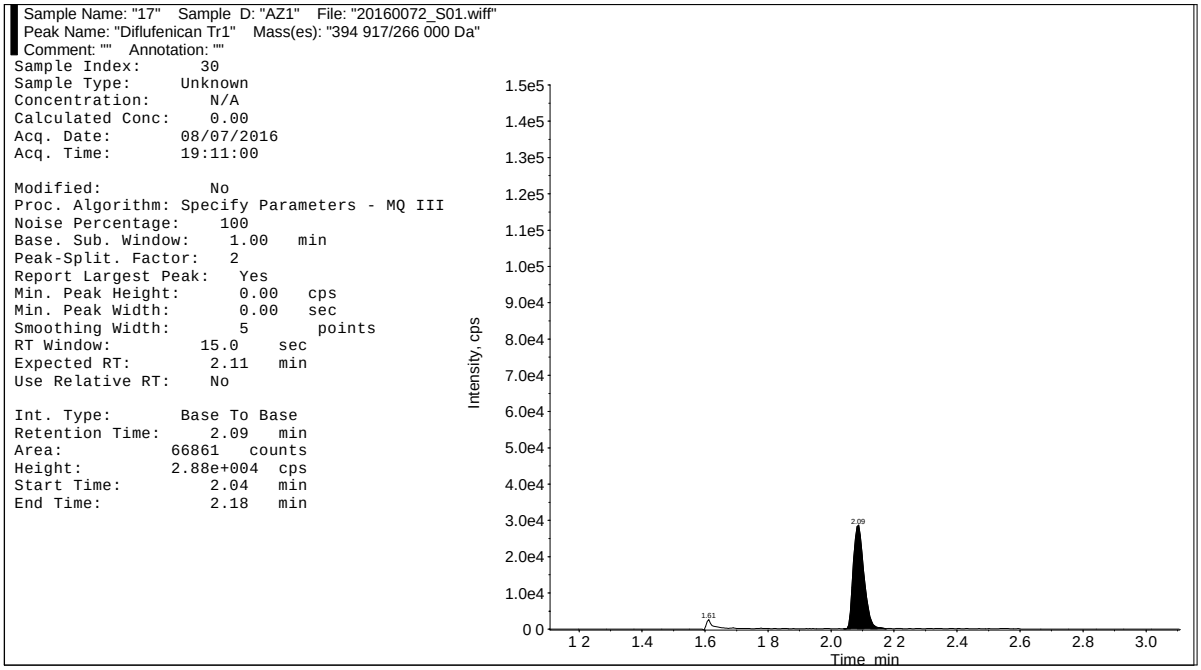
Nominal Concentration: 0.00981 µg Test Item /L

Figure 3 Chromatogram of Calibration Solution (High-Level)



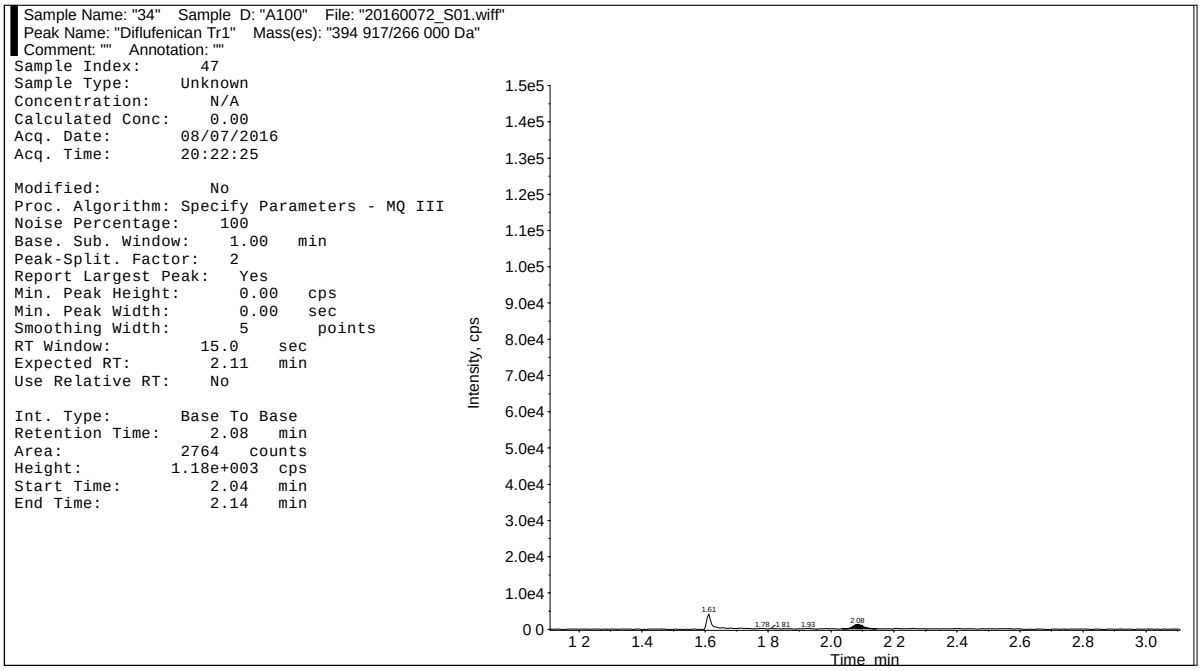
Nominal Concentration: 0.757 µg Test Item /L

