PROSPECTIVE ADDED ENVIRONMENTAL RISK ASSESSMENT FROM RE-SUSPENSION OF CHEMICAL WARFARE AGENTS FOLLOWING THE INSTALLATION OF THE NORD STREAM 2 PIPELINES

Nord Stream 2 added CWA environmental risk assessment

Scientific Report from DCE – Danish Centre for Environment and Energy No. 302 2019

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Abstract:	The report reviews the prospective risk associated the resuspension of chemical warfare agent residues flowing the construction and placement of the Nord Stream 2 gas pipeline in the Baltic Sea

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Summary

The sampling campaigns included in this report are the latest of the Nord Stream (2008–2012) and Nord Stream 2 (2015–2016) campaigns and cover the planned extension of the Nord Stream pipeline system. The samplings were conducted in October 2015 and March 2016. They comprised the preferred route (ES) and two alternative pipeline route options (FS and RA) that were developed based on bathymetric and geological data together and other factors (DHI, 2015). Two pipeline paths (ES and FS) are outside the dumpsite and one (RA) is going through the secondary dumpsite on a number of sampling stations. 17 sediment samples from a total of 121 were therefore taken within the known dump site where there are currently activity restrictions of e.g. anchoring and fishing.

Improved analytical methods resulted in higher detection frequencies and concentrations found than during the previous investigations (Sanderson et al., 2014). It is notable that novel compounds were detected, i.e. cyclic dissipation products of mustard gas as well as parent mustard gas. The former are unknown to the field of environmental toxicology and risk assessment. Hence, an investigation of their environmental toxicity following OECD and GLP test guidelines were commissioned. The most hazardous compound 1-Oxa-4,5-dithiepane was used in chronic tests for algae and daphnia, respectively, to derive an assessment factor of 500 resulting in a PNEC of 0.0165 mg/L.

The risk quotient (RQ) for single chemical warfare agents (CWAs) and for the sum of CWAs was calculated as the CWA concentration divided by the toxicity threshold value. The risk from the inherent, i.e. present and undisturbed CWA concentrations in sediment and bulk water, were quantified together with the added risk from sediment agitation in different distances from the pipeline. Added sediment concentrations in the lower bulk water layer arise from sweeping that occurs along the entire pipeline on all three routes, trenching that occurs at three sections and involves only some sampling stations, and rock placement that takes place near one sampling station.

In summary, the mean and maximum added RQs from pipeline installation for the sum of chemicals are below one (< 0.003) for all three routes indicating a negligible environmental risk, and the two routes ES and RA have approximately the same maximum added RQs, whereas the value for route FS is a factor of approximately 20 lower. Clearly, the route RA with the highest single finds of parent and degradation CWA is less favourable relative the ES route from a CWA exposure point of view. Hence, in conclusion the FS or ES route is recommended from a CWA exposure avoidance perspective.

Sammenfatning

I denne rapport er de seneste af Nord Stream (2008–2012) og Nord Stream 2 (2015–2016) prøvetagningsrunderne, der omfatter den påtænkte udvidelse af rørledningerne, inkluderet. Målingerne blev foretaget i oktober 2015 og marts 2016 for den foretrukne rute (ES) og to alternative ruter (FS og RA), der er udvalgt på baggrund af batymetriske og geologiske forhold sammen med andre faktorer (DHI, 2015). To ruter (ES og FS) ligger uden for dumpningsområderne og én rute (RA) går igennem det sekundære dumpningsområde, hvor der er placeret en række prøvetagningsstationer. 17 sedimentprøver ud af totalt 121 er således taget inden for det kendte dumpningsområde, hvor der er restriktioner mht. bl.a. ankring og fiskeri.

Forbedrede analysemetoder har resulteret i flere fund og højere koncentrationer af krigsgasser (chemical warfare agents; CWAer) end i de tidligere undersøgelser (Sanderson et al., 2014). Det er bemærkelsesværdigt, at der er påvist nye forbindelser, navnlig cykliske nedbrydningsprodukter af sennepsgas samt også selve sennepsgas. De cykliske nedbrydningsprodukter er ikke tidligere undersøgt i forbindelse med miljøgiftighed og risikovurdering. Derfor blev der bestilt en undersøgelse af deres miljøgiftighed i henhold til retningslinjerne beskrevet i OECD og GLP. Den mest skadelige forbindelse, 1-Oxa-4,5-dithiepane, blev anvendt i en kronisk test for henholdsvis alger og dafnier, hvilket gav en sikkerhedsfaktor på 500 og en PNEC på 0.0165 mg/L.

Risikokvotienten (RQ) for enkelt CWA'er og for summen af CWA'er blev beregnet som CWA-koncentrationen divideret med predicted no-effect concentration (PNEC), dvs. den koncentration, hvor man skønner, at stoffet ikke giver anledning til effekter. Risiko for den eksisterende uforstyrrede CWA-koncentration i sedimentet og i det ovenfor liggende vandgrænselag blev beregnet sammen med den ekstra miljørisiko fra rørlægningsaktiviteterne i forskellige afstande fra rørene.

De beregnede middel og maksimum RQ'ere fra rørlægningsaktiviteter for summen af CWA'er er mindre end én (<0.003) for alle tre ruter, hvilket indikerer en negligeabel miljørisiko. Ruterne ES og RA har sammenlignelige maksimum RQ fra rørlægning, hvorimod FS har en maksimum RQ fra rørlægning der er en faktor 20 lavere. Rute RA har de højeste fund af enkelt CWA'er og CWA-nedbrydningsprodukter, og er derfor mindre favorabel sammenlignet med ES-ruten. Som konklusion anbefales FS- og ES-ruterne fremfor RA-ruten ud fra et CWA-eksponeringssynspunkt.

Introduction

Following the end of World War II and as a result of the Potsdam Conferences, the Allied Forces ordered the destruction of Germany's approximately 65,000 tonnes of stockpiled chemical warfare agent (CWA) munitions during the second half of 1947. Significant amounts of these munitions were dumped in the Bornholm Deep. Concerns have been raised with regard to the environmental risks associated with perturbation of sediment containing traces of dumped CWA during the building of the planned Nord Stream gas pipelines in the vicinity of the Bornholm Deep. Risk assessments have been performed for the first two pipelines and reported by Sanderson et al. (2014). A sampling campaign along the Nord Stream 2 route was conducted during the fall of 2015; a total of 103 sediment samples were collected for chemical analyses at VERIFIN. For details on the compounds and analyses, see Appendix C of the DHI (2015) report. The list of analytes has increased relative to the Nord Stream 1 pipeline assessments as methods have been developed for more degradation products, moreover the sensitivity of the analytical methods have also increased resulting in lower levels of quantification and higher detection frequencies. Literature-based and calculated sorption coefficients were used to transform measured sediment concentrations into bio-available pore water concentration for fish. For organ arsenic CWAs the previously used fish community HC5 (0.29 mg/L) value is used (Sanderson et al. 2014). However, for the newly detected degradation products as well as parent mustard gas we have generated new environmental toxicity values, as these are not described for the dissipation products. The potential direct environmental risks towards fish communities from CWA dumped following World War II associated with the construction of the proposed Nord Stream gas pipeline 2 are assessed following the principles in the previous reports and papers (i.e. Sanderson et al. 2014).

Methods

Data generation

Quantitative chemical analysis of target CWAs in sediment samples were done to estimate the presence of dumped chemicals and / or their degradation products and to support the risk assessment for the marine construction works. Sediment samples for chemical analysis of CWAs and their degradation products and other contaminants were taken with a Haps core sampler from the upper 5 cm of the core per station together with samples for quantification of benthos and background parameters. A sampling campaign was carried out October 2015 along sampling stations situated on three routes running from the northeast to the southeast of Bornholm as follows:

- Route D-ES: 61 stations (21 single stations and eight transects of five stations each)
- Route D-FS: 15 stations (five single stations and two transects of five stations each)
- Route D-RA: 27 stations (27 single stations)

As evident from the Figure 1 below D-RA (green line) stations five to 22 consists of samples from within the secondary dumpsite where the probability of detecting CWA is greater than outside the secondary dumpsite.

A supplementary sampling campaign comprising 18 sediment samples was carried out in March 2016 on six sampling stations situated along the D-ES route, see Figure 2. It comprised three sections of the route where trenching might be carried out during the lay of the pipelines:

- Section 1: three stations (D-ES_100, D-ES_101 and D-ES_102)
- Section 2: two stations (D-ES_103 and D-ES_104)
- Section 3: one station (D-ES_105)

For a detailed description of the sampling and chemical analytical methods for the CWA analysis as well as biota and physical-chemical characterisations, please see DHI (2015 & 2016) and Söderström et al. (2016).

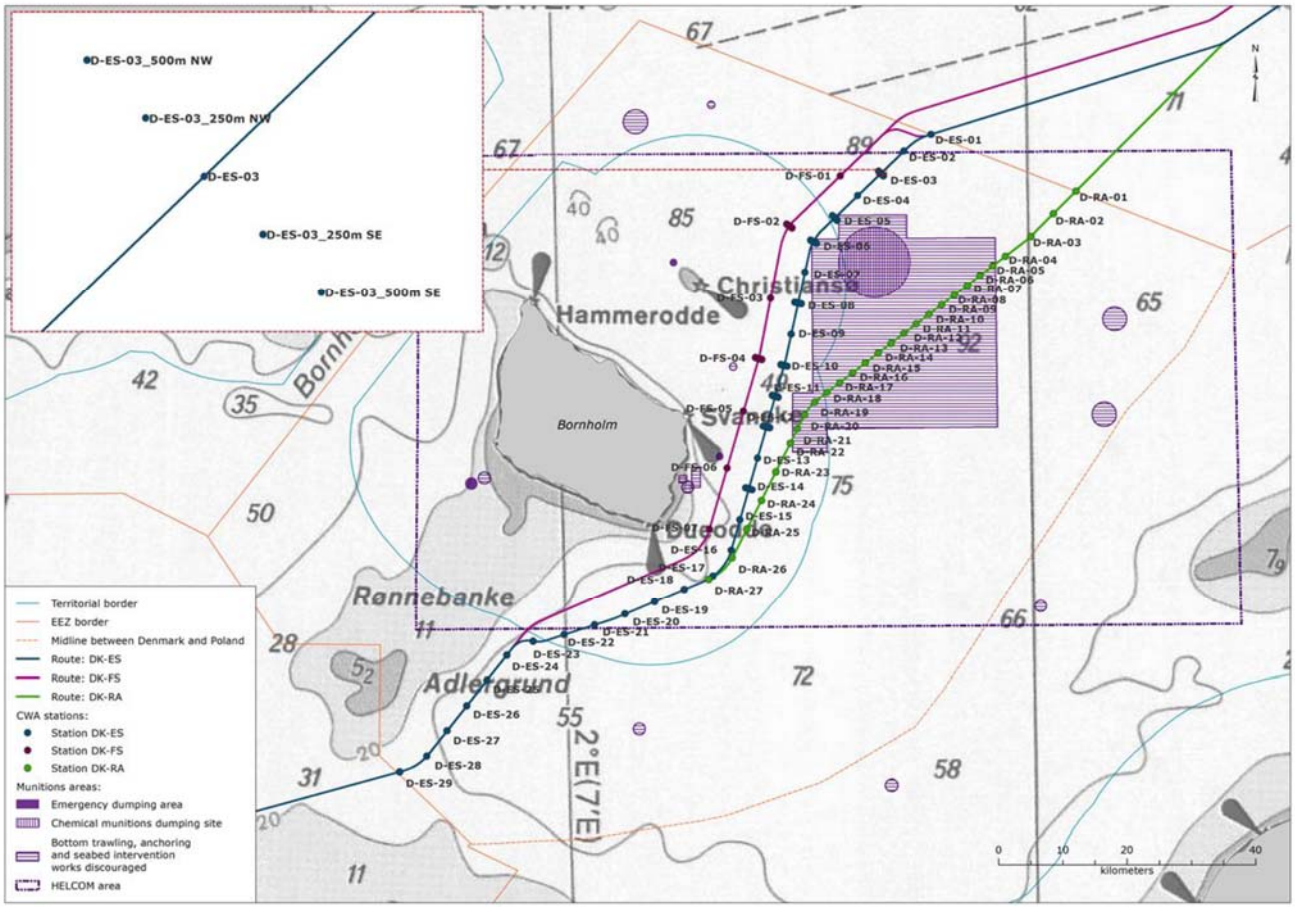

Figure 1 Chemical sampling stations along the Nord Stream route near Bornholm in October 2015.

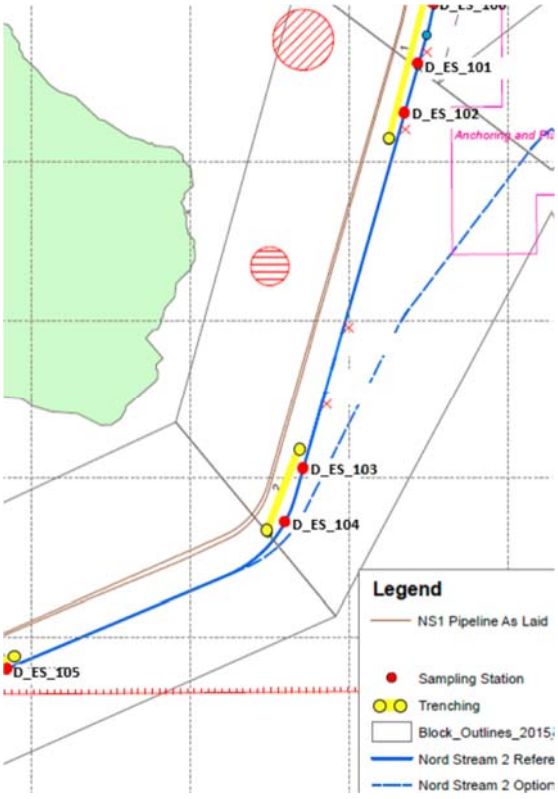

Figure 2 Supplementary sampling stations for CWAs in sediments in March 2016.

Measurements

Chemical analysis were performed for the following parent compounds, which have been dumped in the Bornholm basin: mustard gas (1), Adamsite (2), Clark I (3a), Clark II (3b), triphenyl arsine (4), α-chloroacetophenone (6), tabun (9), Lewisite I and II (7 & 8), components of arsine oil, which contains (4), phenyldichloroarsine (5) and trichloroarsine (10). The following hydrolysis products of sulphur mustard (1) were also analysed: thiodiglycol (1.1) and thiodiglycol sulfoxide (1.1O), as well as degradation products of (2) (3a & 3b), (4), (7 & 8) and arsine oil components (5, 10) including their hydrolysis products.

See Table 1 for all analysed chemicals (Appendix D in DHI, 2015). For each of the three routes (ES, FS and RA) the detection frequencies, maximum inherent sediment concentrations as µg/kg dry weight sediment (± standard deviation) and sampling station for maximum measured inherent concentration are stated. All measured concentrations can be found in DHI (2015 & 2016).

Table 1 Analysed chemicals, CAS numbers, detection frequency relative to the total number of samples (ES=79, FS=15, RA=27), measured maximum inherent sediment concentrations in µg/kg dry weight (± standard deviation) and maximum sampling station. The first number in # describes the dumped parent compound and the following numbers and letters indicate hydrolysis, oxidation or degradation products of parent compounds.

#	Chemical CAS	Detection frequency	Max inherent sediment conc (µg/kg dw) (Maximum sampling station)
1	Mustard gas 505-60-2	ES: 1.3 % FS: Not detected RA: 7.4 %	ES: 0.6±0.035 (ES_10_500m_NW) FS: Not detected RA: 1.2±0.067 (RA_14)
1.1	Thiodiglycol 111-48-8	Not detected	Not detected
1.1O	Thiodiglycol sulfoxide 3085-45-8	Not detected	Not detected
1.2	1,4-Dithiane 505-29-3	ES: 3.8 % FS: 6.7 % RA: 15 %	ES: 0.34±0.018 (ES_06) FS: 0.36±0.019 (FS_02_500m_NW) RA: 1.8±0.094 (RA_14)
1.2O	1,4-Dithiane oxide 19087-70-8	Not detected	Not detected
1.3	1,4-Oxathiane 15980-15-1	Not detected	Not detected
1.4	1,4,5-Oxadithiepane 3886-40-6	ES: 13 % FS: 6.7 % RA: 11 %	ES: 0.44±0.025 (ES_08_500m_SE) FS: 0.45±0.026 (FS_02_500m_NW) RA: 2.9±0.16 (RA_16)
1.5	1,2,5-Trithiepane 6576-93-8	ES: 18 % FS: 33 % RA: 33 %	ES: 1.6±0.18 (ES_06_250m_NW) FS: 1.5±0.16 (FS_02_500m_NW) RA: 16±1.7 (RA_16)
2	Adamsite 578-94-9	ES: 41 % FS: 53 % RA: 63 %	ES: 2000±130 (ES_11_250m_NW) FS: 200±13 (FS_02_500m_SE) RA: 3400±220 (RA_10)
2O	5,10-Dihydrophenarsazin- 10-ol 10-oxide 4733-19-1	ES: 35 % FS: 20 % RA: 41 %	ES: 580±43 (ES_11_250m_NW) FS: 170±12 (FS_05) RA: 250±18 (RA_10)
3a	Clark I 712-48-1	Not detected	Not detected
3b	Clark II 23525-22-6	Not detected	Not detected
3O	Diphenylarsinic acid 4656-80-8	ES: 39 % FS: 27 % RA: 48 %	ES: 1800±140 (ES_11_250m_NW) FS: 21±1.6 (FS_06) RA: 4400±340 (RA_19)
3T	Diphenylpropylthioarsine 17544-92-2	ES: 28 % FS: 13 % RA: 22 %	ES: 59±5.4 (ES_11_250m_NW) FS: 3±0.28 (FS_02_250m_SE) RA: 31±2.8 (RA_16)
4	Triphenylarsine 603-32-7	ES: 16 % FS: 20 % RA: 30 %	ES: 13±1 (ES_10_500m_NW) FS: 4.5±0.35 (FS_02_500m_NW) RA: 30±2.3 (RA_16)

Continued

4+4O	Triphenylarsine oxide (4O) 1153-05-5	ES: 29 % FS: 27 % RA: 41 %	ES: 230±18 (ES_05_500m_SE) FS: 7.3±0.56 (FS_02_500m_SE) RA: 130±10 (RA_19)
5	Phenyldichloroarsine 696-28-6	Not detected	Not detected
5O	Phenylarsonic acid 98-05-5	ES: 30 % FS: 20 % RA: 37 %	ES: 150±9.9 (ES_10) FS: 11±0.76 (FS_04_250m_NW) RA: 260±18 (RA_16)
5T	Dipropyl phenylarsonodithioite 1776-69-8	ES: 29 % FS: 27 % RA: 26 %	ES: 98±11 (ES_10_500m_NW) FS: 2.9±0.32 (FS_06) RA: 91±10 (RA_14)
6	α-Chloroacetophenone 532-27-4	ES: 1.3 % FS: Not detected RA: Not detected	ES: 2.3±0.14 (ES_06_500m_SE) FS: Not detected RA: Not detected
7	Lewisite I 541-25-3	Not detected	Not detected
7T	Dipropyl (2-chlorovinyl) arsonodithioite 677354-97-1	Not detected	Not detected
8	Lewisite II 40334-69-8	Not detected	Not detected
8O	Bis(2-chlorovinyl)arsenic acid 157184-21-9	Not detected	Not detected
8T	Bis(2-chlorovinyl)propylthioarsine 677355-04-3	Not detected	Not detected
9	Tabun 77-81-6	Not detected	Not detected
10	Trichloroarsine 7784-34-1	Not detected	Not detected
10T	Tripropyl arsenotrithioite 5582-57-0	ES: 1.3 % FS: Not detected RA: Not detected	ES: 3.5±0.30 (ES_10_500m_NW) FS: Not detected RA: Not detected
10A	Tripropyl arsenite 15606-91-4	Not detected	Not detected

A total of 121 sediment samples were analysed and 83 of these samples contained measurable concentrations (above the limits of quantitation) for one or more CWAs and/or degradations products. The highest detection frequencies were either found along the middle and northern parts of the ES route (1.4, 3T, 5T, 6 and 10T) or the RA route (remaining nine chemicals). The maximum sediment concentrations were found either along the ES route (2O, 3T, 4+4O, 5T, 6 and 10T) or the RA route (remaining eight chemicals). The southern part of ES route and FS route had a comparatively low degree of exposure to CWAs.

Intact chemical warfare agents were found in 62 of 121 samples; sulphur mustard (1) was found at low level (0.34–1.2 µg/kg dw) in three samples; Adamsite (2) was found in 57 of 121 samples where the two samples with the highest concentrations were 2000 and 3400 µg/kg dw; triphenyl arsine (4) was detected in 24 samples with concentrations ranging from 0.55 to 30 µg/kg dw; α-chloroacetophenone (6) was found in one sample at low level (2.3 µg/kg dw).

Degradation products were found for sulphur mustard (30 samples), Adamsite (42 samples) and Clark I or II (55 samples). The highest concentration of detected degradation product was 4400 µg/kg dw for Clark I/II. Arsine oil components, either the parent compound or degradation products, were found in 61 samples. No traces of the parent compounds or corresponding degradation products were found for Tabun, Lewisite I or Lewisite II.

It is notable that the highest concentration detected in sediment for a single compound (Adamsite at 3400 µg/kg dw) was within the dump site (RA route), and that 2/3 of the positive samples for parent mustard gas were also detected within the dump site (RA route). Only two parent compounds were found in higher concentrations outside the dumpsite (CAP and TCA) with one detection each.

Predicted environmental concentrations (PEC) of CWAs

In order for chemicals to be incorporated into organisms, such as fish and thereby exert toxicity, they generally need to be in solution. Hence, the measured inherent CWA concentrations in sediment, Cs(CWA) (mg/kg dw), will be used to calculate pore water CWA concentrations, Cpw(CWA) (mg/L), which are bioavailable to the organisms, based on adapted equilibrium partitioning (DiToro, 1991 and Sanderson et al., 2008), cf. Eq. 1.

$$Cs(CWA) = Cpw(CWA) \cdot Rs/Xs = Cpw(CWA) \cdot (\theta + Kd \cdot Xs)/Xs \qquad \text{(Eq. 1)}$$

Where; $Rs = (\theta + Kd * Xs)$ is the retention factor, $\theta = 0.55$ is the pore volume fraction in the sediment (Forster et al., 2003), $Kd = foc \cdot Koc$ is the partitioning coefficient between dry matter and water in L/kg dw, $foc = 0.0775$ is the fraction of organic carbon in particulate matter (Emelyanov, 1996), Koc is the partitioning coefficient (sorption coefficient) between organic matter and water (L/kg OM) based on Molecular Connectivity Index (MCI) in KOCWIN v2.00. $Xs = 1.2$ kg dw/L is the density of sediment (Forster et al., 2003).

The measured sediment concentration and calculated pore water concentrations will be used as the predicted fish community CWA exposure concentration (PEC) for the quasi steady-state risk analysis, where it is assumed that the fish water exposure concentration equals the pore water concentration.

In addition to the risk from inherent CWA concentrations, there is a contribution of suspended sediment in the lower bulk water layer from installing the pipelines. Modelling of the agitation and sediment dispersion from trenching and rock placement has been made for a winter situation with rough weather conditions, which is representative for all the considered weather conditions. Maximum suspended sediment concentrations from trenching are shown in Table 2 for the distances 200, 500 and 1000 m from the pipeline, where 200 m is the minimum model grid size (Rambøll, 2016). Maximum sediment concentration values are shown for each of the three trenching sections and the rock placement section together with the sampling stations they involve. The basic assumptions and calculations related to the suspension of sediment particles in the lower bulk water from trenching and rock placement during construction of one gas pipeline are found in Rambøll (2016).

Table 2 Maximum suspended sediment concentrations, Cw(sed), in mg/L in lower bulk water for three trenching sections and rock placement section at three distances from the pipeline and the involved sample stations.

Seabed intervention works	Route section	Affected sample stations	Max. sediment concentration at specific distances from pipelines		
			200 m	500 m	1000 m
	km		mg/L	mg/L	mg/L
Trenching 1	11,6	ES_10 (5 stations), ES_11 (5 stations) FS_04 (5 stations), FS_05	62,3	33,0	14,1
Trenching 2	5,5	ES_15, ES_16 FS_0,7 RA_25, RA_26	33,2	20,4	6,8
Trenching 3	1,6	ES_20, ES_21	43,3	23,1	6,9
Rock placement	4 locations	ES_23	0,78	0,54	0,52

Pipe-laying directly on the seabed give rise to only small amounts of sediment that will be suspended during pipe-laying directly on the seabed for worst-case scenarios where the pipeline is placed on soft clay. Sediment suspension during pipe-laying is negligible compared with suspension during trenching and is therefore not accounted for (Rambøll, 2008b).

Handling of 12 anchors, each weighing 25 tonnes, causes sediment suspension from laying anchor, lifting anchor and sweeping anchor wires across the seabed. The sweeping process is most predominant with respect to sediment disturbance, and the total release to the bulk water is 10 - 38 tonnes sediment / km of the pipeline in areas with soft sediment (Rambøll, 2008a). The release area is approximately 2 % (0.04 km^2 / km) of the anchor corridor. This gives a sediment concentration in the release area and lower 10 m (release water volume) of Cw(sed,sweeping) = 25 - 95 mg sediment / L. When assuming that sediment particles from the release area are spread to the total anchor corridor area, the average sediment concentration is approximately 0.5 - 2 mg / L (Rambøll, 2008a).

The following six release scenarios are considered for the added sediment concentrations in the release area and lower 10 m (release water volume):

- S1 (worst-case): Sweeping high (95 mg / L), trenching (200 m), rock placement (200 m)
- S2: Sweeping high (95 mg / L), trenching (500 m), rock placement (500 m)
- S3: Sweeping high (95 mg / L), trenching (1000 m), rock placement (1000 m)
- S4: Sweeping low (25 mg / L), trenching (200 m), rock placement (200 m)
- S5: Sweeping low (25 mg / L), trenching (500 m), rock placement (500 m)
- S6: Sweeping low (25 mg / L), trenching (1000 m), rock placement (1000 m).

Sweeping occurs along the entire pipeline, whereas trenching occurs only at some sampling stations and rock placement takes place near one sampling station, see Table 2.

The worst-case scenario for additional concentrations in bottom-layer bulk water from pipeline installations assumes that once sediment particles are suspended to the bulk water all the sorbed CWAs are instantaneously released and mixed within a release area. The total CWA concentration, Cw(CWA), in the bottom-layer bulk water from inherent and added sediment contributions is thus:

$$Cw(CWA) = (Xs / Rs + Cw(sed)_{added}) \times Cs(CWA)\ (\mu g\ CWA / L) \qquad \text{(Eq. 2)}$$

Where Cs(CWA) is the measured CWA concentration in sediment, in mg/kg dw, and $Cw(sed)_{added}$ is the added sediment concentrations in lower bulk water in mg sediment/L for scenario S1 to S6, respectively. Concentrations are calculated for single CWAs and sum of CWAs, for each pipeline route.

Predicted CWA HC5 fish community concentrations

The environmental toxicity associated with most physical-chemical properties of CWAs has not been thoroughly investigated with modern methods and reported in the public literature; hence modelling of these properties is warranted to derive comparable datasets (Sanderson et al., 2014).

The predicted environmental concentrations (PECs) will be compared with toxicologically acceptable exposure concentrations towards the fish communities as reported in Sanderson et al. (2014; 2015), using the fish community extrapolated HC5 value for the organoarsenic CWA products. In the absence of high quality environmental toxicity data for the multitude of arsenic compounds, the known most toxic compound is used (inorganic As_{III}). HC5 (hazard concentration 5 %) represents the concentration where 95 % of the acute LC50 of the fish species in the community is not exceeded; or, in other words, a potential risk for the community of 5 % is accepted. The toxicity of organoarsenic CWA (with inorganic As_{III} as surrogate) was derived from the US National Library of Medicine Hazardous Substances Data Base (HSDB: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB). For these compounds, the data was used to derive a species sensitivity distribution for 12 fish species (adult and juvenile). The resulting acute HC5 value (protective of 95 % of the community) equals 0.29 mg/L. The relative risk of each CWA and the total risk-assuming additivity of the CWAs are calculated for the fish community. The assessment factors associated with HC5 values derived by Species Sensitivity Distribution (SSD) range typically between 1 and 5, and have not been included in this analysis, as determination of the size of the factor is a subjective matter of negotiation between the decision-makers and stakeholders, derived on a case-by-case basis. Mustard gas will be reviewed separately for its environmental toxicity (https://pubchem.ncbi.nlm.nih.gov/compound/Mustard_gas#section=Top

The acute fish community for α-chloroacetophenone (CAP (CN)) has been previously determined to be 0.5 mg/L by Sanderson et al. (2008) based on extrapolated value with 1 mg/L as the entry point for bluegill sunfish, which was used based on the H400 classification the compound has received (see Figure 3 below).

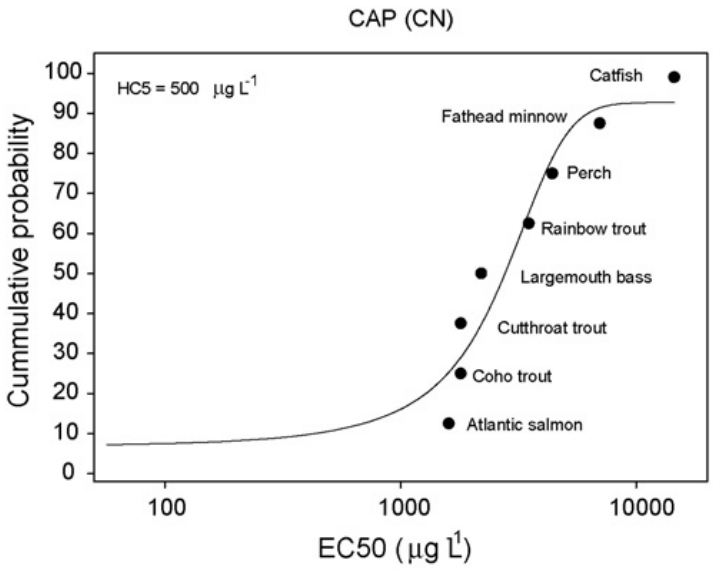

Figure 3 CAP fish community SSD (Sanderson et al. 2008).

Results

Predicted environmental concentrations (PEC)

In Table 3 the calculated means (for each route) for the inherent CWA parent and degradation product concentrations for the sediment pore water are shown for all chemicals, which is assumed to be equal to the lower bulk water layer. Furthermore, the calculated mean (for each route) added lower bulk water concentrations for the worst-case scenario (S1) with high sediment re-suspension from sweeping and distance 200 m from the pipeline, are shown.

Table 3 Calculated mean inherent pore water (assumed bulk water) concentrations (from Eq. 1 and Eq. 2) and mean added worst-case (S1) concentrations to bulk water (µg/L) (± standard deviation). Sorption coefficient between organic matter (OM) and water (Koc based on Molecular Connectivity Index (MCI) in KOCWIN v2.00).

#	Chemical CAS	Koc (L/kg OM)	Calculated mean inherent pore water (bulk water) concentration (µg/L)	Calculated worst-case (S1) mean added bulk water concentration (µg/L)
1	Mustard gas 505-60-2	243	ES: 0.031±0.002 FS: Not detected RA: 0.040±0.002	ES: 0,000094±0.000006 FS: Not detected RA: 0.000073±0.000003
1.2	1,4-Dithiane 505-29-3	1	ES: 0.566±0.017 FS: 0.672±0.035 RA: 1.77±0.058	ES: 0.000029±0.000001 FS: 0.000034±0.000002 RA: 0.000090±0.000003
1.4	1,4,5-Oxadithiepane 3886-40-6	36	ES: 0.098±0.002 FS: 0.139±0.008 RA: 0.519±0.020	ES: 0.000030±0.000001 FS: 0.000043±0.000002 RA: 0.00016±0.000006
1.5	1,2,5-Trithiepane 6576-93-8	265	ES: 0.044±0.001 FS: 0.027±0.002 RA: 0.125±0.009	ES: 0.000089±0.000003 FS: 0.000055±0.000003 RA: 0.00025±0.000019
2	Adamsite 578-94-9	4401	ES: 0.360±0.012 FS: 0.205±0.007 RA: 0.749±0.038	ES: 0,0169±0.00066 FS: 0,0089±0.00029 RA: 0.0243±0.0012
2O	5,10-Dihydrophenarsazin-10-ol 10-oxide 4733-19-1	335583	ES: 0.0023±0.00007 FS: 0.0026±0.0002 RA: 0.0020±0.00007	ES: 0,0080±0.00027 FS: 0,0106±0.00064 RA: 0.0049±0.00018
3O	Diphenylarsinic acid 4656-80-8	520715	ES: 0.0021±0.0001 FS: 0.0002±0.00001 RA: 0.0091±0.0007	ES: 0,0122±0.00071 FS: 0,00084±0.00004 RA: 0,0351±0.0025
3T	Diphenylpropylthioarsine 17544-92-2	29188	ES: 0.0046±0.0002 FS: 0.0012±0.0001 RA: 0.0062±0.0003	ES: 0,0015±0.00006 FS: 0.00027±0.00002 RA: 0.0013±0.00006
4	Triphenylarsine 603-32-7	335583	ES: 0.0002±0.000006 FS: 0.0001±0.000005 RA: 0.0004±0.00002	ES: 0.00057±0.00002 FS: 0.00030±0.00001 RA: 0.00087±0.00004
4+4O	Triphenylarsine oxide (4O) 1153-05-5	520715	ES: 0.0006±0.00002 FS: 0.0002±0.00001 RA: 0.0008±0.00003	ES: 0.0022±0.00008 FS: 0.00069±0.00003 RA: 0.0031±0.00012
5O	Phenylarsonic acid 98-05-5	1001	ES: 0.307±0.007 FS: 0.110±0.005 RA: 0.672±0.025	ES: 0.0033±0.00008 FS: 0.0012±0.00005 RA: 0.0050±0.00019
5T	Dipropyl phenylarsonodithioite 1776-69-8	2017	ES:0.073±0.004 FS: 0.0091±0.0006 RA: 0.160±0.012	ES: 0.0015±0.00008 FS: 0.00017±0.00001 RA: 0.0024±0.00016
6	α-Chloroacetophenone 532-27-4	99	ES: 0.283±0.017 FS: Not detected RA: Not detected	ES:0.00022±0.00001 FS: Not detected RA: Not detected
10T	Tripropyl arsenotrithioite 5582-57-0	4815	ES: 0.0094±0.0008 FS: Not detected RA: Not detected	ES: 0.00055±0.00005 FS: Not detected RA: Not detected

Predicted no effect concentration (PNEC)

The environmental toxicity of mustard gas has been reviewed by Opresko et al. in 1998. The reviewed studies demonstrated that sulfur mustard is extremely toxic to all species, but its environmental action is limited by its low solubility. Results of the studies, involving a variety of aquatic organisms, showed that fish are the most sensitive species (compared with phytoplankton and higher aquatic plants). Mustard gas added to fish aquaria at 250 ppm formed globules on the bottom of the tanks, and amounts equivalent to 25-50 ppm were required for lethality in fish. For the three most sensitive species of fish, bluegill sunfish (*Lepomis macrochirus*), red-eared sunfish (*Lepomis microlophus*), and black bullheads (*Ameiurus melas*), the 30-day toxicity threshold was 2 mg/L. At a concentration of 1,000 mg/L, thiodigycol (TDG) was not toxic to juvenile bluegill sunfish within a 42-day observation period (Munro et al. 1999) – we therefore set the HC5 for TGD to 1000 mg/L. Based on the available literature the chronic EC50 for mustard gas is set to 2 mg/L. This value was used to derive a species sensitivity distribution for 14 different fish species using the USEPA extrapolation tool WEB ICE (https://www3.epa.gov/ceampubl/fchain/webice/index.html) with the most sensitive species, Bluegill sunfish, as the surrogate species. We applied as evident stringent taxonomic and statistical criteria for inclusion of species in the distribution. Table 4 shows a summary of the results leading to a mustard gas fish community HC5 of 0.69 mg/L as with the organoarsenicalswithout an assessment factor.

Table 4 WEB ICE output SSD table HC5 = 0.69 mg/L (CI = 0.27 - 0.88 mg/L).

Common Name	Scientific	Estimated Toxicity (µg/L)	95 % Confidence Intervals (µg/L)	Surrogate	Degrees of Freedom (N-2)	R2	p-value	Mean Square Error	Cross-validation Success (%)	Taxonomic Distance	Slope	Intercept
Atlantic salmon	*Salmo salar*	1142.57	703.59 - 1855.42	Bluegill (*Lepomis macrochirus*)	9	0.96	<1E-05	0.08	100.00	4	1.12	-0.64
Brook trout	*Salvelinus fontinalis*	1070.14	609.64 - 1878.50	Bluegill (*Lepomis macrochirus*)	16	0.88	<1E-05	0.20	88.88	4	1.05	-0.44
Brown trout	*Salmo trutta*	1320.28	725.92 - 2401.28	Bluegill (*Lepomis macrochirus*)	12	0.94	<1E-05	0.12	100.00	4	1.06	-0.39
Channel catfish	*Ictalurus punctatus*	3192.69	2171.92 - 4693.20	Bluegill (*Lepomis macrochirus*)	71	0.75	<1E-05	0.44	76.71	4	0.79	0.87
Chinook salmon	*Oncorhynchus tshawytscha*	557.05	169.06 - 1835.42	Bluegill (*Lepomis macrochirus*)	4	0.90	0.0035	0.20	83.33	4	1.34	-1.69
Coho salmon	*Oncorhynchus kisutch*	1614.65	812.40 - 3209.11	Bluegill (*Lepomis macrochirus*)	11	0.91	<1E-05	0.15	92.30	4	0.98	-0.04
Common carp	*Cyprinus carpio*	3207.82	1483.08 - 6938.33	Bluegill (*Lepomis macrochirus*)	17	0.82	<1E-05	0.37	84.21	4	0.85	0.67
Fathead minnow	*Pimephales promelas*	2894.85	2011.69 - 4165.72	Bluegill (*Lepomis macrochirus*)	66	0.79	<1E-05	0.43	75.00	4	0.84	0.66
Goldfish	*Carassius auratus*	4299.28	2180.67 - 8476.20	Bluegill (*Lepomis macrochirus*)	21	0.80	<1E-05	0.42	86.95	4	0.81	0.93
Guppy	*Poecilia reticulata*	3483.19	1488.44 - 8151.21	Bluegill (*Lepomis macrochirus*)	15	0.83	<1E-05	0.44	76.47	4	0.80	0.89
Lake trout	*Salvelinus namaycush*	863.02	495.58 - 1502.89	Bluegill (*Lepomis macrochirus*)	20	0.70	<1E-05	0.27	86.36	4	0.68	0.68
Rainbow trout	*Oncorhynchus mykiss*	1486.67	1317.93 - 1677.01	Bluegill (*Lepomis macrochirus*)	307	0.88	<1E-05	0.21	90.61	4	0.93	0.08
Sheepshead minnow	*Cyprinodon variegatus*	2560.64	1814.03 - 3614.53	Bluegill (*Lepomis macrochirus*)	70	0.71	<1E-05	0.39	86.11	4	0.80	0.75
White sucker	*Catostomus commersonii*	1406.96	327.90 - 6036.99	Bluegill (*Lepomis macrochirus*)	2	0.97	0.0141	0.04	100.00	4	1.16	-0.68

For the new detected parent mustard gas and dissipation products of mustard gas, we have conducted new OECD standardized GLP tests (algae (*Raphidocelis subcapitata*) and crustacean *(Daphnia magna)*) as well as on a marine bacteria (*Allivibiro fischerei*) in Microtox™. We chose to test the compound 1-Oxa-4,5-dithiepane as a representative for the cyclic mustard gas dissipation products based on the initial screening of these in Microtox™ where we found it to be one of the most toxic of the compounds at 1.7 mg / L (range in Microtox™ = 1.2 – 47.4 mg / L). Moreover, the compound is among the most frequently detected compounds of the cyclic mustard gas degradation products and also found at the highest concentrations in the sediment of the Baltic Sea (Christensen et al. 2016). We applied an assessment factor of 500 to the derived NOECs from the tests in accordance with EU guidelines (EU TGD 2003) as we have two long term freshwater NOECs representing two trophic levels (algae and crustacean) the predicted no effect concentration (see Table 5 (see Appendix 1).

Table 5 Assessment factors proposed for deriving $PNEC_{water}$ for saltwater for different data sets.

Data set	Assessment factor
Lowest short-term L(E)C50 from freshwater or saltwater representatives of three taxonomic groups (algae, crustaceans and fish) of three trophic levels	10,000 [a]
Lowest short-term L(E)C50 from freshwater or saltwater representatives of three taxonomic groups (algae, crustaceans and fish) of three trophic levels, + two additional marine taxonomic groups (e.g. echinoderms, molluscs)	1000 [b]
One long-term NOEC (from freshwater or saltwater crustacean reproduction or fish growth studies)	1000 [b]
Two long-term NOECs from freshwater or saltwater species representing two trophic levels (algae and/or crustaceans and/or fish)	500 [c]
Lowest long-term NOECs from three freshwater or saltwater species (normally algae and/or crustaceans and/or fish) representing three trophic levels	100 [d]
Two long-term NOECs from freshwater or saltwater species representing two trophic levels (algae and/or crustaceans and/or fish) + one long-term NOEC from an additional marine taxonomic group (e.g. echinoderms, molluscs)	50
Lowest long-term NOECs from three freshwater or saltwater species (normally algae and/or crustaceans and/or fish) representing three trophic levels + two long-term NOECs from additional marine taxonomic groups (e.g. echinoderms, molluscs)	10

The chronic algae test resulted in a growth rate and yield $NOEC_{72hr}$ of >8.41 mg/L (see Figure 4.

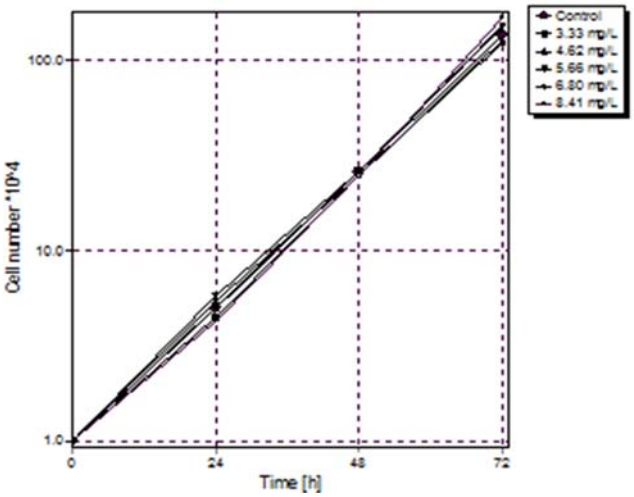

Figure 4 Result of chronic algae growth inhibition test result of 1-Oxa-4,5-dithiepane.

The *Daphnia magna* test resulted in a NOEC of 0.825 mg/L. The resulting PNEC is thus 8.41/500 for algae and for *Daphnia magna;* 0.825/500 mg/L = 0.016 and 0.00165 mg/L, for 1-Oxa-4,5-dithiepane. These toxicity values were also assigned to the other detected cyclic mustard gas dissipation products detected in this report. The PNEC results are summarized in Table 6.

Table 6 PNECs for detected CWAs (mg/L).

Compound	PNEC
Mustard gas	0.69
Organoarsenic CWAs	0.29
Thiodigycol	1000
Cyclic mustard gas products	0.016* / 0.00165**
α-chloroacetophenone	0.5

*) Chronic **Raphidocelis subcapitata* and ***Daphnia magna* test.

Predicted fish community and environmental risk (RQ)

In Table 7, the calculated mean (for each route) inherent environmental daphnia and algae (in italics) risk quotient (RQ) for cyclic mustard degradation products (1.2, 1.4 and 1.5) and fish community RQ for all other chemicals are shown. Furthermore, the calculated mean (for each route) added environmental daphnia and algae (in italics) RQ for the worst-case scenario (S1), with high sediment resuspension from sweeping and distance 200 m from pipeline, are shown for cyclic mustard degradation products (1.2, 1.4 and 1.5) and fish community RQ for all other chemicals.

In Table 8, the calculated maximum inherent RQ and maximum worst-case (S1) added RQ are shown. The conditions are the same as in Table 7.

Table 7 Calculated mean inherent RQ and worst-case (S1) mean added RQ. Mean RQ are for all stations along a route. Total RQ = inherent RQ + added RQ. For cyclic mustard degradation products (1.2, 1.4 and 1.5) the environmental daphnia RQ and environmental algae RQ (in italics) are shown. For all other chemicals, the fish community RQ is shown.

#	Chemical CAS	Calculated mean inherent RQ	Calculated worst-case (S1) mean added RQ
1	Mustard gas 505-60-2	ES: 0.00005±0.000003 FS: Not detected RA: 0.00006±0.000003	ES: <1E-05 FS: Not detected RA: <1E-05
1.2	1,4-Dithiane 505-29-3	ES: 0,34±0,01 *(0,035±0,001)* FS: 0.41±0.02 *(0,042±0,002)* RA: 1.07±0.04 *(0,11±0,004)*	ES: 0,00002±0.0000005 *(<1E-05)* FS: 0.00002±0.000001 *(<1E-05)* RA: 0.00006±0.000002 *(<1E-05)*
1.4	1,4,5-Oxadithiepane 3886-40-6	ES: 0.059±0.001 *(0,0061±0,0001)* FS: 0.085±0.005 *(0,0087±0,0005)* RA: 0.31±0.012 *(0,032±0,001)*	ES: 0.00002±0.0000003 *(<1E-05)* FS: 0.00003±0.000002 *(<1E-05)* RA: 0.0001±0.000004 *(<1E-05)*
1.5	1,2,5-Trithiepane 6576-93-8	ES: 0.027±0.0009 *(0,0028±0,00009)* FS: 0.017±0.001 *(0,0017± 0,0001)* RA: 0.076±0.006 *(0,0078± 0,0006)*	ES: 0.00005±0.000002 *(<1E-05)* FS: 0.00003±0.000002 *(<1E-05)* RA: 0.0002±0.00001 *(0,00002±0,000001)*
2	Adamsite 578-94-9	ES: 0.0012±0.00004 FS: 0.0007±0.00002 RA: 0.0026±0.0001	ES: 0.00006±0.000002 FS: 0.00003±0.000001 RA: 0.00008±0.000004
2O	5,10-Dihydrophenarsazin-10-ol 10-oxide 4733-19-1	ES: <1E-05 FS: <1E-05 RA: <1E-05	ES: 0,00003±0,0000009 FS: 0,00004±0,000002 RA: 0,00002±0,0000006
3O	Diphenylarsinic acid 4656-80-8	ES: <1E-05 FS: <1E-05 RA: 0.00003±0.000002	ES: 0,00004±0,000003 FS: <1E-05 RA: 0,0001±0,000009
3T	Diphenylpropylthioarsine 17544-92-2	ES: 0.00002±0.0000006 FS: <1E-05 RA: 0.00002±0.000001	ES: <1E-05 FS: <1E-05 RA: <1E-05
4	Triphenylarsine 603-32-7	ES: <1E-05 FS: <1E-05 RA: <1E-05	ES: <1E-05 FS: <1E-05 RA: <1E-05
4+4O	Triphenylarsine oxide (4O) 1153-05-5	ES: <1E-05 FS: <1E-05 RA: <1E-05	ES: <1E-05 FS: <1E-05 RA: 0,00001±0,0000004
5O	Phenylarsonic acid 98-05-5	ES: 0.0011±0.00002 FS: 0.0004±0.00002 RA: 0.0023±0.00009	ES: 0,00001±0,0000003 FS: <1E-05 RA: 0,00002±0,0000006
5T	Dipropyl phenylarsonodithioite 1776-69-8	ES: 0.0003±0.00001 FS: 0.00003±0.000002 RA: 0.0006±0.00004	ES: <1E-05 FS: <1E-05 RA: <1E-05
6	α-Chloroacetophenone 532-27-4	ES: 0.0006±0.00003 FS: Not detected RA: Not detected	ES: <1E-05 FS: Not detected RA: Not detected
10T	Tripropyl arsenotrithioite 5582-57-0	ES: 0.00003±0.000003 FS: Not detected RA: Not detected	ES: <1E-05 FS: Not detected RA: Not detected

Table 8 Calculated maximum inherent RQ and worst-case (S1) maximum added RQ. Number of sampling stations with RQ>1 are stated for each sampling route. For cyclic mustard degradation products (1.2, 1.4 and 1.5) the environmental daphnia RQ and environmental algae RQ (in italics) are shown. For all other chemicals, the fish community RQ is shown.