Figure 4 Chromatogram of Spiked Sample



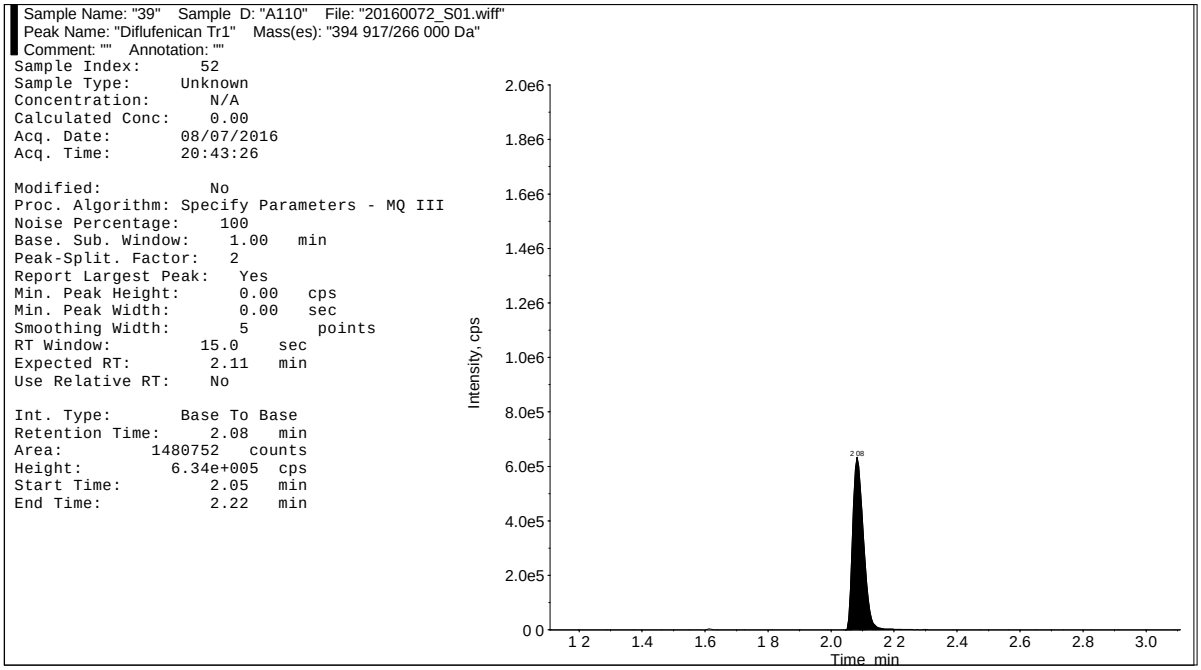
Nominal Concentration in Sample: 0.0196 µg Test Item /L
Nominal Concentration after Dilution: 0.0098 µg Test Item /L