#	Chemical CAS	Calculated max inherent RQ (number of stations with RQ>1)	Calculated max worst-case (S1) added RQ
1	Mustard gas 505-60-2	ES: 0.00005±0.000003 FS: Not detected RA: 0.00009±0.000005	ES: <1E-05 FS: Not detected RA: <1E-05
1.2	1,4-Dithiane 505-29-3	ES: 0.39±0.02 *(0,040±0.002)* FS: 0.41±0.02 *(0,042±0.002)* RA: 2.04±0.11 (2 stations) *(0,21±0.011)*	ES: 0,00002±0.000001 *(<1E-05)* FS: 0.00002±0.000001 *(<1E-05)* RA: 0.0001±0.000005 *(0.00001±0.0000006)*
1.4	1,4,5-Oxadithiepane 3886-40-6	ES: 0.083±0.005 *(0,0085±0.0005)* FS: 0,085±0.005 *(0,0087±0.0005)* RA: 0,54±0.03 *(0,056±0.0003)*	ES: 0.00003±0.000001 *(<1E-05)* FS: 0.00003±0.000002 *(<1E-05)* RA: 0.0002±0.000009 *(0.00002±0.000001)*
1.5	1,2,5-Trithiepane 6576-93-8	ES: 0,046±0.005 *(0,0048±0.0005)* FS: 0,043±0.005 *(0,0045±0.0005)* RA: 0,46±0.05 *(0,048±0.005)*	ES: 0.00009±0.00001 *(<1E-05)* FS: 0.00009±0.000009 *(<1E-05)* RA: 0.0009±0.0001 *(0,0001±0,00001)*
2	Adamsite 578-94-9	ES: 0,020±0.001 FS: 0,0020±0.0001 RA: 0,034±0.002	ES: 0.0011±0.00007 FS: 0.00009±0.000006 RA: 0.0011±0.00007
2O	5,10-Dihydrophenarsazin-10-ol 10-oxide 4733-19-1	ES: 0.00008±0.000006 FS: 0.00002±0.000002 RA: 0.00003±0.000002	ES: 0,0003±0,00002 FS: 0,00009±0,000007 RA: 0,00008±0,000006
3O	Diphenylarsinic acid 4656-80-8	ES: 0,0002±0.00001 FS: <1E-05 RA: 0.0004±0.00003	ES: 0,0010±0,00008 FS: <1E-05 RA: 0,0014±0,0001
3T	Diphenylpropylthioarsine 17544-92-2	ES: 0.00009±0.000008 FS: <1E-05 RA: 0.00005±0.000004	ES: 0.00003±0.000003 FS: <1E-05 RA: 0.00001±0.0000009
4	Triphenylarsine 603-32-7	ES: <1E-05 FS: <1E-05 RA: <1E-05	ES: <1E-05 FS: <1E-05 RA: <1E-05
4+4O	Triphenylarsine oxide (4O) 1153-05-5	ES: 0.00002±0.000002 FS: <1E-05 RA: 0.00001±0.0000009	ES: 0.00008±0.000006 FS: <1E-05 RA: 0,00004±0,000003
5O	Phenylarsonic acid 98-05-5	ES: 0,0066±0.0004 FS: 0,0005±0.00003 RA: 0,011±0.0008	ES: 0,00008±0,000005 FS: <1E-05 RA: 0,00009±0,000006
5T	Dipropyl phenylarsonodithioite 1776-69-8	ES: 0,0022±0.0002 FS: 0.00006±0.000007 RA: 0,0020±0.0002	ES: 0.00005±0.000006 FS: <1E-05 RA: 0.00003±0.000003
6	α-Chloroacetophenone 532-27-4	ES: 0,0006±0.00003 FS: Not detected RA: Not detected	ES: <1E-05 FS: Not detected RA: Not detected
10T	Tripropyl arsenotrithioite 5582-57-0	ES: 0.00003±0.000003 FS: Not detected RA: Not detected	ES: <1E-05 FS: Not detected RA: Not detected

In Table 9, the calculated maximum added RQ for sediment release scenarios S1 (worst-case) to S6, see Section 2.3, are shown. RQ are summed for all chemicals. For the cyclic mustard degradation products (1.2, 1.4 and 1.5), the environmental daphnia RQ and environmental algae RQ (in italics) are used. For all other chemicals, the fish community RQ is used. The sampling station with the maximum added RQ and the pipeline activity at that station is shown.

Table 9 Calculated maximum added RQ for sediment release scenarios S1 (worst-case) to S6, summed for all chemicals. Sampling station with maximum RQ are stated for each sampling route as well as the pipeline activity at that station. For cyclic mustard degradation products (1.2, 1.4 and 1.5) the environmental daphnia RQ and environmental algae RQ (in italics) are used. For all other chemicals, the fish community RQ is used.

Sediment release scenario (see section 2.3)	S1 (worst-case)	S2	S3	S4	S5	S6
Calculated maximum added RQ (sum chemicals)	**ES:** 0,0024±0.0001 *(0,0024±0.00001)*	**ES:** 0,0020±0,00009 *(0,0020±0.00009)*	**ES:** 0,0017±0,00007 *(0,0017±0.00007)*	**ES:** 0,0013±0,00006 *(0,0013±0.00006)*	**ES:** 0,0009±0,00004 *(0,0009±0.00004)*	**ES:** 0,0006±0,00003 *(0,0006±0.00003)*
	FS: 0,0001±0.00001 *(0,0001±0.000007)*	**FS:** 0,0001±0,00001 *(0,0001±0.000006)*	**FS:** 0,0001±0,00001 *(0,00009±0.000005)*	**FS:** 0,00007±0,000004 *(0,00007±0.000004)*	**FS:** 0,00005±0,000003 *(0,00005±0.000003)*	**FS:** 0,00004±0,000002 *(0,00002±0.000003)*
	RA: 0,0016±0.0001 *(0,0016±0,0001)*	**RA:** 0,0016±0.0001 *(0,0016±0.0001)*	**RA:** 0,0016±0.0001 *(0,0016±0.0001)*	**RA:** 0,0004±0.00003 *(0,0004±0.00003)*	**RA:** 0,0004±0.00003 *(0,0004±0.00003)*	**RA:** 0,0004±0.00003 *(0,0004±0.00003)*
Sampling stations with maximum added RQ (sum chemicals)	**ES:** ES_11_250m_NW, sweeping and trenching *ES_11_250m_NW, sweeping and trenching)*	**ES:** ES_11_250m_NW, sweeping and trenching *(ES_11_250m_NW, sweeping and trenching)*	**ES:** ES_11_250m_NW, sweeping and trenching *(ES_11_250m_NW, sweeping and trenching)*	**ES:** ES_11_250m_NW, sweeping and trenching *(ES_11_250m_NW, sweeping and trenching)*	**ES:** ES_11_250m_NW, sweeping and trenching *(ES_11_250m_NW, sweeping and trenching)*	**ES:** ES_11_250m_NW, sweeping and trenching *(ES_11_250m_NW, sweeping and trenching)*
	FS: FS_02_500m_NW, sweeping *(FS_05, sweeping and trenching)*	**FS:** FS_02_500m_NW, sweeping *(FS_05, sweeping and trenching)*	**FS:** FS_02_500m_NW, sweeping *(FS_05, sweeping and trenching)*	**FS:** FS_05, sweeping and trenching *(FS_05, sweeping and trenching)*	**FS:** FS_05, sweeping and trenching *(FS_05, sweeping and trenching)*	**FS:** FS_02_500m_NW, sweeping *(FS_05, sweeping and trenching)*
	RA: RA_19, sweeping *(RA_19, sweeping)*	**RA:** RA_19, sweeping *(RA_19, sweeping)*	**RA:** RA_19, sweeping *(RA_19, sweeping)*	**RA:** RA_19, sweeping *(RA_19, sweeping)*	**RA:** RA_19, sweeping *(RA_19, sweeping)*	**RA:** RA_19, sweeping *(RA_19, sweeping)*

Discussion

Since 2008, changes have been made in the chemical analysis methodologies that have improved the lowest limits of quantitation (LLOQ) and increased the frequency of findings from the sediment considerably. The highest LLOQ values in 2008–2012 were approximately at 20 µg/kg dw. Thus, the methods used in 2008–2012 would not have detected all findings below this level. Therefore, if the results from 2015–2016 need to be compared with the results from 2008–2012, only results above 20 µg/kg dw should be included (Söderström et al., 2016).

From the previous analyses, only once a sulphur mustard gas-related chemical was found in the sediment samples even though sulphur mustard had been the most frequently dumped agent. To better be able to locate mustard gas dumping areas and detect residues of CWAs several new chemicals were introduced in the analytical analyses, i.e. the cyclic degradation products for sulphur mustard: 1.2–1.6 and oxidation product for triphenyl arsine: 4O (Söderström et al., 2016).

It is notable that several samples contained multiple different CWA residues, suggesting a more homogeneous distribution of the exposure with time. It is assumed that the identified mustard gas were present as micro-particles in the sample, rather than a dissolved amount of the compound (pers comm. Martin Søderstrøm, VERIFIN). This could explain why several samples taken from a small area showed that some of these samples contained very high concentrations (over 1000 µg/kg) while at neighbouring stations there were no findings.

The inclusion of cyclic degradation products for sulphur mustard in the chemical analysis gave detection of compounds that are new to the field of environmental toxicology and risk assessment. Hence, we had to commission an investigation of their environmental toxicity following OECD and GLP test guidelines. As indicated above we chose to test the most risky (hazardous) compound, 1-Oxa-4,5-dithiepane, based on preliminary data from the NATO SPS MODUM project (Christensen et al. 2016) with two chronic tests to derive an assessment factor of 500 resulting in a PNEC of 0.0165 mg/L. This data point could be improved by additional testing and subsequent lowering of the assessment factor. In addition, the assessment could be improved by testing all the cyclic mustard gas compounds for their environmental toxicities.

Moreover, the assessment for the cyclic mustard gas compounds are not fish community specific as for the rest of the compounds, but rather an overall marine environmental risk screening.

Conclusions

The inherent mean RQ (for all stations along any route) is up to a factor of 1000 higher than the worst-case added mean RQ from pipeline installations, see Table 3.5. Chemicals with high sorption coefficients (Koc) have a relatively higher impact on the added RQ due to the larger amount of chemical that is sorbed to the re-suspended particles. However, for these chemicals, i.e. (2O), (3O), (4+4O), the measured concentrations are relatively low, toxicity threshold values are relatively high and the added RQ therefore generally low.

The maximum added RQ for single chemicals is 0.001 for (2) and (3O) occurring at the ES and RA routes, see Table 3.6. The value is found at stations RA_10 for (2) and RA_19 for (3O) both situated within the secondary dumpsite only affected by sweeping, and at station ES_11_250m_NW for both (2) and (3O) in the vicinity of the western border of the secondary dumpsite affected by sweeping and trenching. For all other chemicals and sampling stations along all three routes the added RQ for single chemicals is <.0.0009

The maximum added RQ for the sum of chemicals is 0.0024 for route ES, in the interval 0.0012-0.0016 for route RA and 0.0001 for route FS, see Table 9. These values are found at sampling stations ES_11_250m_NW affected by sweeping and trenching, and situated in close proximity to the secondary dumpsite, RA_10, RA_16 and RA_19 affected only by sweeping situated within the secondary dumpsite, and FS_02 affected by sweeping and trenching close to the western border of the secondary dumpsite. For all other sampling stations along all three routes the added RQ for the sum of chemicals is <0.0006.

Furthermore, the added RQ decreases with distance from the pipeline, i.e. at distances 200 m (S1), 500 m (S2) and 1000 m (S3) the maximum added RQ for the sum of chemicals for the ES route is 0.0024, 0.0020 and 0.0017, respectively. The decrease of added RQ with distance is most predominant for the ES route because the concentrations of (2O), (3O) and (4+4O) that have the relative highest Koc values are highest for the ES route.

In summary, the mean and maximum added RQs from pipeline installation for the sum of chemicals are below one (<0.003) for all three routes indicating a negligible risk. The two routes ES and RA have approximately the same maximum added RQs, whereas the value for route FS is a factor of approximately 20 lower. Clearly, the route RA with the highest single finds of parent and degradation CWA is less favourable relative to the ES route from a CWA exposure point of view. Hence, in conclusion the FS or ES route is recommended from a CWA exposure avoidance perspective.

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Fraunhofer
IME

Study Report

Freshwater Alga, Growth Inhibition Test (OECD 201)

1-Oxa-4,5-dithiepane: Effects on *Raphidocelis subcapitata*

GLP Study Code of Test Facility: AAR-001/4-10/A

Sponsor
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Study director
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Number of pages
43

June 30, 2016

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GLP certificate

Ministerium für Arbeit, Integration und Soziales
des Landes Nordrhein-Westfalen

Fürstenwall 25, 40219 Düsseldorf — Aktenzeichen III 5 – 8673.79

Gute Laborpraxis/Good Laboratory Practice
GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

Fraunhofer Institut für
Molekularbiologie und Angewandte Oekologie IME
Auf dem Aberg 1
57392 Schmallenberg

Prüfungen nach Kategorien (gemäß ChemVwV-GLP Nr. 5.3/OECD guidance)	**Areas of Expertise** (according ChemVwV GLP Nr. 5.3/OECD guidance)
Kategorie 1 Prüfungen zur Bestimmung der physikalisch-chemischen Eigenschaften und Gehaltsbestimmungen	category 1 physical-chemical testing
Kategorie 4 Ökotoxikologische Prüfungen zur Bestimmung der Auswirkungen auf aquatische und terrestrische Organismen	category 4 environmental toxicity studies on aquatic and terrestrial organisms
Kategorie 5 Prüfungen zum Verhalten im Boden, im Wasser und in der Luft; Prüfungen zur Bioakkumulation und zur Metabolisierung	category 5 studies on behaviour in water, soil and air; bioaccumulation

GLP certificate (continued)

Kategorie 6 Prüfungen zur Bestimmung von Rückständen	category 6 residue studies
Kategorie 7 Prüfungen zur Bestimmung der Auswirkungen auf Mesokosmen und natürliche Ökosysteme	category 7 studies on effects on mesocosms and natural ecosystems

Datum der Inspektion

16. Dezember 2015

Die/Der genannte Prüfeinrichtung/Prüfstandort befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Düsseldorf, 31. Mai 2016
Im Auftrag

(Steffen Röddecke)

Date of Inspection

16th December 2015

The above mentioned test facility/ test site is included in the national GLP Compliance Programme and is inspected on a regular basis.

Based on the inspection report it can be confirmed, that this test facility/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Land Nordrhein-Westfalen · Ministerium für Arbeit, Integration und Soziales

Dienstsiegel/official-seal

2/2

Statement of GLP compliance

Title of the study:	Freshwater Alga, Growth Inhibition Test (OECD 201). 1-Oxa-4,5-dithiepane: Effects on *Raphidocelis subcapitata*.
Test item:	1-Oxa-4,5-dithiepane
GLP code:	AAR-001/4-10/A

This study was conducted in compliance with Good Laboratory Practice Regulations (GLP). The German requirements [1] are based on the OECD Principles of Good Laboratory Practice [2], which are accepted by regulatory authorities throughout the European Community [3], the United States of America (FDA and EPA) and Japan (MHLW, MAFF and METI) on the base of intergovernmental agreements.

We hereby attest to the authenticity of the study and guarantee that the data are correct, and that the study was performed by the procedures described. This report accurately reflects the raw data. There were no known circumstances which may have affected the integrity of the study.

Schmallenberg,

June 30, 2016 — A. Wenzel

Date — Study director (Dr. Andrea Wenzel)

June 30, 2016

Date — Chemical investigator (Dr. Matthias Kotthoff)

July 01, 2016

Date — Test facility management (Prof. Dr. Christoph Schäfers)

Statement of the Quality Assurance Unit

Title of the study:	Freshwater Alga, Growth Inhibition Test (OECD 201). 1-Oxa-4,5-dithiepane: Effects on *Raphidocelis subcapitata.*
Test item:	1-Oxa-4,5-dithiepane
GLP code:	AAR-001/4-10/A

Date of study plan revision:	May 09, 2016
Audit of the biological test:	
Determination of cell density at test termination	May 12, 2016
Audit report to study director:	May 12, 2016
Audit report to management:	May 12, 2016
Intervals of inspections:	Laboratories, three months
Audit of the final report:	June 29, 2016
Study completion:	June 30, 2016

The Quality Assurance Unit of the test facility inspected the study, audited the final report, and reported possible findings to the Study Director and to the management.

The Quality Assurance Unit found no discrepancies between final report and raw data and confirms that the methods, procedures, and observations are accurately and completely described, and that the reported results accurately and completely reflect the raw data of the study.

Schmallenberg,

June 30, 2016 — Lars Wiedemann-Krantz

Date — Quality Assurance Unit (Lars Wiedemann-Krantz)

Study Plan Amendments / GLP Deviations

No GLP deviations occurred. There was no amendment to the study plan and no deviation from the OECD guideline.

Distribution list

Sponsor:	1 original (1st of two), electronic (pdf) file of original
GLP-archive:	1 original (2nd of two)
Study director:	electronic (pdf) file of original
Chemical Investigator:	electronic (pdf) file of original

Archiving

An aliquot of the test item, the test protocols, all raw data and all records necessary to reconstruct the study are archived in the GLP archive of the Fraunhofer Institute for Molecular Biology and Applied Ecology, 57392 Schmallenberg, Germany, to be kept for 15 years following internal SOPs, according to the Principles of Good Laboratory Practice [1], [2]. The sponsor is obliged to notify the GLP archive of the Fraunhofer IME of any change of address to ensure that test item and records can be returned after the end of the archiving period, if desired. After the end of the archiving period Fraunhofer IME will send a letter to the indicated address to clarify the further use of the test item and records. These will be discarded after 16 years if Fraunhofer IME has not received a written notice giving instruction to either return test item and records or to further archive them in the GLP archive of the Fraunhofer IME.

List of archived records:

- Data specifying the test item
- Data concerning the test organism (origin, stock culture)
- Original raw data of test (test conditions, i.e. pH-values, temperature, records of chemical analysis)
- Records of statistical evaluation
- Original study plan
- Original final report

Summary

A study was performed at the Fraunhofer Institute for Molecular Biology and Applied Ecology IME to determine the effects of 1-Oxa-4,5-dithiepane on the growth of the uni-cellular fresh-water green algal species *Raphidocelis subcapitata*. Exponentially-growing cultures of the alga were exposed to various concentrations of the test item over several generations under defined conditions for 72 hours according to the OECD guideline 201 [4].

The nominal test concentrations were prepared in sterile growth medium under sterile conditions. For the determination of alga growth eight replicates of the control (test medium only) and four replicates of each test concentration were exposed to the following five nominal concentrations: Control, 4.10, 5.12, 6.40, 8.00 and 10.0 mg/L.

The concentrations of the test item in the test media were determined by chemical analysis of the test item in the aqueous phase of all treatment levels using GC-MS at start and at the end of the growth test (LOQ 0.1 mg/L). The nominal concentrations of 1-Oxa-4,5-dithiepane had been confirmed by the analytical measurements at test start; the concentrations were 3.33, 4.62, 5.66, 6.80 and 8.41 mg/L (81.2 – 90.0% of nominal). After 72 hours the concentrations decreased slightly to 3.15, 4.12, 5.22, 6.37 and 7.60 mg/L (75.9 – 81.3% of nominal). Since the test item concentrations were partly below 80% of nominal at test end but was found to be stable during the test period (89.3 - 94.5% of measured initial concentrations), the test evaluation was based on the measured initial concentrations.

There were no concentration-dependent inhibiting effects on the growth of the green algae over the range of the tested concentrations. The 72 hour $E_rC_{50,\ 20,\ 10}$ and $E_yC_{50,\ 20,\ 10}$ for growth rate and yield were above the highest test concentration of 8.41 mg/L.
The NOEC for both growth rate and yield was calculated to be ≥8.41mg 1-Oxa-4,5-dithiepane item/L (Table 1).

Table 1: Effective concentrations of 1-Oxa-4,5-dithiepane for the exposure of *Raphidocelis subcapitata* for 72 hours.

Parameter*		EC_{50}	EC_{20}	EC_{10}	LOEC	NOEC
		Measured initial concentrations [mg test item/L]				
Growth rate (r)	Value	>8.41	>8.41	>8.41	>8.41	≥8.41
	95 %-cl lower	-	-	-		
	95 %-cl upper	-	-	-		
Yield (y)	Value	>8.41	>8.41	>8.41	>8.41	≥8.41
	95 %-cl lower	-	-	-		
	95 %-cl upper	-	-	-		

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Definitions/Abbreviations

Biomass	Cell number
$EC_{10/20/50}$	(Effective level) is the level of the test item, which results in a 10, 20 or 50 per cent reduction in the measured parameter relative to the control
EPA	United States Environmental Protection Agency
FDA	Food and Drug Administration (USA)
GLP	Good Laboratory Practice
Growth rate (r)	Logarithmic algal cell number increase (average growth rate) during the exposure period
GC-MS	Gas Chromatography - Mass Spectrometry
LOEC	(Lowest observed effect concentration) is the lowest concentration tested at which the measured parameter shows significant inhibition relative to the control
LOD	Limit of Detection
LOQ	Limit of Quantitation
MAFF	Ministry of Agriculture, Forestry and Fisheries (Japan)
METI	Ministry of Economy, Trade and Industry (Japan)
MHLW	Ministry of Health, Labour and Welfare (Japan)
NOEC	(No observed effect concentration) is the highest concentration tested at which the measured parameter shows no significant inhibition relative to the control
OECD	Organisation for Economic Co-operation and Development
SOP	Standard Operation Procedure
Yield (y)	Algal biomass (cell counts/mL) at the end of the exposure period minus the algal biomass at the start of the exposure

1 Test identification

Freshwater Alga, Growth Inhibition Test.
1-Oxa-4,5-dithiepane: Effects on *Raphidocelis subcapitata*

Test item: 1-Oxa-4,5-dithiepane
GLP study code: AAR-0012/4-10/A

1.1 Sponsor

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Division Applied Ecology

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Study director:	Dr. Andrea Wenzel
Deputy:	Dr. Karsten Schlich
Chemical investigator:	Dr. Matthias Kotthoff
Deputy:	Stephan Hennecke
Quality Assurance Unit:	Dr. Cornelia Bernhardt
	Dr. Ursula Wahle
	Jennifer Teigeler
	Lars Wiedemann-Krantz
	Karin Fink

1.4 Sub-contracting

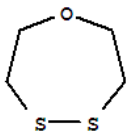
No sub-order was awarded in this study.

1.5 Schedule

Growth test:	May 09 – 12, 2016
Chemical analysis:	May 09 – 15, 2016
Study completion:	June 30, 2016

2 Test item

The test item was provided by the sponsor before the start of the study. By signing the study plan, the sponsor confirmed his agreement with the fact that the chemical identity, purity and stability of the test item under storage conditions (stated in accordance with OECD Principles of Good Laboratory Practice [2] were not examined analytically by the test facility. Test item which will not be needed for further testing and archiving will be stored at Fraunhofer-IME and will be disposed of as hazardous waste according to local regulations after expiry date.

Test item name:	**1-Oxa-4,5-dithiepane**
Synonyms:	1-Oxa-4,5-dithiacycloheptane
CAS number:	3886-40-6
Structural formula:	
State of matter and appearance:	very faint yellow oil
Batch/Lot Number:	EN1502b
Purity:	99.8 % (CoA, see A.6)
Molecular formula:	$C_4H_8OS_2$
Molecular mass:	136.24 g/mol
Vapour pressure:	No information available
Solubility in water:	soluble
Storage conditions:	Store at -20 °C. Store in conditions where escape to the environment by leakage is prevented.
Safety data sheet:	no
Quality test/Release date of CoA:	January 09, 2015
Origin of the test item:	Sponsor
Date of receipt:	March 03, 2016
Expiry date:	January 2017 (fixed by the study director: 2 years after date of CoA)

3 Test principle

The objective of this study was the assessment of the effects of the test item on the growth of the uni-cellular freshwater green alga *Raphidocelis subcapitata*.
The algae were exposed to various concentrations of the test item and control (growth medium without test item) under static conditions for a period of 72 hours. The algae were continuously illuminated and shaken. Cell densities were determined daily and the inhibitions of growth and yield were calculated. Growth is expressed as the logarithmic increase in biomass (average specific growth rate) during the exposure time. The test was performed in accordance with the OECD guideline 201 [4]. The concentrations of the test item were assessed by chemical analysis at start and end of the growth test.

4 Materials and methods

4.1 Test organism

4.1.1 Justification

The unicellular green alga *Raphidocelis subcapitata* was selected as a test organism representative of primary producers in freshwater, according to the OECD guideline 201 [4].

4.1.2 Specification

Species: *Raphidocelis subcapitata*, Chlorophycea, Chlorophyta, (former names *Pseudokirchneriella subcapitata*, *Selenastrum capricornutum*).

Origin: SAG, Culture Collection of Algae at Pflanzenphysiologisches Institut of the University at Göttingen, Albrecht von Haller Institut, Untere Klarspüle 2, D-37073 Göttingen, Catalog No 61.81 SAG.

Stock culture: The stock cultures are maintained fulfilling the criteria of the OECD guideline (culture medium recommended by Bringmann und Kühn (1980) [5]).

Pre-culture: Prior to testing a pre-culture was established in standard OECD growth medium to obtain exponentially-growing algae for the test. The culture duration of the pre-cultures was 3 days.

4.1.3 Reference substance

The sensitivity of the test organism is routinely checked using 3,5-dichlorophenol as primary standard following internal SOPs in a non-GLP test twice a year. The latest (February 2016)

nominal E_rC_{50} value of 2.83 mg/L (2.79 - 2.88 mg/L 95 % confidence limits) is in good agreement with the results of an international ring test with E_rC_{50} of 3.38 ± 1.30 mg/L [6].

4.2 Test procedure

4.2.1 Growth medium

The sterilised synthetic OECD medium according to OECD 201 [4] was used as growth medium (Table 2).
All stock solutions and the medium were prepared with purified water processed using an ELGA „PURELAB S7 + Classic UVF“. The pH of the medium was obtained at equilibrium between the carbonate system of the medium and the partial pressure of CO_2 in atmospheric air.

Table 2: Growth medium according to OECD 201, final concentrations

	mg/L		mg/L
$NaHCO_3$	50	H_3BO_3	0.185
NH_4Cl	15	$MnCl_2$ x 4 H_2O	0.415
KH_2PO_4	1.6	$ZnCl_2$	0.003
$MgSO_4$ x 7 H_2O	15	$CoCl_2$ x 6 H_2O	0.0015
$MgCl_2$ x 6 H_2O	12	$CuCl_2$ x 2 H_2O	0.00001
$CaCl_2$ x 2 H_2O	18	Na_2MoO_4 x 2 H_2O	0.007
$FeCl_3$ x 6 H_2O	0.064	Na_2EDTA x 2 H_2O	0.10
		pH, at test start	7.5 - 8.0

4.2.2 Test vessels

The test vessels used were 250 mL conical glass flasks covered with air-permeable silicone-sponge caps. Test vessels were cleaned using a dishwasher and additionally rinsed with purified water. Test vessels and caps were sterilised by autoclaving prior to use.

4.2.3 Test conditions

The culture vessels were incubated at 21 to 24°C, controlled at ± 2°C with a light intensity (day light: OSRAM “day light”) adjusted between 60 - 120 µE m^{-2} s^{-1} close to the surface of the liquid (equivalent 4440 and 8880 lux). Light measurements [µE m^{-2} s^{-1}] were made using a cosine (2 π) receptor (LI-250A with radiation sensor, LI-COR). Measurements were made of the direct light above the tray of the incubator. The cultures were oscillated by continuously stirring on a laboratory shaker with 150 rpm (Incubation Shaker Multitron®, INFORS, Switzerland).

During the exposure period, the incubation temperature was measured daily in an additionally prepared control vessel kept under the same conditions. The pH values were measured in the additionally prepared replicate at the beginning of the test and directly in the test vessels at the end of the test.

4.2.4 Cell counting

The cell concentrations were determined in the inoculum culture prior to the addition to the test vessels at test start and after 24, 48 and 72 h in the test cultures.
The cell density was measured using an electronic particle counter (CASY® TT, Innovatis, Germany).

4.2.5 Test concentrations / Range-finder

Range-finder test

A non-GLP range-finder test with nominal test concentrations of 0.1, 2 and 5 mg/L was performed without chemical analysis of the test item. Compared to controls there was no inhibition of growth rate and yield up to the highest test concentration.
The stability of the test item (0.141, 1.126 and 5.63 mg test item/L) in growth medium without algae under test conditions during the 3-day test period was confirmed in separate pre-test. All measured concentrations were between 96.0 and 108% of the nominal values.

Definitive test

The concentrations to be tested in the definitive test were selected on the basis of the results from the range-finding test and agreed upon with the monitor. Since only a limited test item quantity was available, 10 mg test item per litre was used as highest test concentration.

Control, 4.10, 5.12, 6.40, 8.00 and 10.0 mg/L (separation factor 1.25).

The concentrations of the test item in the test media were assessed by chemical analyses in the aqueous phase at start and end of the definitive growth-inhibition test. For chemical analysis see Annex A.5.

4.2.6 Preparation of the test media

Since the test item was water soluble, the highest test concentration was prepared by adding the required amount of the test item (20.04 mg, considering a purity of 99.8%) to 2 L sterile growth medium under sterile conditions to obtain a nominal concentration of 10.0 mg/L. The solution was stirred vigorously using a magnetic stirring bar for about 2 h at room temperature (ca. 20 °C). An aliquot of the clear test solution was then be diluted with sterile growth

medium to obtain the other four test concentrations. All dilution work was conducted under a clean bench using sterile medium and sterile equipment.

Table 3: Preparation of the test item solutions

Concentration number	Nominal concentrations [mg test item/L]	Volume of test concentration [mL]	Volume of growth medium [mL]
5	10.0	(20.0 mg)-	2000
4	8.00	1600 mL of concentration 5	400
3	6.40	1600 mL of concentration 4	400
2	5.12	1600 mL of concentration 3	400
1	4.10	1600 mL of concentration 2	400
Control	-	-	1000

4.2.7 Set up of the growth inhibition test

For the growth inhibition test there were four replicates of each test concentration and eight replicates of the control filled with 100 mL of the respective test medium. The algal pre-culture (754 µL at a cell density of 1.327 x 10^6 cells/mL) was added to the test vessels to achieve the initial cell concentration of 10,000 cells/mL.
At the beginning of the test, the initial cell concentration was calculated based on the cell number of the pre-culture. During the test, the cell concentrations were determined after 24, 48 and 72 hours.
All test preparations were performed under sterile conditions.

4.3 Sampling and chemical analysis of the test item

The concentrations of the test item in the water phase were assessed by chemical analysis using LC/MS (details of chemical analysis see Annex A.5). 2 x 5 mL samples were taken from all test solutions and control and at the beginning of the exposure period prior to addition of the algae. After 72 hours representative replicates were sampled for chemical analysis. 2 x 5 mL were taken from every test concentration level. The samples were frozen immediately until analysis. The samples were analysed May 15, 2016.

4.4 Evaluation and statistics

Numerical values in this report are frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in results obtained from calculations with such rounded values in comparison to those obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and thus not of practical concern.

- The evaluation of the test was based on measured initial concentrations of the test item since the measured concentrations of 1-Oxa-4,5-dithiepane were below 80% of nominal at test and but did not decrease more than 20 % during the test.
- The mean values of the cell counts for each concentration plot of the exposure test were used for plotting growth curves.
- For the growth test, mean average growth rates were calculated (entire exposure period of 0 - 3 d).
- For the growth test, calculation of the percent inhibition compared to controls of growth rate [r] and yield [y] for the exposure period were performed according to the guideline [4] and listed in a table.
- For the growth test, the percent inhibition values of growth rate and yield were plotted as a function of the test item concentration.
- Since the test results of the growth inhibition test (exposure period) did not show any inhibitions compared to controls, they were not statistically analysed to determine EC-values.
- The NOEC values were determined using Williams` Multiple Sequential t-Test [7] [8].
- The computer program ToxRat [9] was used for statistical evaluations.

5 Results

5.1 Validity of the test

The test fulfils all validity criteria of the OECD guideline 201 [4] as:

- The cell number in the control cultures increased by a factor of 135.8 within the 72 hour test period (see Table 10; validity criterion: > 16).
- Evaluation of the sectional growth rates of the controls: The mean of the replicate coefficients of variations (CV %) in the section-by-section growth rate of controls was 10.7 % during the test period (validity criterion ≤ 35 %, Table 11).
- The coefficient of variation of average specific growth rate at test end in replicate control cultures was 3.30 % (see Table 12; validity criterion ≤ 7 %).

5.2 Test conditions

Light intensity ranged from 101.62 µE m^{-2} s^{-1} to 103.66 µE m^{-2} s^{-1} and temperature was 22.0 C during the test (Table 8). The pH of the controls increased slightly by 0.28 units during the test (7.81 at test start and 8.09 (mean) at test end). In the test cultures the initial pH ranged between 7.66 and 7.81 and between 8.07 and 8.38 (mean values) at test termination. The data are compiled in Table 9 in chapter A.1.1 (Annex 1).

5.3 Chemical analysis

The test item was quantified by GC-MS measurements in samples of freshly prepared test solutions and controls prior to adding into the test vessels. At test end samples were taken from representative test replicates per treatment level and control. The level of quantification (LOQ) was ste at 0.1 mg/L. The samples were frozen immediately after sampling until analysis at -20°C. The samples were analysed May 15, 2016
Details of the analytical method and results are presented in Annex A.5.

The nominal concentrations of 1-Oxa-4,5-dithiepane had been confirmed by the analytical measurements at test start; the concentrations were 3.33, 4.62, 5.66, 6.80 and 8.41 mg/L (81.2 – 90.0% of nominal). After 72 hours the concentrations decreased slightly to 3.15, 4.12, 5.22, 6.37 and 7.60 mg/L (75.9 – 81.3% of nominal) (Table 4). Since the test item concentrations were partly below 80% of nominal at test end but was found to be stable during the test period (89.3 - 94.5% of measured initial concentrations), the test evaluation was based on the measured initial concentrations.

Table 4: Measured concentrations of 1-Oxa-4,5-dithiepane in the test vessels at start and end of the test (0-72 hours).

Nominal test item [mg/L]	Test start		Test end (72h)	
	measured [mg/L]	% of nominal	measured [mg/L]	% of nominal
Control	< LOQ	-	< LOQ	-
4.11	3.33	81.2	3.15	76.6
5.13	4.62	90.0	4.12	80.4
6.42	5.66	88.2	5.22	81.3
8.02	6.80	84.9	6.37	79.5
10.02	8.41	83.9	7.60	75.9

LOQ: Limit of quantification 0.1 mg/L

5.4 Growth inhibition test

5.4.1 Growth curves

The effect of the test item on the growth of *Raphidocelis subcapitata* was tested with five graded concentrations over an exposure period of 72 hours. There were no effects on the growth of the green alga over the range of the tested concentrations (Figure 1).

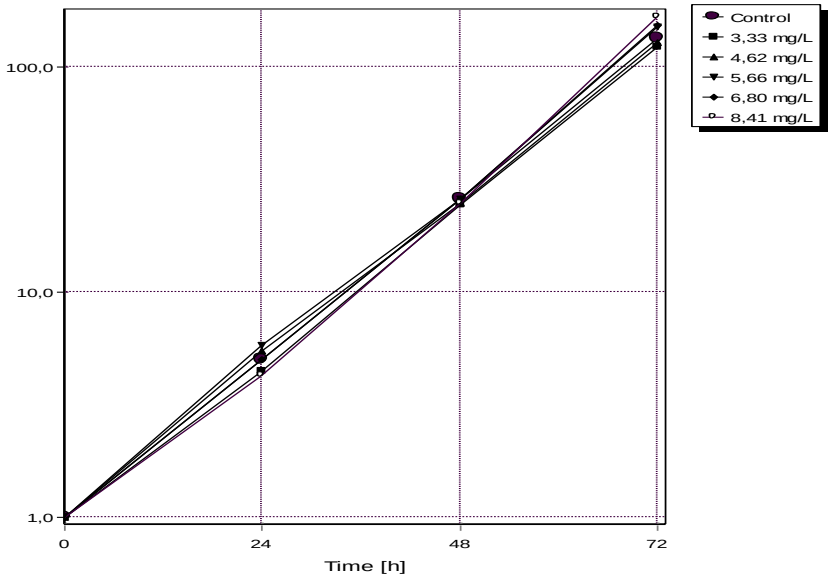


Figure 1: Cell number (x 10^4) of *Raphidocelis subcapitata* dependent on measured initial concentrations of the test item

5.4.2 Effective concentrations

The percent inhibition of yield and growth rate compared to the control are summarised in Table 5 and Figure 2 and Figure 3.

Since the test results of the growth inhibition test did not show any inhibition compared to the control, they were not statistically analysed to determine EC-values.
The NOEC values for both growth rate and yield were calculated to be ≥8.41mg 1-Oxa-4,5-dithiepane/L (Table 6).

Table 5: Percent inhibition of growth rate and yield compared to controls after 72 hours

Nominal test item [mg/L]	% Inhibition of growth rate	% Inhibition of yield
Control	-	-
3.33	1,6 (-)	8,40 (-)
4.62	1,0 (-)	5,40 (-)
5.66	-2,1 (-)	-11,2 (-)
6.80	-2,7 (-)	-13,2 (-)
8.41	-4,5 (-)	-24,1 (-)

(+) statistically significant difference between controls / (-) no significant difference between controls and treatments. Williams t-test (growth rate and yield), significance level 0.05, one-sided smaller.

Table 6: Effective concentrations of 1-Oxa-4,5-dithiepane for the exposure of *Raphidocelis subcapitata* for 72 hours

Parameter*		EC_{50}	EC_{20}	EC_{10}	LOEC	NOEC
		Measured initial concentrations [mg test item/L]				
Growth rate (r)	Value	>8.41	>8.41	>8.41	>8.41	≥8.41
	95 %-cl lower	n.d.	n.d.	n.d.		
	95 %-cl upper	n.d.	n.d.	n.d.		
Yield (y)	Value	>8.41	>8.41	>8.41	>8.41	≥8.41
	95 %-cl lower	n.d.	n.d.	n.d.		
	95 %-cl upper	n.d.	n.d.	n.d.		

n.d.: not determined due to mathematical reasons or inappropriate data (no inhibition > 50%)

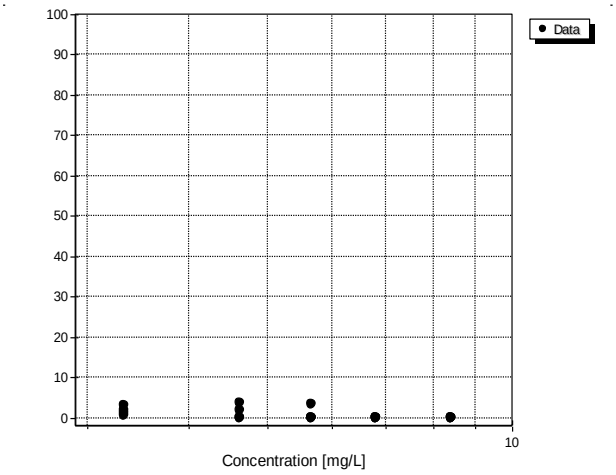


Figure 2: Influence of measured initial test item concentrations on percent inhibition of growth rate after 72 h

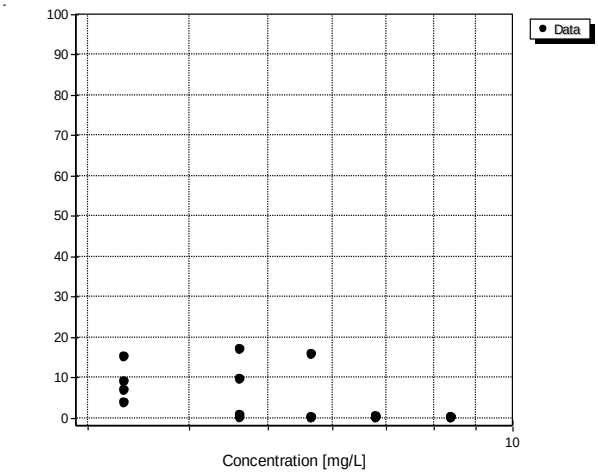


Figure 3: Influence of measured initial test item concentrations on percent inhibition of yield after 72 h

5.4.3 Other observations

Microscopic observation were performed to verify a normal and healthy appearance of the inoculum culture and to observe any abnormal appearance of the algae (as may be caused by the exposure to the test substance) at the end of the test.

Table 7: Microscopic observation of the cell cultures

Date	Observations
Test start, day 0	Inoculum culture: normal appearance of intact cells
Test end, day 3	
Control	Normal appearance of intact cells, almost no cell debris
3.33	Normal appearance of intact cells, almost no cell debris
4.62	Normal appearance of intact cells, almost no cell debris
5.66	Normal appearance of intact cells, almost no cell debris
6.80	Normal appearance of intact cells, almost no cell debris
8.41	Normal appearance of intact cells, almost no cell debris

5.4.4 Conclusion growth test

The 72 hour toxicity of 1-Oxa-4,5-dithiepane to the uni-cellular green alga *Raphidocelis subcapitata* was determined in a static system (OECD 201) exposed to nominal concentrations of 4.10, 5.12, 6.40, 8.00 and 10.0 mg/L plus control.

The nominal concentrations of 1-Oxa-4,5-dithiepane had been confirmed by the analytical measurements at test start (80 - 120% of nominal). At test termination, the measured concentrations were partly below 80% of nominal at test and but did not decrease more than 20% during the test. The test was evaluated using the measured initial concentrations.

There were no concentration-dependent inhibiting effects on the growth of the green algae over the range of the tested concentrations. The 72 hour $E_rC_{50,\ 20,\ 10}$ and $E_yC_{50,\ 20,\ 10}$ for growth rate and yield were above the highest test concentration of 8.41 mg/L.
The NOEC for both growth rate and yield was calculated to be ≥8.41mg 1-Oxa-4,5-dithiepane item/L.

6 References

[1] Gesetz zum Schutz vor gefährlichen Stoffen (Chemikaliengesetz - ChemG) in der Fassung der Bekanntmachung vom 28. August 2013 (BGBl. I S. 3498, 3991), das zuletzt durch Artikel 7 des Gesetzes vom 4. April 2016 (BGBl. I S. 569) geändert worden ist (Anhang 1 zu § 19a Abs. 1 Grundsätze der Guten Laborpraxis (GLP)).

[2] OECD Principles of Good Laboratory Practice (as revised in 1997), Paris, 1998.

[3] EEC (2004). Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

[4] OECD Guideline for Testing of Chemicals 201, Sect. 2: Effects on Biotic Systems, Freshwater Alga and Cyanobacteria, Growth Inhibition Test. 23 March 2006. Annex 5, corrected 28 July, 2011.

[5] Bringmann, G. and Kühn, R. (1980). Comparison of the toxicity thresholds of water pollutants to bacteria, algae, and protozoa in the cell multiplication inhibition test. Water Research 14(3), 231-241.

[6] International Organisation for Standardisation (2010). ISO 8692 Water quality - Fresh-water algal growth inhibition test with unicellular green algae. Draft International Standard ISO/DIS 8692.

[7] Williams, D.A. (1971): A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics 27, 103-117.

[8] Williams, D.A. (1972): The comparison of several dose levels with a zero dose control. Biometrics 28, 519-531.

[9] ToxRat® Professional 3.2.1. ToxRat® Solutions GmbH, Naheweg 15, 52477 Alsdorf, Germany.

[10] SANCO/3029/99 rev.4 (11/07/2000): European Commission. 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. Working document.

ANNEXES

A.1 Annex 1: Test conditions

Table 8: Light intensities and temperature during the growth inhibition test

Date	Test duration	Light [µE $^{1}m^{-2}$ s^{-}]	Temperature [°C]
May 09, 2016	Test start	101.62	22.0
May 10, 2016	Day 1	103.39	22.0
May 11, 2016	Day 2	103.66	22.0
May 12, 2016	Day 3	103.59	22.0

Table 9: The pH at start and end of the growth inhibition test

	Concentrations nominal [mg test item/L]					
	Control	3,33	4,62	5,66	6,80	8,41
Test start	7.81	7.81	7.70	7.68	7.66	7.72
Test end	8.02	8.07	8.04	8.14	8.26	8.42
	8.08	8.02	8.01	8.11	8.20	8.30
	8.10	8.16	8.12	8.07	8.29	8.43
	8.11	8.08	8.12	8.16	8.38	8.38
	8.17					
	8.16					
	8.10					
	8.04					
Mean value	8.09	8.08	8.07	8.12	8.28	8.38
Minimum	8.02	8.02	8.01	8.07	8.2	8.30
Maximum	8.17	8.16	8.12	8.16	8.38	8.43

A.2 Annex 2: Cell number

Table 10: Cell number (x 10^4) per mL dependent on test item and time

Treatment mean measured [mg test item/L]	Control	3.33	4.62	5.66	6.80	8.41
0 h	1	1	1	1	1	1
24 h	4.12	4.43	5.12	4.42	4.46	4.11
	4.17	4.63	5.99	6.24	5.37	4.38
	6.93	4.54	5.92	5.83	5.78	4.31
	4.70	4.32	4.88	6.74	4.63	4.41
	5.54					
	6.59					
	4.20					
	3.84					
Replicates	8	4	4	4	4	4
Mean:	5.01	4.48	5.48	5.81	5.06	4.30
Std.Dev.:	1.20	0.14	0.56	1.00	0.62	0.13
CV:	23.9	3.09	10.2	17.2	12.3	3.10
48 h	23.9	23.7	24.7	20.7	23.3	27.8
	20.5	27.3	27.4	25.6	26.3	28.0
	27.3	24.0	25.5	28.2	28.1	22.7
	27.2	23.4	22.1	30.0	26.6	21.0
	30.4					
	36.7					
	23.6					
	18.2					
Replicates	8	4	4	4	4	4
Mean:	26.0	24.6	25.0	26.1	26.1	24.9
Std.Dev.:	5.85	1.83	2.19	4.02	1.98	3.54
CV:	22.5	7.43	8.77	15.4	7.60	14.2
72 h	126.6	115.8	123.2	114.9	135.5	160.0
	123.8	130.9	142.8	144.2	161.6	191.9
	152.5	124.1	135.3	163.1	164.9	161.1
	155.6	127.0	113.1	181.6	152.3	160.4
	139.7					
	166.3					
	120.5					
	101.7					
Replicates	8	4	4	4	4	4
Mean:	135.8	124.5	128.6	151.0	153.6	168.4
Std.Dev.:	21.5	6.40	13.11	28.47	13.18	15.71
CV:	15.8	5.15	10.2	18.9	8.58	9.33

Mean: arithmetic mean; Std. Dev.: standard deviation; n: number of replicates; CV: coefficient of variation

A.3 Annex 3: Validity criterion: Section by section growth rate

Table 11: Mean coefficient of variation for section-by-section growth rates.