Figure 5 Chromatogram of Biological Control Sample A100



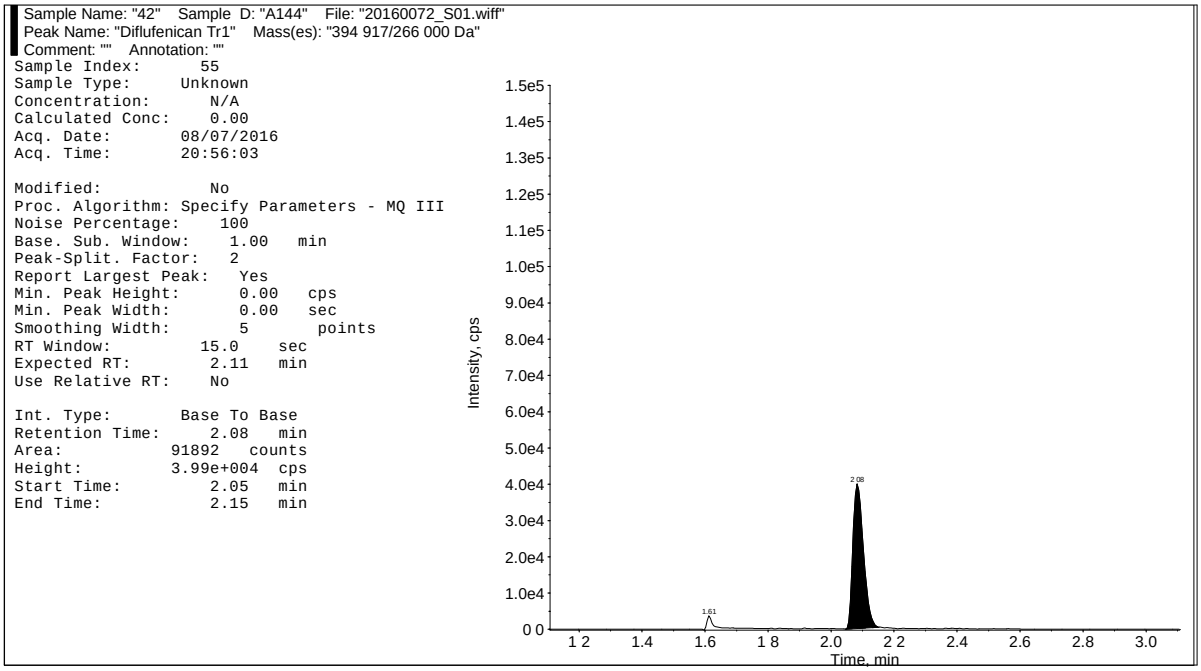
Measured Concentration: < LOD

Figure 6 Chromatogram of Fresh Test Sample A110



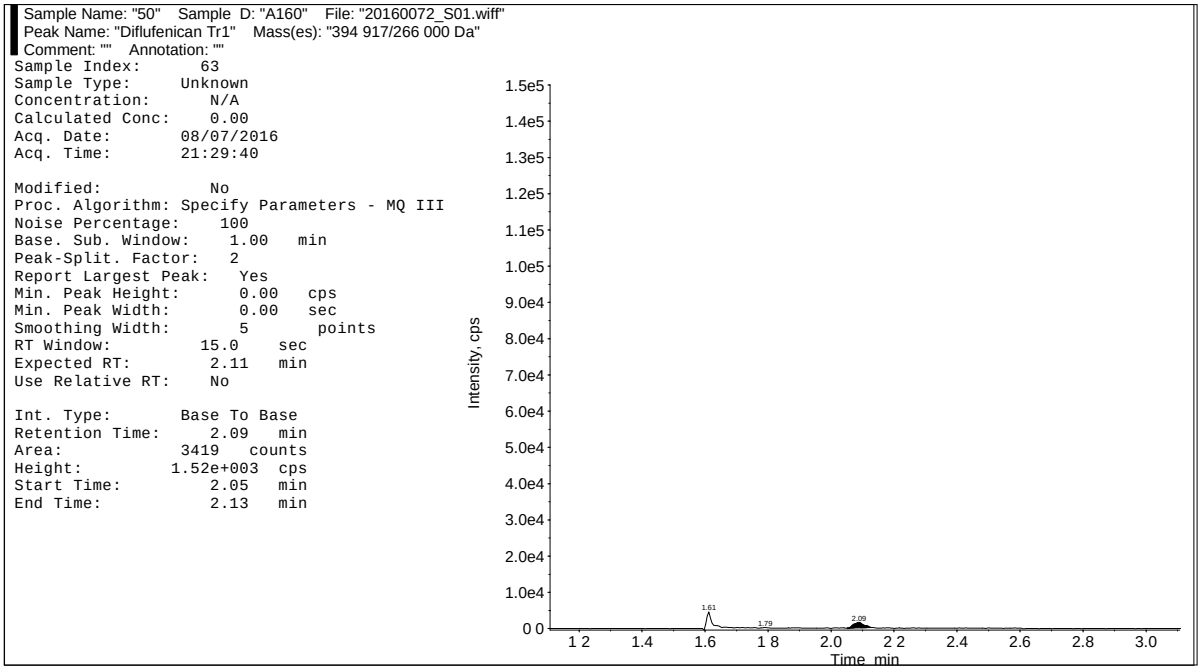
Nominal Concentration in Sample: 0.46 µg Test Item /L
Nominal Concentration after Dilution: 0.23 µg Test Item /L