Sectional growth [1/d] rates of controls			
Time:	**0 - 24 h**	**24 - 48 h**	**48 - 72 h**
Replicate 1	1.416	1.756	1.668
Mean Replicate1	1.61		
CV Replicate1	10.94		
Replicate 2	1.428	1.591	1.799
Mean Replicate2	1.61		
CV Replicate2	11.57		
Replicate 3	1.936	1.369	1.722
Mean Replicate3	1.68		
CV Replicate3	17.09		
Replicate 4	1.548	1.754	1.746
Mean Replicate4	1.68		
CV Replicate4	6.92		
Replicate 5	1.711	1.705	1.524
Mean Replicate5	1.65		
CV Replicate5	6.46		
Replicate 6	1.885	1.718	1.511
Mean Replicate6	1.71		
CV Replicate6	11.00		
Replicate 7	1.436	1.723	1.633
Mean Replicate7	1.60		
CV Replicate7	9.19		
Replicate 8	1.346	1.555	1.722
Mean Replicate8	1.54		
CV Replicate8	12.23		

	Required	**Achieved**
Mean Growth rate of mean replicate	**0.92/d**	**1.633 /d**
Mean CV Replicate	**35**	**10.7**

Mean Repl.: mean replicate sectional GR over time; CV Repl.: coefficient of variation of replicate over time;
Mean of mean Repl.: mean of 'mean replicate sectional GR over time;
Mean CV Repl.: mean of the coefficients of variation of replicate over time.

A.4 Annex 4: Test data and statistical evaluation

A.4.1 Growth rate data

Table 12: Growth rates (1/d) dependent on the test item and time

Treatment, measured initial [mg/L]	Control	3.33	4.62	5.66	6.80	8.41
24 h	1.416	1.488	1.633	1.486	1.495	1.414
	1.428	1.533	1.790	1.831	1.682	1.476
	1.936	1.514	1.777	1.762	1.754	1.460
	1.548	1.462	1.585	1.907	1.533	1.484
	1.711	-	-	-	-	-
	1.885	-	-	-	-	-
	1.436	-	-	-	-	-
	1.346	-	-	-	-	-
Mean:	1.588	1.499	1.696	1.747	1.616	1.459
Std.Dev.:	0.2277	0.0310	0.1028	0.1835	0.1220	0.0314
n:	8	4	4	4	4	4
CV:	8	4	4	4	4	4
	14.34	2.07	6.06	10.50	7.55	2.15
48 h	1.586	1.583	1.604	1.516	1.575	1.663
	1.510	1.653	1.656	1.621	1.634	1.665
	1.653	1.592	1.620	1.669	1.667	1.561
	1.651	1.576	1.549	1.700	1.640	1.523
	1.708	-	-	-	-	-
	1.801	-	-	-	-	-
	1.580	-	-	-	-	-
	1.450	-	-	-	-	-
Mean:	1.617	1.601	1.607	1.626	1.629	1.603
Std.Dev.:	0.1111	0.0357	0.0445	0.0807	0.0389	0.0721
n:	8	4	4	4	4	4
CV:	6.87	2.23	2.77	4.96	2.39	4.50
72 h	1.614	1.584	1.605	1.581	1.636	1.692
	1.606	1.625	1.654	1.657	1.695	1.752
	1.676	1.607	1.636	1.698	1.702	1.694
	1.682	1.615	1.576	1.734	1.675	1.693
	1.646	-	-	-	-	-
	1.705	-	-	-	-	-
	1.597	-	-	-	-	-
	1.541	-	-	-	-	-
Mean:	1.633	1.608	1.618	1.668	1.677	1.708
Std.Dev.:	0.0541	0.0174	0.0343	0.0655	0.0294	0.0298
n:	8	4	4	4	4	4
CV:	3.31	1.08	2.12	3.93	1.75	1.74

Mean: arithmetic mean; Std. Dev.: standard deviation; n: number of replicates; CV: coefficient of variation

A.4.1.1 Inhibition of growth rate

Table 13: Growth rate (G) and its inhibition relative to control (% I) as computed from the raw data for growth test intervals selected

Treatment, measured initial [mg/L]	0-24 h		0-48 h		0-72 h	
	G	% I	G	% I	G	% I
Control	1.588	0.0	1.617	0.0	1.633	0.0
3.33	1.499	5.6	1.600	1.1	1.608	1.6
4.62	1.696	-6.8	1.607	0.6	1.618	1.0
5.66	1.747	-10.0	1.626	-0.6	1.668	-2.1
6.80	1.616	-1.7	1.629	-0.7	1.677	-2.7
8.41	1.459	8.2	1.603	0.9	1.708	-4.5

- negative inhibition indicates increase in the observed parameter

A.4.1.2 Effective concentrations for growth rate (E_rC_x), 72 h

Since the test results of the growth inhibition test (exposure period) did not show any inhibitions compared to controls, they were not statistically analysed to determine EC-values.

Table 14: Effective levels and their 95%-confidence limits (growth rate after 72 h)

Toxicity metric	E_rC_{10}	E_rC_{20}	E_rC_{50}
Value measured initial [mg test item/L]	>8.41	>8.41	>8.41
lower and upper 95% - confidence limit	-	-	-

A.4.1.3 NOEC determination for growth rate, 72 h

Normal distribution (Shapiro-Wilk´s Test) and variance homogeneity (Levene's test) requirements were fulfilled. The parametric Williams t-test was advisable. The analysis of contrasts revealed a linear trend, thus the selected Williams test was performed. The NOEC was calculated to be ≥8.41mg 1-Oxa-4,5-dithiepane item/L.

Table 15: Williams Multiple Sequential t-test Procedure, growth rate 72 h

Treatment, measured initial [mg/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
Control	1.633	0.04424						
3,33	1.608	0.04424	22	1.656	-2.85	0.82	-1.72	-
4,62	1.618	0.04424	22	1.656	-2.96	0.82	-1.78	-
5,66	1.668	0.04424	22	1.656	-2.99	0.82	-1.81	-
6,80	1.677	0.04424	22	1.656	-3.01	0.82	-1.81	-
8,41	1.708	0.04424	22	1.656	-3.02	0.82	-1.82	-

+: significant; -: non-significant

A.4.2 Yield data

Table 16: Yield (cell number increase) dependent on the test item and time

Treatment, measured initial [mg/L]	Control	3.33	4.62	5.66	6.80	8.41
24 h	3.12	3.43	4.12	3.42	3.46	3.11
	3.17	3.63	4.99	5.24	4.37	3.38
	5.93	3.54	4.91	4.82	4.78	3.31
	3.70	3.32	3.88	5.74	3.63	3.41
	4.53	-	-	-	-	-
	5.59	-	-	-	-	-
	3.20	-	-	-	-	-
	2.84	-	-	-	-	-
Mean:	4.01	3.48	4.48	4.81	4.06	3.30
Std.Dev.:	1.199	0.138	0.559	0.996	0.620	0.133
n:	8	4	4	4	4	4
CV:	29.88	3.98	12.49	20.72	15.26	4.04
48 h	22.87	22.69	23.74	19.72	22.33	26.82
	19.48	26.30	26.42	24.56	25.25	26.95
	26.25	23.13	24.51	27.16	27.06	21.69
	26.16	22.36	21.14	28.96	25.60	20.04
	29.44	-	-	-	-	-
	35.70	-	-	-	-	-
	22.55	-	-	-	-	-
	17.18					
Mean:	24.95	23.62	23.95	25.10	25.06	23.88
Std.Dev.:	5.846	1.814	2.187	4.016	1.982	3.541
n:	8	4	4	4	4	4
CV:	23.43	7.68	9.13	16.00	7.91	14.83
72 h	125.60	114.80	122.20	113.90	134.50	159.00
	122.80	129.90	141.80	143.20	160.60	190.90
	151.50	123.10	134.30	162.10	163.90	160.10
	154.60	126.00	112.10	180.60	151.30	159.40
	138.70	-	-	-	-	-
	165.30	-	-	-	-	-
	119.50	-	-	-	-	-
	100.70	-	-	-	-	-
Mean:	134.84	123.45	127.60	149.95	152.58	167.35
Std.Dev.:	21.520	6.404	13.114	28.473	13.178	15.707
n:		8	4	4	4	4
CV:	15.96	5.19	10.28	18.99	8.64	9.39

Mean: arithmetic mean; Std. Dev.: standard deviation; n: number of replicates; CV: coefficient of variation

A.4.2.1 Inhibition of yield

Table 17: Yield (Y) and its inhibition relative to control (% I) as computed from the raw data for growth test intervals selected

Treatment, measured initial [mg/L]	0-24 h		0-48 h		0-72 h	
	Y	% I	Y	% I	Y	% I
Control	4.01	0.00	24.95	0.00	134.84	0.00
3.33	3.48	13.24	23.62	5.34	123.45	8.45
4.62	4.48	-11.57	23.95	4.01	127.60	5.37
5.66	4.81	-19.79	25.10	-0.59	149.95	-11.21
6.80	4.06	-1.23	25.06	-0.43	152.58	-13.15
8.41	3.30	17.72	23.88	4.32	167.35	-24.11

- negative values indicate increase in the observed parameter.

A.4.2.2 Effective concentrations for yield (E_yC_x), 72 h

Since the test results of the growth inhibition test (exposure period) did not show any inhibitions compared to controls, they were not statistically analysed to determine EC-values.

Table 18: Effective levels and their 95%-confidence limits (yield after 72 h)

Toxicity metric	E_yC_{10}	E_yC_{20}	E_yC_{50}
Value measured initial [mg test item/L]	>8.41	>8.41	>8.41
upper and lower 95% - confidence limit	-	-	-

A.4.2.3 NOEC determination for yield, 72 h

Normal distribution (Shapiro-Wilk´s Test) and variance homogeneity (Levene's test) requirements were fulfilled. The parametric Williams t-test was advisable. The analysis of contrasts revealed a linear trend, thus the selected Williams test was performed. The NOEC was calculated to be ≥8.41mg 1-Oxa-4,5-dithie¬pane item/L.

Table 19: Williams Multiple Sequential t-test Procedure, yield 72 h

Treatment, measured initial [mg/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
Control	134.84	18.5548						
3.33	123.45	18.5548	22	144.19	-14.47	0.82	-1.72	-
4.62	127.60	18.5548	22	144.19	-15.02	0.82	-1.78	-
5.66	149.95	18.5548	22	144.19	-15.21	0.82	-1.81	-
6.80	152.58	18.5548	22	144.19	-15.28	0.82	-1.81	-
8.41	167.35	18.5548	22	144.19	-15.35	0.82	-1.82	-

+: significant; -: non-significant

A.5 Annex 5: Analytical report - Details of method and results

A.5.1 Preface and scope

The purpose of the analytical part of the study was to develop quantitative residue analytical methods for the determination of the test item 1-Oxa-4,5-dithiepane in water.
The analytical methods for the determination of 1-Oxa-4,5-dithiepane in water using GC-MS were developed in experiments described here.
The LOQ was set at 0.1 mg/L. The quantitative measurements were carried out by gas chromatography (GC) coupled to a triple quadrupole mass spectrometer (MS) using electrospray ionization positive (ESI); the mass spectrometer (MS) was operated in the single ion monitoring mode (SIM).1,4-Oxathian was used as internal standard.

A.5.2 Chemicals: Reagents and analytical equipment

- Analytical standard (= test item), 1-Oxa-4,5-dithiepane. Purity > 99.8 %, Batch/Lot-No EN1502b (see CoA of the test item)
- Internal standard, 1,4-Oxathian Purity 98% (Sigma Aldrich)
- Cyclohexane (JT. Baker)
- Acetone, min. 99.95% (ChemSolute)
- Algal growth medium (OECD 201 [4])
- GC-MS Agilent inert 5793
- Balance Mettler AT 201
- Balance Mettler PM 2000
- Pipet Microman 0-25 µL
- Pipet Microman 0-50 µL
- Pipet Microman 0-250 µL
- Pipet Gilson 0-1000 µL
- Pipet Gilson 0-5000 µL
- Freezer Liebherr Comfort

A.5.3 GC-MS measurement (water analysis)

Details of instrumental analysis

GC-MS-System:	GC 6890N with MSD 5973 inert (Agilent)
Auto sampler:	MPS 2 with 10µL-liquid injection unit (Gerstel)

Column:	Rtx-1701; 30 m, 0.25 mm ID, 0.5 µm film (Agilent)
Oven:	1.0 min 50°C, then 15°C/min -> 140°C, then 25°C/min 230°C hold for 2 min
Carrier gas:	Helium, constant flow, 1.0 mL/min
Inlet:	Splitless Inlet -> 250°C
Acquisition mode:	SIM-Mode
SIM masses:	1-Oxathian
	(internal Standard; Target ion m/z 46.0)
	1-Oxa-4,5-dithiepane
	(analyte; Target ion m/z 136.0)
MS source:	250°C
MS Quadrupole:	150°C
Solvent delay:	4 min
Run time:	12.6 min

A.5.4 Sample preparation

A.5.4.1 Water samples

Samples with nominal concentrations of 4.11 mg/L, 5.13 mg/L, 6.42 mg/L, 8.02 mg/L and 10.02 mg/L were analysed for their actual concentrations. For each sampling the sample was diluted with algal growth medium. The amount of sample and of growth medium used can be seen in Table 20. 20 µL of the internal standard solution were added to the samples before analysis via GC-MS.

Table 20: Nominal sample composition

Sample	Nominal concentration [mg/L]	Volume of sample [mL]	Volume of growth medium [mL]
1	0	4	0.0
2	4.11	0.5	3.5
3	5.13	0.5	3.5
4	6.42	0.5	3. 5
5	8.02	0.25	3.75
6	10.02	0.25	3.75

A.5.5 Calibration, quantification and calculation of the analytical results

A.5.5.1 Preparation of stock solutions of the analyte and internal standard

The stock solution of the analytical standard was prepared by weighing 19.85 mg of the test item (purity = 99.8%) directly into a 10 mL volumetric flask and by subsequently filling it up to the ring mark with acetone. Therefore the analyte concentration of the stock solution was 1.981 g/L. The stock solution of the internal standard was prepared by weighing 17.7 mg of the internal standard (purity= 98%) directly into a 10 mL volumetric flask and subsequently filling it up to the ring mark with cyclohexane. Therefore the internal standard concentration of the stock solution was 1.735 mg/L. The prepared stock solutions were stored in a refrigerator at 4 °C.

A.5.5.2 Calibration of the GC-MS system

A.5.5.2.1 Preparation of the calibration solutions (water analysis)

Seven ‘calibration standards', including a zero value with just the internal standard, were produced in the concentration range from 0.05 to 1.24 mg/L by diluting the stock solutions with Acetone in volumetric flasks (Microman pipettes were used for this dilution step).
For preparation of the ‘calibration samples' 4 mL of algal growth medium were pipetted into a 8 mL vial and 2 mL of cyclohexane added. This solution was spiked with 20 µL of the respective spike solution. The prepared solution was shook vigorously for 2 min (Vortex). 1 mL of the organic phase was transferred to a GC micro vial and measured via GC-MS.

A.5.5.2.2 Creating the calibration functions

The GC-MS system was calibrated by measuring the prepared matrix calibration samples. The calibration function was created afterwards by processing (integration) the chromatographic raw data and by plotting the response of the detector against the injected analyte concentrations. With the received data quadratic regression calculations were performed.

A.5.5.3 Quantification and calculation of the analytical results

The quantification data was generated by processing the chromatographic raw data of the measured samples and by subsequent calculation of the quantification results using the respective basic calibration function.

A.5.5.4 Preparation of the LOQ and 10xLOQ samples

4 mL of the growth medium were spiked with 20 µL of the respective spike solution (LOQ: 0.1 mg/L, 10 x LOQ: 1.0 mg/L). 2 mL of cyclohexane were added and the solution shaken vigorously (Vortex) for 2min. 1 mL of the organic phase was transferred to a GC vial and measured via GC-MS.

A.5.5.5 Quality control standards

Two quality control standards were prepared and analysed additionally to the calibration standards to insure the validity of the measurements.

A.5.6 Analytical results

A.5.6.1 Water analysis

A.5.6.1.1 Calibration

The respective calibration function for the analyte 1-Oxa-4,5-dithiepane was determined by processing (integration) the chromatographic raw data and by plotting the response of the detector against the analyte concentration. With the received data quadratic regression calculations were performed. The function was calculated with the Mass Hunter quantification software using quadratic regression model.

A typical calibration function: $y = 0.079038x^2 + 1.263340x - 0.015735$

All derived coefficients of determination are $r^2 > 0.999$

Linearity

Using the quadratic regression model the coefficient of determination for 1-Oxa-4,5-dithiepane was calculated to be greater than 0.999. As the calculated r^2-value was close to 1, the 2nd order fit of the calibration function ($0.079038x^2 + 1.263340x - 0.015735$) was accepted.

Validation

For the validation of the method according to SANCO 3029/99 [10] two stock solutions with concentrations of 0.200 mg/L and 0.798 mg/L were prepared. Five samples with the concentration of the LOQ and five samples with a concentration of ten times the LOQ (0.10 and 1.00 mg/L) were prepared by spiking 4 mL of the growth medium with 20 µL of the standards in cyclohexane, adding 2 mL of cyclohexane, shaking vigorously (Vortex), pipetting 1 mL of the organic phase into GC micro vial and measuring via GC-MS.

A.5.6.1.2 Results of the analysed water samples

Five nominal concentrations of 4.11 mg/L, 5.13 mg/L, 6.42 mg/L, 8.02 mg/L and 10.0 mg/L were applied and one additional test system served as an untreated control. The results of the analysed samples for 1-Oxa-4,5-dithiepane are listed in Table 21 and Table 22.

Table 21: Measured concentration and recovery of 1-Oxa-4,5-dithiepane at test start

Sample	Nominal conc. 1-Oxa-4,5-dithiepane (mg/L water)	Measured conc. 1-Oxa-4,5-dithiepane (mg/L water)	Recovery (%)
Control (Test start)	0	< LOQ	-
Conc.1 (Test start)	4.11	3.33	81.09
Conc.2 (Test start)	5.13	4.62	90.00
Conc.3 (Test start)	6.42	5.66	88.19
Conc.4 (Test start)	8.02	6.80	84.85
Conc.5 (Test start)	10.0	8.41	83.94

Table 22: Measured concentration and recovery of 1-Oxa-4,5-dithiepane at the end of the test

Sample	Nominal conc. 1-Oxa-4,5-dithiepane (mg/L water)	Measured conc. 1-Oxa-4,5-dithiepane (mg/L water)	Recovery (%)
Control (Test end)	0	< LOQ	-
Conc.1 (Test end)	4.11	3.15	76.59
Conc.2 (Test end)	5.13	4.12	80.37
Conc.3 (Test end)	6.42	5.22	81.31
Conc.4 (Test end)	8.02	6.37	79.46
Conc.5 (Test end)	10.0	7.60	75.90

A.5.7 Typical GC-MS chromatograms

Typical chromatograms of calibration samples, LOQ and 10x LOQ samples, a blank, samples taken at the test start and on day 3 (treatments and controls) are shown in Figure 4 to Figure 11. The retention times (t_R) for 1-Oxa-4,5-dithiepane was 5.99 min and 9.28 min for the internal standard.

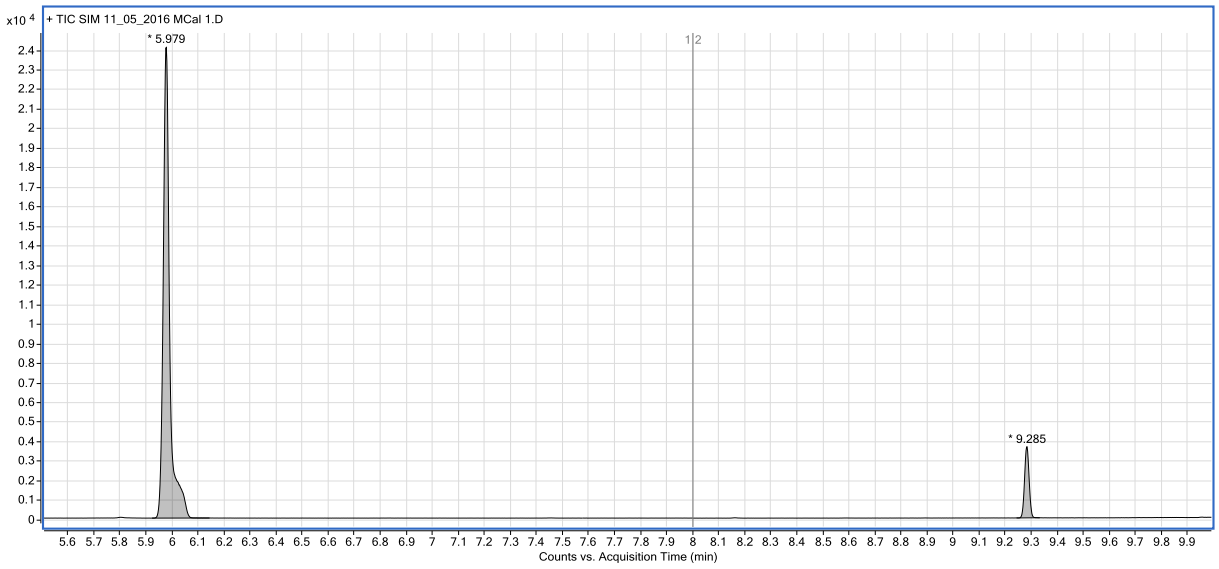


Figure 4: Calibration standard 1 (0.050 mg/L) measured May 15, 2016

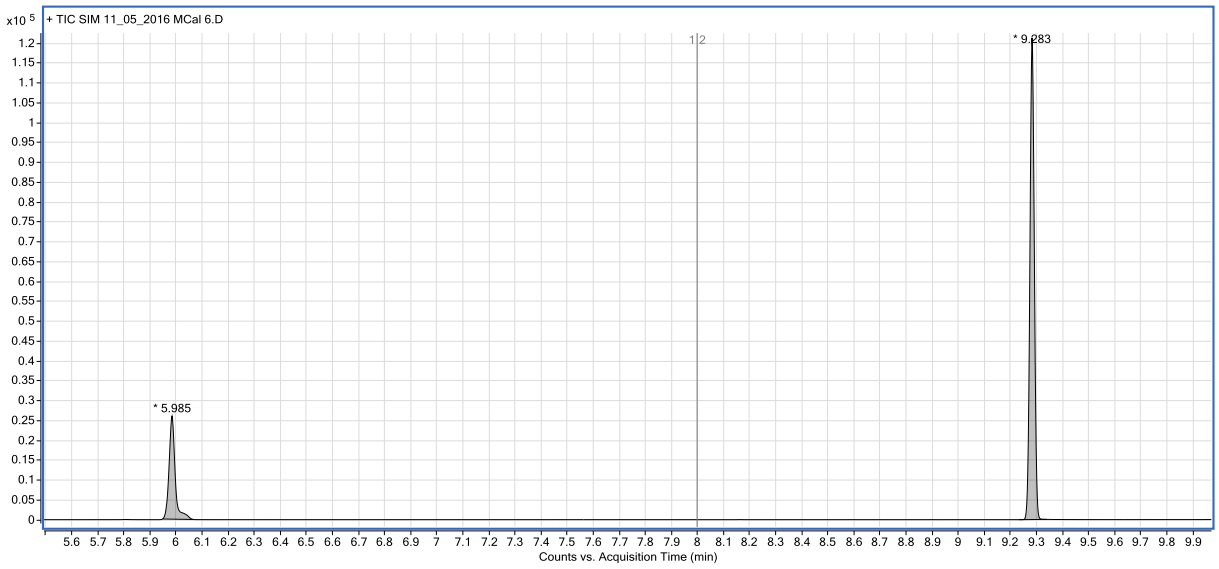


Figure 5: Calibration standard 6 (1.238 mg/L) measured May 15, 2016

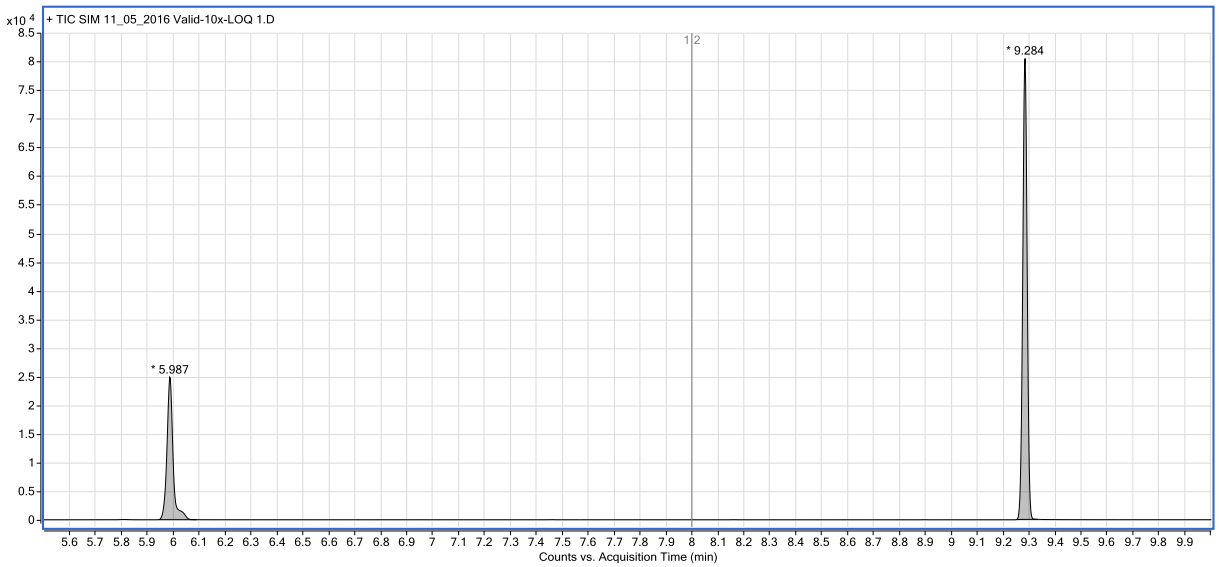


Figure 6: 10x LOQ validation sample, measured May 15, 2016

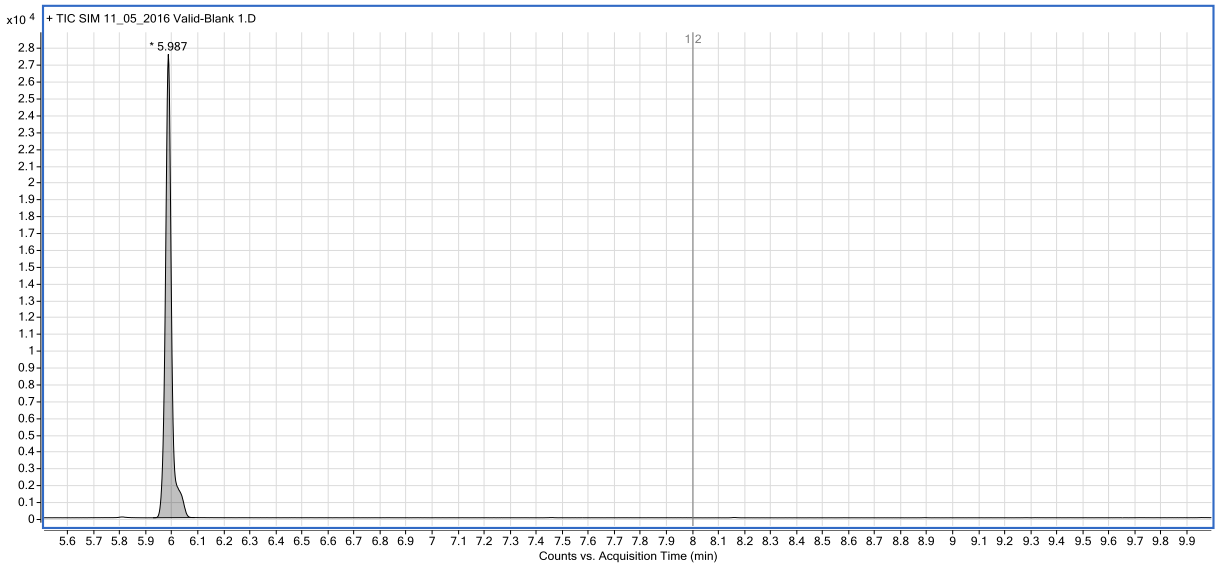


Figure 7: Validation blank sample, measured May 15, 2016

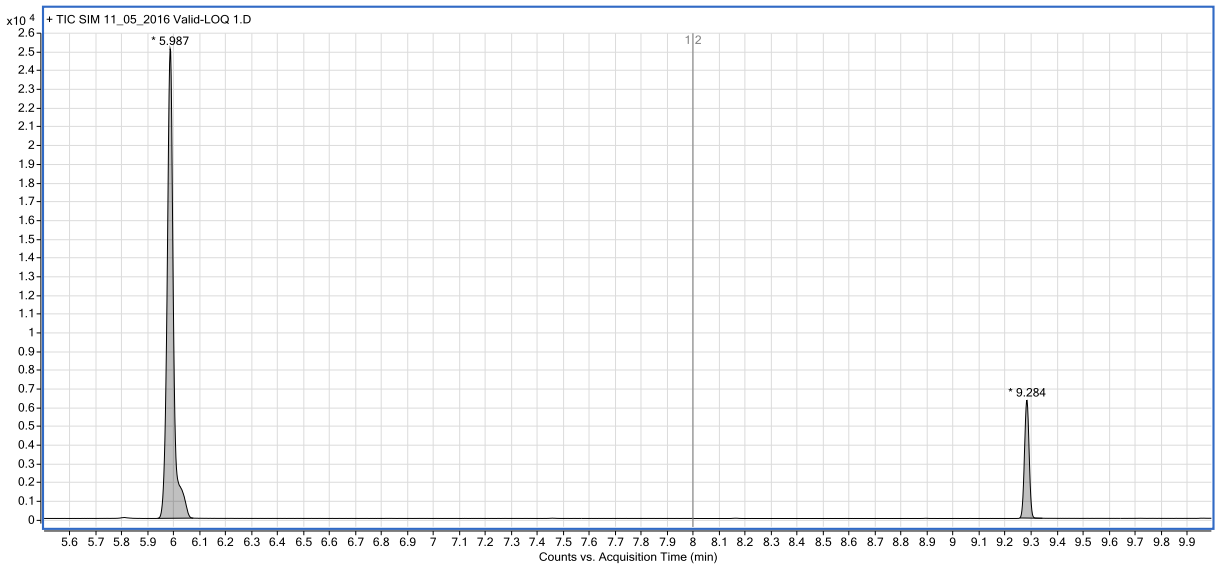


Figure 8: LOQ sample measured May 15, 2016

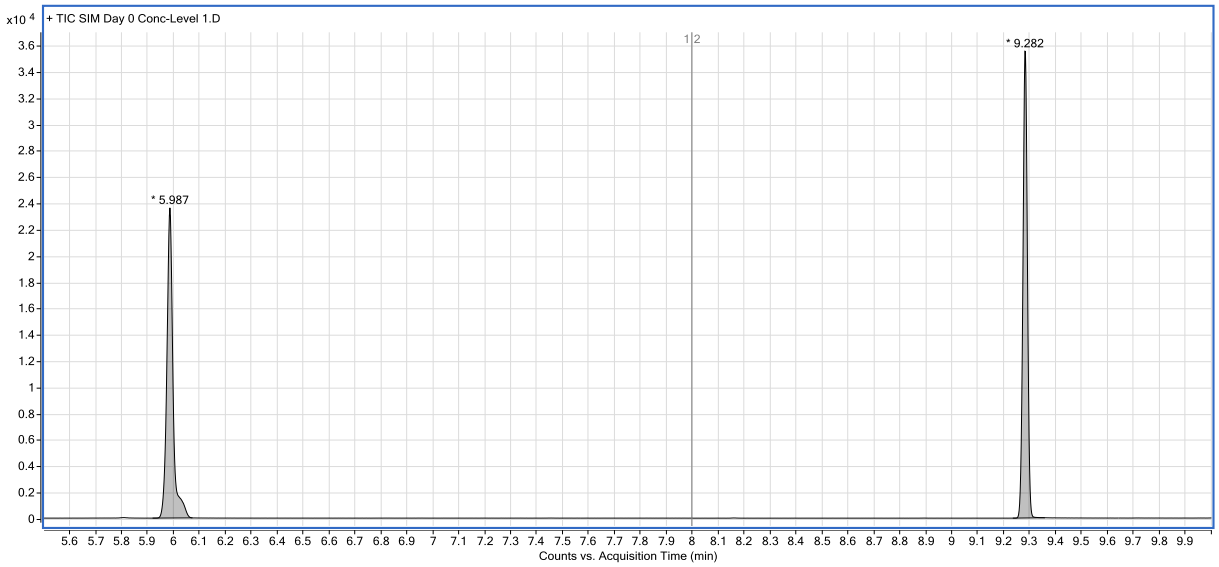


Figure 9: Concentration level 1 (4.11 mg/L) at test start, measured May 15, 2016

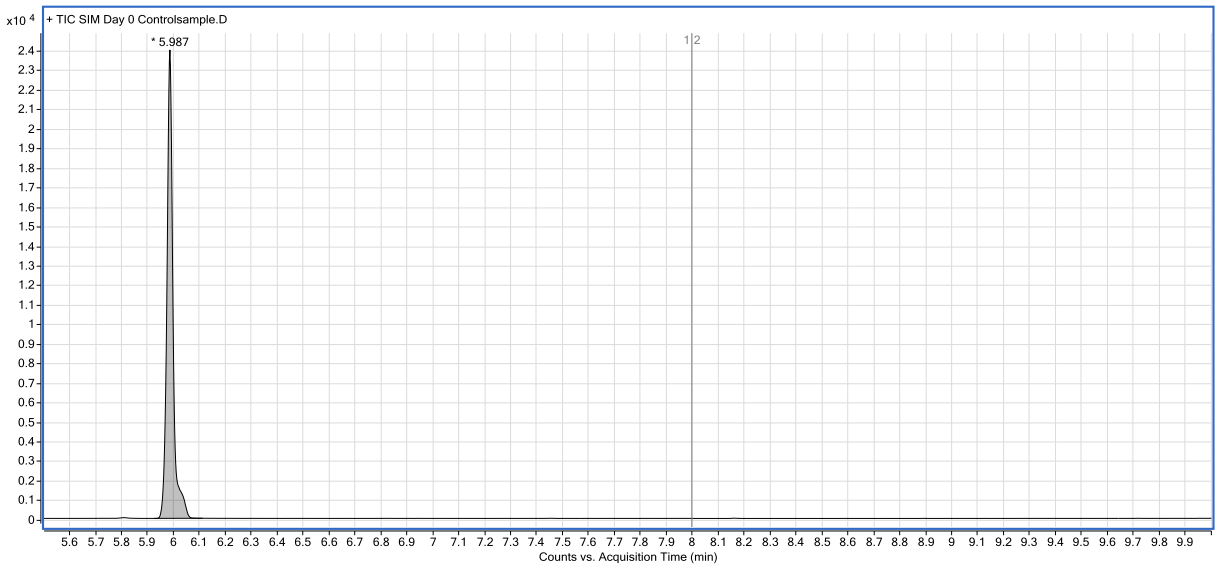


Figure 10: Control sample at test start May 15, 2016

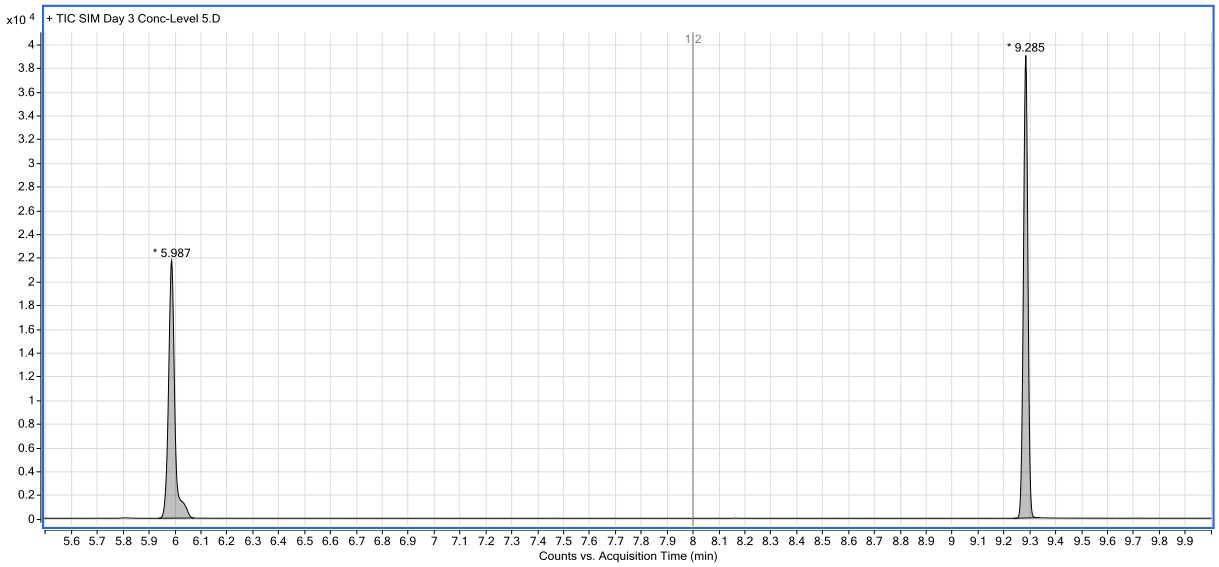


Figure 11: Concentration level 5 (10.02 mg/L) at the end of the test May 15, 2016

A.6 Annex 6: Certificate of Analysis of the test item

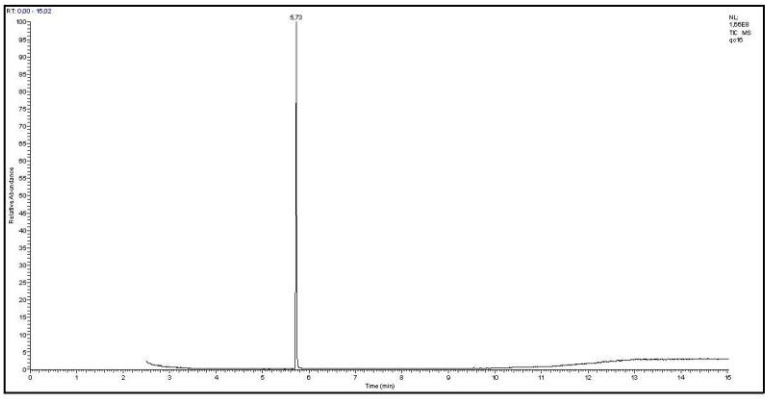
Envilytix GmbH

Certificate of analysis

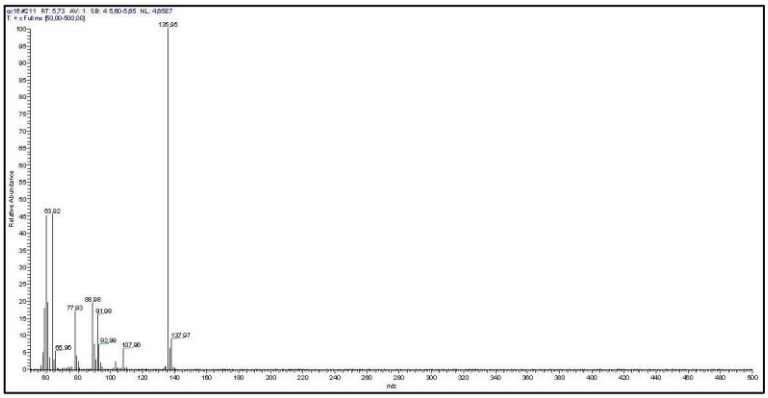
Envilytix GmbH – Rheingaustrasse 190-196 – D-65174 Wiesbaden

Product:	**1-Oxa-4,5-dithiepane**
CAS-No.:	3886-40-6
Empirical formula:	$C_4H_8OS_2$
Molecular weight:	136,24 g/Mol
Lot-No.:	EN1502b
Storage:	Store at -20°C
Appearance:	Very faint yellow oil
Purity (EI-GC/MS):	99,8%
Identity:	EI-MS conforms to structure

Total ion current chromatogram



Mass spectrum



Wiesbaden, 09.01.2015

1 of 1

Fraunhofer
IME

Study Report

Daphnia magna, Reproduction test (OECD 211) Semi-static exposure

Effect of 1-Oxa-4,5-dithiepane on the reproduction of *Daphnia magna*

GLP-Code of Test Facility: AAR – 001 / 4 – 21 / G

Sponsor
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Study Monitor
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Test Facility
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Auf dem Aberg 1
57392 Schmallenberg, Germany

Test Facility Management
Prof. Dr. Christoph Schäfers

Study Director
Dr. Markus Simon

1 of 60 pages

July 06th, 2016

This page was intentionally left blank for statements of the sponsor or submitter.

GLP certificate (2 pages)

Ministerium für Arbeit, Integration und Soziales
des Landes Nordrhein-Westfalen

Fürstenwall 25, 40219 Düsseldorf — Aktenzeichen III 5 – 8673.79

Gute Laborpraxis/Good Laboratory Practice
GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

Fraunhofer Institut für
Molekularbiologie und Angewandte Oekologie IME
Auf dem Aberg 1
57392 Schmallenberg

Prüfungen nach Kategorien (gemäß ChemVwV-GLP Nr. 5.3/OECD guidance)	**Areas of Expertise** (according ChemVwV GLP Nr. 5.3/OECD guidance)
Kategorie 1 Prüfungen zur Bestimmung der physikalisch-chemischen Eigenschaften und Gehaltsbestimmungen	category 1 physical-chemical testing
Kategorie 4 Ökotoxikologische Prüfungen zur Bestimmung der Auswirkungen auf aquatische und terrestrische Organismen	category 4 environmental toxicity studies on aquatic and terrestrial organisms
Kategorie 5 Prüfungen zum Verhalten im Boden, im Wasser und in der Luft; Prüfungen zur Bioakkumulation und zur Metabolisierung	category 5 studies on behaviour in water, soil and air; bioaccumulation

GLP certificate (continued)

Kategorie 6 Prüfungen zur Bestimmung von Rückständen	category 6 residue studies
Kategorie 7 Prüfungen zur Bestimmung der Auswirkungen auf Mesokosmen und natürliche Ökosysteme	category 7 studies on effects on mesocosms and natural ecosystems

Datum der Inspektion

16. Dezember 2015

Die/Der genannte Prüfeinrichtung/Prüfstandort befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Düsseldorf, 31. Mai 2016
Im Auftrag

(Steffen Röddecke)

Date of Inspection

16th December 2015

The above mentioned test facility/ test site is included in the national GLP Compliance Programme and is inspected on a regular basis.

Based on the inspection report it can be confirmed, that this test facility/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Land Nordrhein-Westfalen · Ministerium für Arbeit, Integration und Soziales

Dienstsiegel/official-seal

2/2

http://www.bfr.bund.de/cm/343/verzeichnis-der-pruefeinrichtungen-im-ueberwachungsprogramm.pdf

Statement of GLP-compliance

Title of the study:	*Daphnia magna*, Reproduction test (OECD 211); Semi-static exposure - Effect of 1-Oxa-4,5-dithiepane on the reproduction of *Daphnia magna*
Test item:	1-Oxa-4,5-dithiepane
GLP-Code:	AAR – 001 / 4 – 21 / G

This study was conducted in compliance with Good Laboratory Practice Regulations (GLP). The German requirements [5] are based on the OECD Principles of Good Laboratory Practice [3], which are accepted by regulatory authorities throughout the European Community [4], the United States of America (FDA and EPA) and Japan (MHLW, MAFF and METI) on the base of intergovernmental agreements.

We hereby attest to the authenticity of the study and guarantee that the data are correct, and that the study was performed by the procedures described. This report accurately reflects the raw data. There were no known circumstances which may have affected the integrity of the study.

Schmallenberg, July 06, 2016 Study director: _____
(Dr. Markus Simon)

Schmallenberg, July 06, 2016 Chemical investigator: _____
for (Dr. Matthias Kotthoff)

Schmallenberg, July 06, 2016 Test facility management: _____
(Prof. Dr. Christoph Schäfers)

Statement of the Quality assurance unit

Title of the study:	*Daphnia magna*, Reproduction test (OECD 211); Semi-static exposure - Effect of 1-Oxa-4,5-dithiepane on the reproduction of *Daphnia magna*
Test item:	1-Oxa-4,5-dithiepane
GLP-Code:	AAR – 001 / 4 – 21 / G

Date of study plan revision:	May 12, 2016	
Audit of the test:	June 03, 2016	Transfer of test specimens
Audit report to study director:	June 03, 2016	
Audit report to management:	June 03, 2016	
Audit of the final report:	July 05, 2016	
Intervals of inspections:	Laboratories: Three months	

The Quality Assurance Unit of the test facility inspected the study, audited the final report, and reported possible findings to the Study Director and to the management.
The Quality Assurance Unit found no discrepancies between final report and raw data and confirms that the methods, procedures, and observations are accurately and completely described, and that the reported results accurately and completely reflect the raw data of the study.

Schmallenberg, July 06, 2016

Quality Assurance unit: Lars Wied-Kr

(Lars Wiedemann-Krantz)

Study Plan Amendments / GLP Deviations

No study plan amendments occurred.
No GLP deviations occurred.

Distribution list

Sponsor:	1 original (1st of two), Electronic (PDF) file of original
GLP-archive:	1 original (2nd of two)
Study director:	Electronic (PDF) file of original
Chemical investigator:	Electronic (PDF) file of original

Archiving

An aliquot of the test item, the test protocols, all raw data and all records necessary to reconstruct the study were archived in the GLP archive of the Fraunhofer Institute for Molecular Biology and Applied Ecology, 57392 Schmallenberg, Germany, to be kept for 15 years following internal SOPs, according to the Principles of Good Laboratory Practice [3]. The sponsor is obliged to notify the GLP archive of the Fraunhofer IME of any change of address to ensure that test item and records can be returned after the end of the archiving period, if desired. After the end of the archiving period Fraunhofer IME will send a letter to the indicated address to clarify the further use of the test item and records. These will be discarded after 16 years if Fraunhofer IME has not received a written notice giving instruction to either return test item and records or to further archive them in the GLP archive of the Fraunhofer IME.

List of archived records:

- Data specifying the test item
- Data concerning the test species (origin, species)
- Correspondence between study director and monitor
- Original raw data of test (test conditions, i.e. pH-values, temperature, records of chemical analysis)
- Storage conditions of test item
- Records of statistical evaluation
- Original study plan
- Original final report

Summary

At the Fraunhofer Institute for Molecular Biology and Applied Ecology the influence of 1-Oxa-4,5-dithiepane on the reproduction of aquatic invertebrates, represented by *Daphnia magna*, was investigated. A 21 day semi-static exposure to 1-Oxa-4,5-dithiepane at different concentrations with renewal of the test solutions three times a week was conducted according to the OECD guideline 211. Untreated control replicates were run in parallel. Each treatment group consisted of 10 replicates with one daphnid each (individual exposure). Effects on growth (adult length at test termination) and reproductive performance were investigated. Test item concentrations were measured at representative fresh and aged test solutions.

The mean measured test item concentrations of the freshly prepared test solution of the active ingredient (initial concentrations) were between 96 % and 107 % of nominal concentrations. During the time interval until renewal of the test solution, active ingredient concentrations decreased considerably to 79 – 92 % of nominal. The time weighted average (TWA) of mean measured initial and mean measured aged concentration at test solution renewal were 0.26, 0.47, 0.83, 1.64, and 2.98 mg/L, corresponding with 91, 93, 89, 98, and 99 % of the nominal concentrations.

TWA test concentrations > 0.83 mg test item per liter (NOEC) did affect survival (viability) of adults. A concentration depending effect occurred. An $EC_{10\ survival}$ of 1.32 mg TI/L TWA was estimated. All specimens died in the highest treatment level. No clinical sign was observed for the survived individuals. Adult growth (body length) and age to first brood were unaffected up to and including 1.64 mg TI/L TWA, the highest treatment level with surviving adults. Adult reproduction was unaffected up to and including 0.83 mg TI/L TWA (NOEC). A concentration depending effect occurred. An $EC_{10\ reproduction}$ of 1.12 mg TI/L TWA was estimated. TWA test concentrations > 0.83 mg test item per L (NOEC) did affect survival (viability) of adults. A concentration depending effect occurred. An $EC_{10\ intrinsic\ rate}$ of 1.34 mg TI/L TWA was estimated.