Figure 7 Chromatogram of Aged Test Sample A144



Nominal Concentration in Sample: 0.022 µg Test Item /L
Nominal Concentration after Dilution: 0.011 µg Test Item /L

Figure 8 Chromatogram of Test Sample A160 from Recovery Phase



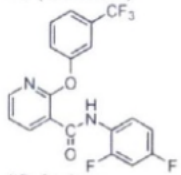
Measured Concentration: < LOQ

Appendix 2 Certificate of Analysis (Test Item)

cerexagri
UPL Group

[redacted] 10469

CERTIFICATE OF ANALYSIS

Sample name		: Diflufenican Tech
Type		: TC (technical)
Structural formula		: 
Common name		: diflufenican
Chemical name	(IUPAC)	: 2',4'-difluoro-2-(α,α,α-trifluoro-*m*-tolyloxy)nicotinanilide
	(C.A., 9CI)	: *N*-(2,4-difluorophenyl)-2-[3-(trifluoromethyl)phenoxy]-3-pyridinecarboxamide
Molecular formula		: $C_{19}H_{11}F_5N_2O_2$
CAS RN		: [83164-33-4]
Received through		: AgriChem B.V., Oosterhout, The Netherlands
Batch number		: DFF-101/12
Appearance		: white powder
Storage conditions		: room temperature
Date of analysis		: 16 October 2014
Expiry date		: 16 October 2016

RESULTS OF ANALYSIS

The analyses have been carried out in Study DL 14-168 according to SOP DLA-292.1 in compliance with Good Laboratory Practice (GLP) standards as defined in:
Directive 2004/10/EC of the European Parliament and the Council, of 11 February 2004.

Diflufenican content : 100 % m/m

estimated error : ± 0.6 % (95 % probability; n=5)

[redacted]
Study Director
Senior Assistant Product Chemistry

[redacted]
Assistant Product Chemistry

[redacted]
Date: 20 October 2014

[redacted]

Appendix 3 GLP Certificate

The Swiss GLP Monitoring Authorities

Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

Swiss Confederation

Federal Department of Home Affairs DHA
Federal Office of Public Health FOPH

Federal Department of the Environment, Transport, Energy and Communications DETEC
Federal Office for the Environment FOEN

swissmedic
Swiss Agency for Therapeutic Products

Statement of GLP Compliance

According to Article 14 paragraph 3 Ordinance on Good Laboratory Practice [OGLP, SR 813.112.1]

The notification authority for chemicals confirms that the following test facility was inspected with respect to the compliance with the Swiss Ordinance on Good Laboratory Practice, adopted on 18th May 2005 [OGLP, SR 813.112.1]. This Ordinance is based on the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted on 26th November 1997 by decision of the OECD Council [C(97)186/Final].

Unequivocal name and address of the test facility:	Areas of expertise according to article 3 paragraph 1 letter b OGLP:
[redacted]	1. physical-chemical testing 4. environmental toxicity studies on aquatic and terrestrial organisms, 5. studies on behaviour in water, soil and air; bioaccumulation 6. residue studies, 8. analytical and clinical chemistry testing, 9. other studies (in vitro metabolism / dermal penetration).

Inspection authority: Federal Office for the Environment (FOEN)

Date of inspection: 29 June – 2 July, 19 and 21 August 2015

Date of decision: 16 November 2015

Based on the above mentioned decision it can be confirmed that the above mentioned test facility is able to conduct studies according to the aforementioned areas of expertise in compliance with the principles of GLP. The above mentioned test facility is listed in the register and GLP list according to the Article 14 OGLP and is inspected on a regular basis according to Article 6 paragraph 2 OGLP.

Swiss Federal Office of Public Health
Consumer protection directorate
Notification authority for chemicals
CH-3003 Bern

FEDERAL OFFICE OF PUBLIC HEALTH

Bern, 16 December 2015, The Head, Dr. Pierre Favre.