Conclusion

1-Oxa-4,5-dithiepane has a chronic adverse effect on adult *Daphnia magna* under the chosen test conditions. Survival of the adults and reproduction rate was affected starting at 1.64 mg test item per liter TWA. The relevant NOEC was found to be 0.83 mg test item per liter TWA.

The relevant EC_{10} in this study is the $EC_{10\ reproduction}$ with 1.12 mg TI/L TWA (per introduced parent).

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Definitions/Abbreviations

CL	Confidence level
CoA	Certificate of analysis
DOC	Dissolved organ carbon
$EC_{10/20/50}$	(effective concentration) is the concentration of the test item, which results in a 10, 20 or 50 per cent reduction in the measured parameter relative to the control
EPA	United States Environmental Protection Agency
ESI	Electrospray ionization positive
FDA	Food and Drug Administration (USA)
GC-MS	Gas chromatography mass spectrometer
GLP	Good laboratory practice
LOD	Limit of detection
LOEC	(lowest observed effect concentration) is the lowest concentration tested at which the measured parameter shows significant inhibition relative to the control
LOQ	Limit of quantification
MAFF	Ministry of Agriculture, Forestry and Fisheries (Japan)
METI	Ministry of Economy, Trade and Industry (Japan)
MHLW	Ministry of Health, Labour and Welfare (Japan)
MSDS	Material safety data sheet
NOEC	(no observed effect concentration) is the highest concentration tested at which the measured parameter shows no significant inhibition relative to the control
NPOC	Non purgeable organic carbon
OECD	Organisation for Economic Co-operation and Development
SIM	Single ion monitoring mode
SOP	Standard operating procedure
TI	Test item
TWA	Time weighted average (test item concentration)

1 Test

Daphnia magna, Reproduction test (OECD 211); Semi-static exposure - Effect of 1-Oxa-4,5-dithiepane on the reproduction of *Daphnia magna*
Test item: 1-Oxa-4,5-dithiepane
GLP-Code: AAR – 001 / 4 – 21 / G

1.1 Sponsor

Jørn Bo Larsen
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1.2 Study Monitor

Hans Sanderson, PhD
Aarhus University
Dept. Environmental Science
Frederiksborgvej 399
4000 Roskilde, Denmark

1.3 Test Facility

Fraunhofer Institute for
Molecular Biology and Applied Ecology (IME)
Auf dem Aberg 1
57392 Schmallenberg, Germany

Division Applied Ecology
Test facility management: Prof. Dr. Christoph Schäfers

Study director:	Dr. Markus Simon
Deputy:	Dr. Karsten Schlich
Chemical investigator:	Dr. Matthias Kotthoff
Deputy:	Stephan Hennecke
Quality Assurance Unit:	Dr. Cornelia Bernhardt
	Dr. Ursula Wahle
	Karin Fink
	Jennifer Teigeler
	Lars Wiedemann-Krantz

1.4 Sub-contracting

No sub-order was awarded in this study.

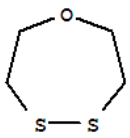
1.5 Schedule

Test (biological phase):	18.05.2016 – 08.06.2016
Test (chemical phase):	19.05.2016 – 08.06.2016
Study completion:	06.07.2016

2 Test item

The test item and certificate of analysis (CoA, dated on January 09, 2015) were provided by the sponsor before the start of the study. Test item information is given by the sponsor. With signing the study plan, the sponsor confirmed his agreement with the fact that the chemical identity, purity and stability of the test item under test and storage conditions (which has to be stated according to OECD Principles of Good Laboratory Practice [3]) were not examined analytically by the test facility. Test item which will not be needed for testing and for archiving will be disposed of as hazardous waste according to local regulations.

2.1 Test item

Test item name:	1-Oxa-4,5-dithiepane
Synonym:	1-Oxa-4,5-dithiacycloheptane
Chemical structure:	
Molecular formula:	$C_4H_8OS_2$
Molecular weight:	136.24 g/mol
CAS-Number:	3886-40-6
Lot/Batch Number:	EN1502b
Purity:	99.8 % (CoA dated on January 09, 2015)
State of matter and appearance:	Very faint yellow oil
Water solubility (preliminary data):	At least 10 mg/L, pH 8
Boiling point:	218.3 °C at 760 mmHg
Flash point:	85.8 °C
Stability in water:	Stable at pH 8 for at least three days
Biodegradability:	Not readily biodegradable
Storage conditions:	Store at -20 °C. Store in conditions where escape to the environment by leakage is prevented.
Safety data sheet available?	Not available

3 Test principle

Young female Daphnia (the parent animals), aged less than 24 hours at the start of the test, were exposed under semi-static conditions to the test item added to water at a range of concentrations for 21 days. Test solution was exchanged three times weekly. At the end of the test, the total number of living offspring produced per parent animal alive at the end of the test was assessed. The test was performed according to the guideline OECD 211 [1]. Using appropriate statistical methods it was analyzed, whether there was a statistical significant difference in immobilisation and reproduction rate between the treatments and the control.

4 Materials and Methods

4.1 Biological material

Test organisms were young specimens of Daphnia magna, 4 – 24 hours old at test start.

Origin of the daphnids: German Federal Environment Agency, Institut für Wasser-, Boden- und Lufthygiene. Specimens used in the test were bred in the laboratory of the Fraunhofer IME.

Breeding conditions: Adult Daphnia, at least 3 weeks old, were separated from the stock population by sieving. Batches of 30 to 50 animals are held at room temperature in ca. 1.8 L dilution water for one week. During this week the daphnids were fed daily with an algal suspension (*Desmodesmus subspicatus*) and LiquizellR (HOBBY). Algae growing in the log-phase were centrifuged and the pellet was re-suspended in a few mL of medium. 30 mL of this suspension was given to 1 L Daphnia medium. The water was changed once per week. Newborn Daphnia were separated by sieving, the first generation was discarded.

4.2 Holding- and dilution-water

Purified drinking water was used as holding- and dilution water. The purification included filtration with activated charcoal, passage through a lime-stone column and aeration. To avoid copper contamination, plastic water pipes are used for the testing facilities.

The following water chemistry data are recorded regularly in the testing facility and are reported: pH, conductivity, dissolved oxygen content, content of nitrate, nitrite, ammonium, phosphate, calcium, magnesium, total hardness, alkalinity, DOC content (or NPOC, as appropriate), content of metals (copper, iron, manganese and zinc). For details see Annex A 1.

4.3 Food

The daphnids are fed during the test with suspensions of unicellular alga *Desmodesmus subspicatus*. The content of food in the test suspensions, measured as at 585 nm, was kept constant at 0.2 mg C/(*Daphnia* x day).

4.4 Primary control

According to the guideline no reference item is required. However, the sensitivity of the test clone was checked twice a year in non-GLP tests by using $K_2Cr_2O_7$ as reference substance. $K_2Cr_2O_7$ is a recommended reference substance for acute effects on daphnids according to the OECD guideline 202 [2]

4.5 Test item

The test item was 1-Oxa-4,5-dithiepane. The nominal concentrations in the test containers with test item were 3.00, 1.67, 0.93, 0.51, 0.29 mg test item/L. The concentrations were selected on the basis of the results from a range-finding test at 0.05, 0.5 and 5.0 mg test item/L and agreed upon with the sponsor. Ten replicates per concentration with individual specimen were conducted.

4.6 Control

The control consists of dilution water only. Ten replicates with individual specimen were conducted.

4.7 Test container

Round glass beakers (50 mL) were used as test vessels. The vessels were filled up with 50 mL test solution. The beakers were covered with glass panes to prevent from evaporation as much as possible, but also permit gaseous exchange between the medium and the atmosphere and access of light.

4.8 Introduction of the test item

The test item was distributed to the replicate beakers using an aqueous stock solution (3 mg/L). The stock solution was prepared in dilution water. The individual test solutions were prepared by dilution with dilution water and distributed to the test beakers. The stock solution was freshly prepared before each renewal.

4.9 Analytical measurement

Concentration of the test substance

Once a week, fresh media and the respective aged media were sampled for verification of test item concentration in the test. For worst case assumption, 3-day incubation periods were selected for sampling. Fresh media were taken from the test solution preparation just before distributing it to the 10 replicates. At the renewals, samples of the aged test solution were taken from each vessel, pooled per concentration and measured. When analysis could not be applied immediately after sampling, samples were stored in a freezer at ≤ -18 °C until analysis. For details see Annex A 3.

Analytical method

The test item was analyzed by measuring the aqueous samples with a GC-MS system and quantified by using an internal standard. The validation was completed before in-life testing is started. The analytical method was validated following SANCO/3029/99 [6]. For details see Annex A 3.

Physical chemical parameters

Oxygen concentration, pH value, and temperature were checked directly before adding the animals and at each water renewal in new and aged test solutions. Hardness was checked once a week at water renewal in new and aged control media and test solutions of the highest treatment. Several water chemistry data of the dilution water were recorded regularly in the testing facility (see point 4.2).

4.10 Test procedure

Daphnia magna less than 24 h old were exposed to five concentrations of the test item under semi-static conditions for a period of 21 days. Individuals applied in the test are transferred with a bore Pasteur pipette a few hours after sieving to ensure applying only healthy specimens.

Test solution was exchanged three times a week. The test solution was distributed to the replicate beakers. Afterwards, test specimens were added (transfer with a bore Pasteur pipette).

The daphnids were exposed without aeration. The daphnia were fed during the test with suspensions of unicellular alga *Desmodesmus subspicatus* at a ration level equivalent to 0.2 mg C/(*Daphnia* x day). To create a food suspension of the desired C concentration, the relationship between algal density (in milligrams of C per litre) and optical absorbance (OD 585 nm) was determined.

The daphnids were subjected to a light/dark cycle of 16/8 hours. The test temperature during the test was 18 – 22°C. The temperature did not vary by more than 2°C within these limits. The light intensity did not exceed 15 -20 µE / (m^2 * s) or 1125 - 1500 lx.

4.11 Observation and biological measurements

The numbers of immobile daphnids were visually determined daily and the immobile daphnids were removed. Any abnormalities in appearance and behaviour were recorded if occurred.

The newborn daphnids per beaker were counted and removed daily until day 10 (after day 10 newborn daphnids were counted and removed three times weekly at each water renewal), abnormalities in condition (including male sex) or presence of winter eggs were checked and recorded.

At study termination, length of the adults up to the highest treatment without significant mortality was measured by digital photography and image analysis.

4.12 Deviations from the guideline/study plan

No deviations occur.

5 Treatment of the results

Data evaluation:

Numerical values in this report are frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in results obtained from calculations with such rounded values in comparison to those obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and thus of no practical concern.

The parental mortality, time to first brood and offspring number were used to calculate the intrinsic rate of population increase r as integrative parameter relevant for population effects. According to OECD 211, it is required to report the NOEC/EC for the cumulative offspring per introduced parent (provided these values are lower than in the cumulative offspring per survivor) if there is a significant trend in mortality of adults.

Statistical calculations:

For each endpoint, the NOEC, LOEC, and, if possible, the EC_{10} was determined. A LOEC was calculated by using ANOVA followed by Dunnett's or Williams' test or an appropriate non-parametric test. For trend analysis, a Contrasts test (Monotonicity of Concentration/Response) for metric data and a Cochran-Armitage test for mortality were applied, respectively. When the test results show a concentration-response relationship, the data is analysed by regression to determine the EC_{10} including the 95 % confidence interval using Probit-analysis assuming log-normal distribution of the values.

The evaluation of the concentration-effect-relationships and the calculations of effect concentrations were based on the mean measured concentrations (time weighted average, TWA).

Results are presented in chapter 6.3 and 6.4, and Annex A 2.

6 Results

6.1 Environmental conditions

With 19.5 – 20.6 °C throughout the test the permitted range of 18 – 22 °C (with a variance of less than 2 °C) was maintained. The oxygen saturation was between 6.4 mg/L and 12.9 mg/L. The pH values throughout the test were within a range of 7.9 – 9.4 at all treatment levels. The light intensity was measured using an illuminance meter (MINOLTA) with photometric sensor in Lux. With 816 – 867 lx (corresponding to 10.9 – 11.6 µE/(m² *s)) the light intensity was below the threshold value of about 1000 - 1500 lx (15–20 µE/(m² *s)) as permitted by OECD guideline 211. Thus, all water quality criteria mentioned in the guideline (13.1) were met. For raw data please refer Annex 1.

6.2 Test concentrations

The mean measured test item concentrations of the freshly prepared test solution (initial concentrations) were between 96 % and 107 % of nominal concentrations (table 2). During the time interval of three days until renewal of the test solution, test item concentrations decreased considerably to 79 – 92 % of nominal. Due to the decrease of the exposure concentrations, time weighted average concentrations were calculated for the evaluation of the biological parameters and the endpoints LOEC/NOEC, EC_X.

According to the guideline [1], following formula was used for the calculation of the time weighted average concentration:

$$\left(\frac{conc0 - conc1}{\ln conc0 - \ln conc1}\right) \times days \qquad = \text{Area}$$

where
conc 0 = concentrations at start of treatment period
conc 1 = concentrations at end of treatment period
days = duration of treatment period

The time-weighted average is the total area divided by the total days.

The time weighted average (TWA) of mean measured initial and mean measured aged concentration at test solution renewal were 0.26, 0.47, 0.83, 1.64, and 2.98 mg/L, corresponding with 91, 93, 89, 98, and 99 % of the nominal concentrations (Table 1). For details see Annex A 3.

Table 1: Concentrations of the test item. Mean measured initial concentrations [mg/L], mean measured aged concentrations [mg/L], time weighted mean concentration [mg/L], and percentage of nominal concentration [%].
TI = Test item; LOQ = Limit of quantification (0.1 mg/L);

Nominal conc.	0.29 mg TI / L	0.51 mg TI / L	0.93 mg TI / L	1.67 mg TI / L	3.00 mg TI / L
Mean measured initial conc.	0.28	0.53	0.92	1.78	3.22
% of nominal	95.8	103.0	99.4	106.9	107.4
Mean measured aged conc.	0.25	0.43	0.73	1.50	2.76
% of nominal	85.9	83.5	78.9	89.9	91.9
Time weighted mean conc.	0.26	0.47	0.83	1.64	2.98
% of nominal	90.6	93.0	88.7	98.1	99.4

6.3 Survival, body growth, physical/pathological symptoms and changes in behaviour of the test organisms

Concentration relating mortality of the adults was observed. The EC_{10} and EC_{50} were estimated at 1.32 and 1.51 mg test item per liter time weighted average (TWA), respectively. The $NOEC_{mortality}$ was found to be 0.83 mg TI/L TWA. At 2.94 mg TI/L TWA no introduced specimen survived. No other clinical signs were observed in any replicate at any concentration tested. Adult body length exhibited no significant differences between treatments ($NOEC_{growth}$ ≥ 1.64 mg TI/L TWA). Due to the lack of a clear dose response relationship, no EC values could be calculated.

6.4 Reproduction and population growth

Age at the first brood was between 8.8 and 9.7 days up to the highest concentration with survivors at test end (1.64 mg TI/L TWA). The difference was not statistically significant (NOEC ≥ 1.64 mg TI/L TWA).

A significant trend in mortality was revealed. According to the guideline OECD 211, cumulative offspring per introduced and per survived parent have to be evaluated.

With 70.2 to 100.8 juveniles, the cumulative number of offspring per introduced parent was comparable with the control up to and including a test concentration of 0.83 mg TI/L TWA (NOEC). With 13.6 and 0.0 juveniles, the cumulative number of offspring per introduced parent at 1.64 and 2.98 mg TI/L TWA, respectively, was significantly reduced. The EC_{10} and EC_{50} were estimated at 1.12 and 1.39 mg test item per liter time weighted mean (TWA), respectively.

With 77.4 to 109.0 juveniles, the cumulative number of offspring per survived parent was comparable with the control up to and including a test concentration of 0.83 mg TI/L TWA (NOEC). With 62.5 juveniles, the cumulative number of offspring per replicate at 1.64 mg TI/L TWA was significantly reduced. At 2.98 mg TI/L TWA no adult female survived the complete test duration. The EC_{10} and EC_{50} were estimated at 1.32 and > 1.64 mg test item per liter time weighted average (TWA), respectively.

According to the guideline, the relevant endpoint is the more sensitive one. Consequently, the relevant endpoint is cumulative number of offspring per introduced parent.

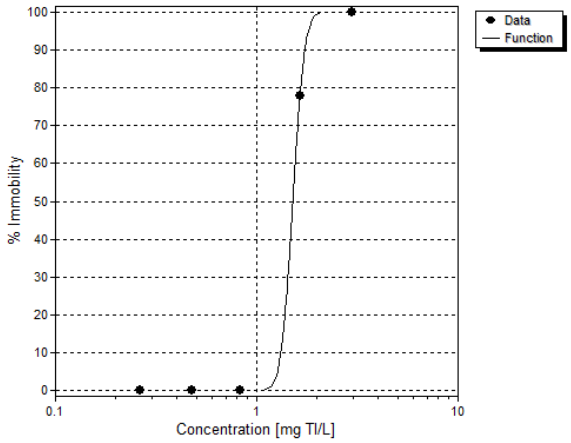
With values of 0.304 to 0.342, the intrinsic rate was comparable with the control up to and including a test concentration of 0.83 mg TI/L TWA (NOEC). With a value of 0.211, the intrinsic rate at 1.64 mg TI/L TWA was significantly reduced. At 2.98 mg TI/L TWA no calculation could be applied due to the lack of offspring data. The EC_{10} and EC_{50} were estimated at 1.34 and > 1.64 mg test item per liter time weighted mean (TWA), respectively.

Table 2: Survival, growth and reproduction data.
SD = standard deviation. For raw data see Annex 1. Number of *D. magna* per concentration: n = 10. TWA = Time weighted average.

Concentration	**Parental survival**	**Growth (length on day 21)**	**Age at first brood**	**Offspring per introduced parent**	**Offspring per survived parent**	**Intrinsic rate of increase**
(mg TI/L TWA)	(%)	Mean ± SD (mm)	Mean ± SD (days)	Mean ± SD (Ind.)	Mean ± SD (Ind.)	Mean ± SD (Ind./day)
Control	90	5.0 ± 0.2	9.7 ± 1.2	83.5 ± 36.6	92.8 ± 23.2	0.304 ± 0.051
0.26	90	5.2 ± 0.2	9.0 ± 0.5	100.8 ± 28.4	109.0 ± 12.3	0.342 ± 0.022
0.47	90	4.8 ± 0.7	9.4 ± 1.1	70.2 ± 44.6	77.4 ± 40.6	0.308 ± 0.055
0.83	100	5.1 ± 0.2	9.8 ± 0.7	93.9 ± 22.2	93.9 ± 22.2	0.323 ± 0.032
1.64	20	4.6 ± 0.5	8.8 ± 0.6	13.6 ± 26.0	62.5 ± 3.5	0.211 ± 0.000
2.98	0	-	-	0.0 ± 0.0	-	-

Table 3: Effect summary table.
Based on concentrations calculated from time weighted average concentrations (TWA).
n.d. = not determined due to mathematical reasons

Concentration	**Parental survival**	**Growth (length on day 21)**	**Age at first brood**	**Offspring per introduced parent**	**Offspring per survived parent**	**Intrinsic rate of increase**
EC_{10} (95% CL)	1.32 (n.d.)	> 1.64 mg/L (n.d.)		1.12 (n.d.)	1.32 (n.d.)	1.34 (n.d.)
NOEC	0.83 mg/L	≥ 1.64 mg/L	≥ 1.64 mg/L	0.83 mg/L	0.83 mg/L	0.83 mg/L



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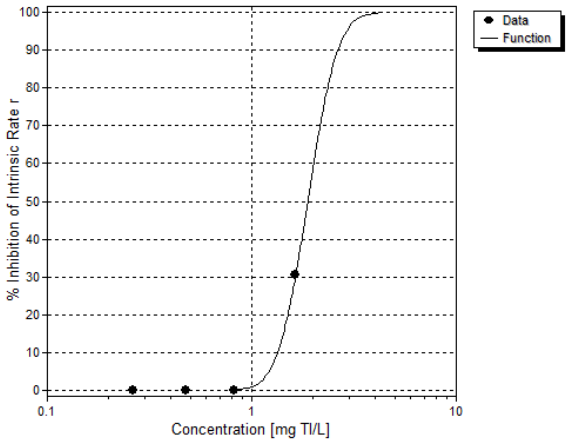

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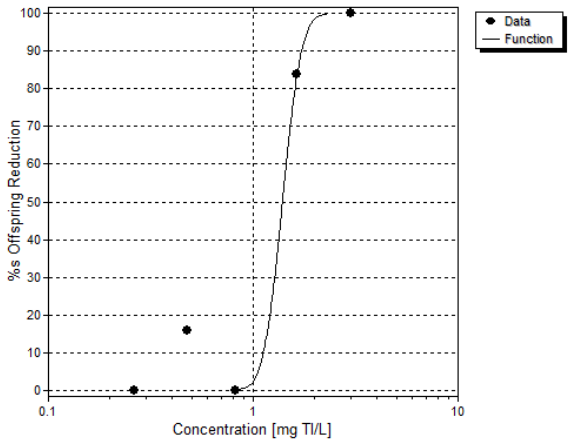

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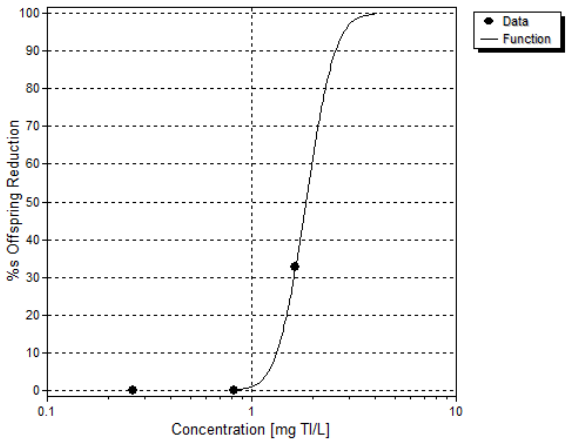


D

Figure 1: Concentration-effect curve showing the influence of the test item on A: immobilization of the adults, B: intrinsic rate, C: number of offsprings per introduced parent and D: number of offsprings per survived parent as observed after 21 d.
Figures were produced by ToxRat Professional. No calculation of convidence limits (CL) possible

6.5 Primary standard

In order to confirm the sensitivity of the test species *Daphnia magna* (clone 5), acute immobilization tests over 24 h with the reference substance (RS) $K_2Cr_2O_7$ are performed in regular intervals, as proposed by OECD 202. The results of the latest reference study (February 2016) are in agreement with historical 24 h EC_{50}-values obtained in this institute.

Immobilization after 24 h:

Control:	0 %		
0.40 mg RS/L:	0 %	related to control:	0 %
0.60 mg RS/L:	0 %	related to control:	0 %
0.90 mg RS/L:	20 %	related to control:	20 %
1.35 mg RS/L:	55 %	related to control:	55 %
2.00 mg RS/L:	75 %	related to control:	75 %

24 h EC_{50} value:

Immobilization: 1.38 mg/L (95% CL: 1.19 – 1.64 mg/L)

7 Validity

The test is considered valid since

- mortality in controls (10%) does not exceed 20%
- the mean number of offspring in the control within the 21 days (83.5) was above the criterion of 60 / introduced female

Also the following additional quality criteria indicated in the OECD guideline were fulfilled.

- The dissolved oxygen concentration was above 3 mg/l at the beginning and during the test.
- The pH should be within the range 6 - 9, and normally it should not vary by more than 1.5 units in any one test.
- The coefficient of variation for the mean number of control offspring per survived female (25%) was ≤ 25%, which is indicated for a well-run test.

8 References

[1] OECD 211 (02 October 2012): OECD guideline for testing of chemicals – *Daphnia magna* Reproduction Test.

[2] OECD 202 (13 April 2004): Guideline for Testing of Chemicals - *Daphnia* sp., Acute Immobilization Test.

[3] OECD Principles of Good Laboratory Practice (as revised in 1997), Paris, 1998.

[4] EEC (2004). Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version).

[5] Gesetz zum Schutz vor gefährlichen Stoffen (Chemikaliengesetz - ChemG) in der Fassung der Bekanntmachung vom 28. August 2013 (BGBl. IS. 3498, 3991), zuletzt durch Artikel 7 des Gesetzes vom 4. April 2016 (BGBl. I S. 569) geändert worden ist (Anhang 1 zu § 19a Abs. 1 Grundsätze der Guten Laborpraxis (GLP)).

[6] European Commission, Directorate General Health and Consumer Protection: SANCO/3029/99 rev.4 (11/07/2000), 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.

[7] ToxRat® Professional 3.2.1 ToxRat® Solutions GmbH. Dr. Monika M. Ratte, Naheweg 15, 52477 Alsdorf, Germany.

A 1 Annex 1: Processed raw data

Table 4: Oxygen saturation of the overlaying water. Values of the parallel test vessels throughout test duration [mg/L].
TI = Test item; Concentrations given as time weighted average (TWA).

Date	Control		0.26 mg TI/L		0.47 mg TI/L		0.83 mg TI/L		1.64 mg TI/L		2.98 mg TI/L	
	aged	new	aged	new	aged	new	aged	new	aged	new	aged	new
Start		6.61		6.62		6.61		6.45		6.47		6.43
Day 2	10.22	8.59	10.11	8.35	9.98	8.40	10.17	8.35	10.20	8.22	10.17	8.04
Day 4	11.38	8.33	11.24	8.32	11.37	8.20	11.14	8.21	11.87	8.21	12.91	8.17
Day 7	10.78	8.96	10.43	8.45	10.14	8.67	10.35	8.39	11.06	8.33	12.12	8.26
Day 9	10.56	9.05	10.56	8.91	10.72	8.94	10.74	8.91	11.51	8.87	10.31	*
Day 11	10.59	9.17	10.83	9.19	11.63	9.18	10.77	9.12	12.14	9.01	*	*
Day 14	8.96	8.39	9.21	8.41	9.23	8.35	9.07	8.38	10.35	8.38	*	*
Day 16	7.86	8.92	7.94	7.91	8.37	7.83	8.44	7.91	8.79	7.91	*	*
Day 18	8.25	8.77	8.56	8.48	9.14	8.50	8.96	8.60	10.74	8.47	*	*
Day 21	9.54		9.73		10.08		9.44		11.19		*	

* No test media prepared since no adult female survived

Table 5: pH of the overlaying water. Values of the parallel test vessels throughout test duration.
TI = Test item; Concentrations given as time weighted average (TWA).

Date	Control		0.26 mg TI/L		0.47 mg TI/L		0.83 mg TI/L		1.64 mg TI/L		2.98 mg TI/L	
	aged	new	aged	new	aged	new	aged	new	aged	new	aged	new
Start		8.50		8.29		8.27		8.27		8.28		8.20
Day 2	8.72	8.04	8.72	7.94	8.70	7.93	8.72	7.93	8.71	7.93	8.73	7.92
Day 4	9.11	8.05	9.08	8.06	9.09	8.05	9.05	8.06	9.18	8.05	9.37	8.04
Day 7	8.84	7.98	8.84	8.00	8.85	7.99	8.82	8.00	9.05	7.98	9.17	7.98
Day 9	8.75	8.06	8.71	8.09	8.77	8.07	8.68	8.08	9.01	8.08	9.05	*
Day 11	8.66	7.95	8.70	7.98	8.88	7.98	8.71	7.98	9.15	7.98	*	*
Day 14	8.35	8.00	8.45	8.04	8.46	8.06	8.33	8.06	8.90	8.06	*	*
Day 16	8.02	7.85	8.03	8.04	8.23	8.09	8.22	8.09	8.53	8.09	*	*
Day 18	7.95	7.93	8.06	8.01	8.29	8.03	8.23	8.02	9.02	8.03	*	*
Day 21	7.91		7.99		8.21		7.95		8.81		*	

* No test media prepared since no adult female survived

Table 6: Hardness of the overlaying water. Values of the parallel test vessels throughout test duration [mmol/L]
TI = Test item; Concentrations given as time weighted average (TWA).

Date	Control		3 mg TI/L	
	aged	new	aged	new
Start	-	1.4	-	1.3
Day 7	1.2	1.1	1.2	1.1
Day 14	1.2	1.3	1.2	1.2
Day 21	1.2	-	1.1	-

Table 7: Temperature of the overlaying water [°C]. Measured in the climatic chamber.

Day 0	**Day 2**	**Day 4**	**Day 7**	**Day 9**
20.1	20.6	20.5	20.6	19.5
Day 11	**Day 14**	**Day 16**	**Day 18**	**Day 21**
20.2	20.1	20.4	20.1	19.9

Table 8: Light intensity [lx] in the climatic chamber.

Day 0	**Day 2**	**Day 4**	**Day 7**	**Day 9**
835	836	816	843	848
Day 11	**Day 14**	**Day 16**	**Day 18**	**Day 21**
817	857	865	841	867

Table 9: Dilution water, chemical properties.
LOD = Limit of detection; LOQ = Limit of quantification.

Conductivity (µS/cm)	**Alcalinity (mmol/l)**	**Tot. hardness (mmol/l)**	**Ca-hardness (mmol/l)**	**Mg-hardness (mmol/l)**	**DOC (NPOC) (mg/L)**	**NO_3 (mg/L)**
207 – 215	1.8	1.1	0.9 – 1.0	0.1 – 0.2	0.80 – 1.05	7 – 10
NO_2 (mg/L)	**NH_4 (mg/L)**	**PO_4 (mg/L)**	**Cl (mg/L)**	**Cd (µg/L)**	**Cr (µg/L)**	**Cu (µg/L)**
< 0.005 (LOQ)	≤ 0.01 (LOQ)	0.11 – 0.45	≤ 0.02	< 0.591 (LOD)	< 0.596 (LOD)	< 4.07 (LOQ)
Fe (µg/L)	**Mn (µg/L)**	**Ni (µg/L)**	**Pb (µg/L)**	**Zn (µg/L)**		
< 3.11 (LOD)	< 8.02 (LOD)	< 1.64 (LOD)	< 1.65 (LOD)	< 3.89 (LOQ)		

Table 10: Offspring per replicate and day.
TI = Test item; Concentrations given as time weighted average (TWA). * Adult female did not survive until test end;

Control										
	Replicate number									
Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
6	0	0	0	*	0	0	0	0	0	0
7	0	0	0	-	0	0	0	0	0	0
8	0	8	0	-	8	0	0	13	0	0
9	10	0	0	-	0	0	12	0	0	8
10	0	0	15	-	0	0	0	0	0	0
11	0	0	0	-	15	5	0	19	2	0
12	26	10	0	-	0	0	12	0	0	21
14	0	0	22	-	0	21	0	26	13	0
16	24	20	0	-	24	0	9	0	0	32
19	35	29	29	-	32	26	7	45	26	42
21	0	0	28	-	22	27	29	41	42	0

* Adult female died during this day;

0.26 mg TI/L										
	Replicate number									
Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
8	10	0	0	0	10	0	8	14	0	15
9	0	15	10	17	0	7	0	0	4	0
10	0	0	0	0	0	0	0	0	0	0
11	16	0	0	0	26	21	0	0	0	20
12	0	29	26	23	0	12	2	0	22	0
14	29	0	0	0	0	0	0	26	0	0
16	0	39	31	29	32	24	17	0	28	30
19	35	41	30	34	41	36	*	32	37	36
21	33	0	0	0	0	24	-	37	0	0

* Adult female died during this day;

- Table 10, to be continued -

Table 10: Offspring per replicate and day (continued).
TI = Test item; Concentrations given as time weighted average (TWA).

0.47 mg TI/L										
	Replicate number									
Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	6	0	0	0	0
8	0	12	5	0	0	0	10	0	12	0
9	0	0	*	10	13	0	0	0	0	7
10	14	0	-	0	0	0	0	0	0	0
11	1	7	-	15	0	5	21	0	15	0
12	0	0	-	0	20	0	0	0	0	10
14	9	27	-	1	0	11	35	0	0	0
16	21	0	-	24	34	7	0	0	24	23
19	31	17	-	32	28	20	43	0	34	30
21	0	0	-	26	0	9	39	0	0	0

* Adult female died during this day;

0.83 mg TI/L										
	Replicate number									
Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
6	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	6	0	0	0	0
8	11	0	0	0	0	0	0	0	0	0
9	0	12	11	0	0	15	0	13	0	16
10	0	0	0	14	10	0	8	0	15	0
11	12	0	0	0	0	0	0	0	0	0
12	0	18	31	0	0	22	0	19	0	28
14	27	0	0	14	19	0	2	0	32	0
16	0	27	29	27	4	1	17	27	0	35
19	32	37	23	0	0	36	0	31	29	34
21	32	0	0	31	25	39	31	0	43	0

- Table 10, to be continued -

Table 10: Offspring per replicate and day (continued).
TI = Test item; Concentrations given as time weighted average (TWA).

1.64 mg TI/L										
	Replicate number									
Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
4	0	0	0	0	0	0	0	0	0	0
5	*	0	*	0	0	0	0	0	0	*
6	-	0	-	0	0	0	0	0	0	-
7	-	*	-	*	0	6	0	*	0	-
8	-	-	-	-	14	0	12	-	0	-
9	-	-	-	-	0	*	0	-	11	-
10	-	-	-	-	0	-	0	-	0	-
11	-	-	-	-	0	-	0	-	0	-
12	-	-	-	-	17	-	19	-	*	-
14	-	-	-	-	0	-	0	-	-	-
16	-	-	-	-	22	-	22	-	-	-
19	-	-	-	-	7	-	12	-	-	-
21	-	-	-	-	0	-	0	-	-	-

* Adult female died during this day;

2.98 mg TI/L										
	Replicate number									
Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	0	0	0	0	0	0	0	0	0	0
2	*	0	*	*	*	*	*	0	0	*
3	-	0	-	-	-	-	-	0	0	-
4	-	0	-	-	-	-	-	0	0	-
5	-	0	-	-	-	-	-	*	*	-
6	-	0	-	-	-	-	-	-	-	-
7	-	0	-	-	-	-	-	-	-	-
8	-	0	-	-	-	-	-	-	-	-
9	-	*	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-

* Adult female died during this day;

Table 11: Parental lengths at day 21 [mm].
TI = Test item; Concentrations given as time weighted average (TWA). * Adult female did not survive until test end; ** No adult female survived until test end;

Replicate	Control	0.26 mg TI/L	0.47 mg TI/L	0.83 mg TI/L	1.64 mg TI/L	2.98 mg TI/L
1	5.05	5.28	4.84	*	*	*
2	5.06	4.96	4.72	4.96	*	*
3	4.93	5.59	*	4.97	*	*
4	*	5.23	5.09	5.39	*	*
5	5.46	5.12	5.09	5.08	4.21	*
6	4.97	4.98	5.30	5.25	*	*
7	5.06	*	5.06	4.77	4.93	*
8	5.21	5.17	2.91	5.10	*	*
9	4.67	4.96	5.21	4.96	*	*
10	4.84	5.11	4.52	5.53	*	*
Mean	0.47	0.46	0.45	0.45	0.49	**
SD	0.02	0.04	0.05	0.05	0.00	**

A 2 Annex 2: Statistical evaluation

A 2.1 Relation of *Daphnia magna* Endpoints on Concentration

Summary of Results for all Endpoints: Critical effect and threshold concentration as observed at end of experimental time; EC: Effective concentration for xx% reduction; 95%-CL: 95% Confidence limits; LOEC: Lowest observed effect concentration; NOEC: No observed effect concentration

Critical Conc.s [mg TI/L] 0-21 d

Cumulative offspring per introduced parent (21 d)			
	EC10	**1.12**	**relevant EC10**
95%-CL	lower	n.d.	
	upper	n.d.	
	LOEC	1.64	
	NOEC	**0.83**	**relevant NOEC**
Cumulative offspring per survived parent (0 - 21 d)			
	EC10	1.32	
95%-CL	lower	n.d.	
	upper	n.d.	
	LOEC	>1.64	
	NOEC	>=1.64	
Immobility (0 - 21 d)			
	EC10	1.32	
95%-CL	lower	n.d.	
	upper	n.d.	
	LOEC	1.64	
	NOEC	0.83	
Length (21 d)			
	EC10	>1.64	
95%-CL	lower	n.d.	
	upper	n.d.	
	LOEC	n.d.	
	NOEC	n.d.	
Age of first reproduction			
	LOEC	>1.64	
	NOEC	>=1.64	
Intrinsic rate r			
	EC10	1.34	
95%-CL	lower	n.d.	
	upper	n.d.	
	LOEC	1.64	
	NOEC	0.83	

n.d.: not determined due to mathematical reasons or inappropriate data

A 2.2 Cumulative Offspring per Introduced Parent at 21 d

Cumulative Offspring per Introduced Parent in Daphnia magna after 21 d.

%Inhibition of cumulative offspring of introduced caused by the test item after 21 d.

Treatm.[mg TI/L]	Mean	Std. Dev.	n	%Reduction
Control	83.5	36.61	10	
0.263	100.8	28.41	10	-20.7
0.47	70.2	44.61	10	15.9
0.83	93.9	22.16	10	-12.5
1.64	13.6	26.03	10	83.7
2.98	0.0	0.00	10	100.0

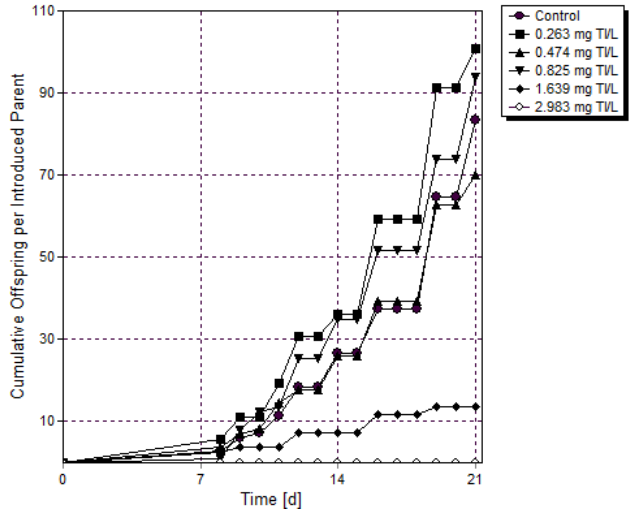


Figure 2: Cumulative offspring per Introduced Parent of *Daphnia magna* as observed under presence of the test item after 21 d.

Effective Concentrations (ECx) with Cumulative Offspring of Introduced Parent in *Daphnia magna* after 21 d.

Results of the probit analysis

Results of the probit analysis: Selected effective concentrations (ECx) of the test item and their 95%-confidence limits (according to Fieller`s theorem).

Toxicity Metric	EC10	EC20	EC50
Value [mg TI/L]	1.119	1.205	1.389
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

n.d.: not determined due to mathematical reasons or inappropriate data

Slope function after Litchfield and Wilcoxon: 1.184 Inhibitions lower equal 0% or greater equal 100.0% were replaced by 0.100 and 99.9%, respectively.Computation was adjusted to metric data (Christensen & Nyholm 1984); variance and thus confidence limits were corrected by covariance with the control (Draper & Smith 1981)The probability p(F) is greater than 0.05; i.e. the slope was not significantly different from zero. The shown toxic metrics could be meaningless. (The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

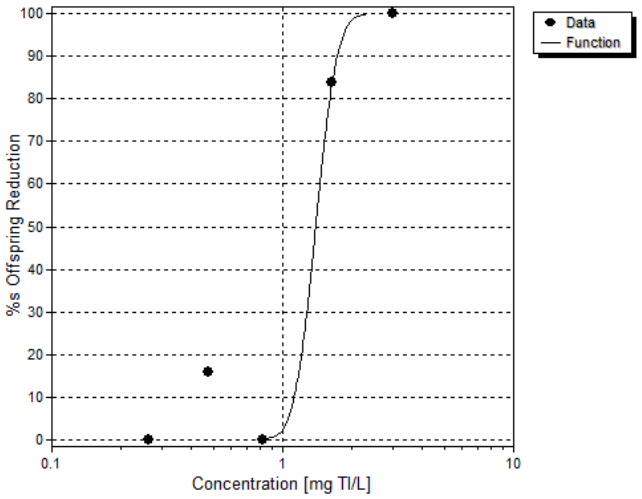


Figure 3: Concentration-effect curve showing the influence of the test item on mean cumulative offspring per Introduced Parent of *Daphnia magna* as observed after 21 d.

Threshold Concentrations (NOEC) with Cumulative Offspring of Introduced Parent at 21 d

Statistical characteristics of the sample

Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [mg TI/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	83.5	88.5	0.0	144.0	10	36.61	43.8	11.58	13.9	57.3	109.7
0.26	100.8	106.0	27.0	124.0	10	28.41	28.2	8.98	8.9	80.5	121.1
0.47	70.2	73.0	0.0	148.0	10	44.61	63.5	14.11	20.1	38.3	102.1
0.83	93.9	94.0	58.0	119.0	10	22.16	23.6	7.01	7.5	78.0	109.8
1.64	13.6	0.0	0.0	65.0	10	26.03	191.4	8.23	60.5	-5.0	32.2
2.98	0.0	0.0	0.0	0.0	10	0.00	n.d.	0.00			

Shapiro-Wilk´s Test on Normal Distribution

Normality check was passed (p > 0.01).

Levene´s Test on Variance Homogeneity (with Residuals)

Variance homogeneity check was passed (p > 0.01).

Normal-distribution and variance-homogeneity requirements are fulfilled. A parametric multiple test is advisable. To justify the use of Williams test at first a trend analysis by contrasts is performed.

Trend analysis by Contrasts (Monotonicity of Concentration/Response)

The linear trend is significant ($p \leq 0.05$), thus the selected Williams test was performed.

Williams Multiple Sequential t-test Procedure

Comparison of treatments with "Control" by the t test procedure after Williams. Significance was Alpha = 0,05, one-sided smaller; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: $\mu 1 = \mu 2 = ... = \mu k$; the differences are significant in case $|t| > |t^*|$ (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [mg TI/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
Control	83.5	29.739						
0.26	100.8	29.739	54	100.8	-26.7	1.30	-1.67	-
0.47	70.2	29.739	54	82.1	-27.9	-0.11	-1.75	-
0.83	93.9	29.739	54	82.1	-28.2	-0.11	-1.77	-
1.64	13.6	29.739	54	13.6	-28.4	-5.26	-1.78	+
2.98	0.0	29.739	54	0.0	-28.5	-6.28	-1.79	+

+: significant; -: non-significant

A NOEC of 0.83 mg TI/L is suggested by the program.

A 2.3 Cumulative Offspring per Survived Parent at 21 d

Cumulative Offspring per Survived Parent in *Daphnia magna* after 21 d.

%Inhibition of cumulative offspring of survivors caused by the test item after 21 d.

Treatm.[mg TI/L]	Mean	Std. Dev.	n	%Reduction
Control	92.8	23.23	9	
0.263	109.0	12.32	9	-17.5
0.474	77.4	40.60	9	16.5
0.825	93.9	22.16	10	-1.2
1.639	62.5	3.54	2	32.6

Treatments with no survivors were excluded from further analysis.

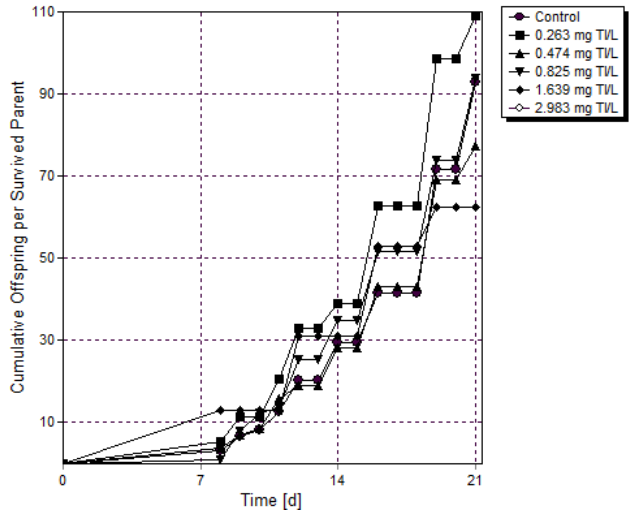


Figure 4: Cumulative offspring per Survived Parent of *Daphnia magna* as observed under presence of the test item after 21 d.

Effective Concentrations (ECx) with Cumulative Offspring of Survived Parent in *Daphnia magna* after 21 d.

Results of the probit analysis

Results of the probit analysis: Selected effective concentrations (ECx) of the test item and their 95%-confidence limits (according to Fieller`s theorem).

Toxicity Metric	EC10	EC20	EC50
Value [mg TI/L]	1.320	1.480	1.842
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

n.d.: not determined due to mathematical reasons or inappropriate data

Slope function after Litchfield and Wilcoxon: 1.297 Inhibitions lower equal 0% or greater equal 100.0% were replaced by 0.100 and 99.9%, respectively.Computation was adjusted to metric data (Christensen & Nyholm 1984); variance and thus confidence limits were corrected by covariance with the control (Draper & Smith 1981)The probability p(F) is greater than 0.05; i.e. the slope was not significantly different from zero. The shown toxic metrics could be meaningless. (The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

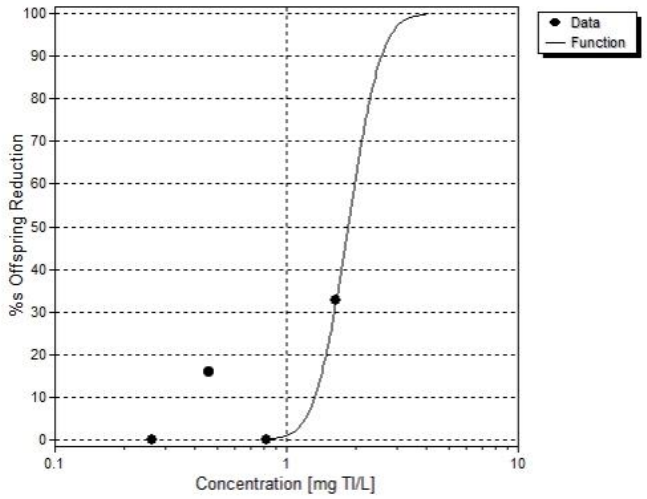


Figure 5: Concentration-effect curve showing the influence of the test item on mean cumulative offspring per Introduced Parent of *Daphnia magna* as observed after 21 d.
Data of the second treatment concentration was neglected for a better fitting.

Threshold Concentrations (NOEC) with Cumulative Offspring of Survived Parent at 21 d

Statistical characteristics of the sample

Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [mg TI/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	92.8	94.0	67.0	144.0	9	23.23	25.0	7.74	8.3	74.9	110.6
0.26	109.0	109.0	91.0	124.0	9	12.32	11.3	4.11	3.8	99.5	118.5
0.47	77.4	76.0	0.0	148.0	9	40.60	52.4	13.53	17.5	46.2	108.7
0.83	93.9	94.0	58.0	119.0	10	22.16	23.6	7.01	7.5	78.0	109.8
1.64	62.5	62.5	60.0	65.0	2	3.54	5.7	2.50	4.0	30.7	94.3

Treatments with no survivors were excluded from further analysis.

Shapiro-Wilk´s Test on Normal Distribution

Normality check was passed (p > 0.01).

Levene´s Test on Variance Homogeneity (with Residuals)

Variance homogeneity check was passed (p > 0.01).

Normal-distribution and variance-homogeneity requirements are fulfilled. A parametric multiple test is advisable. To justify the use of Williams test at first a trend analysis by contrasts is performed.

Trend analysis by Contrasts (Monotonicity of Concentration/Response)
The linear trend is significant (p ≤ 0.05), thus the selected Williams test was performed.

Williams Multiple Sequential t-test Procedure
Comparison of treatments with "Control" by the t test procedure after Williams. Significance was Alpha = 0,05, one-sided smaller; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; %MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: μ1 = μ2 = ... = μk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [mg TI/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
Control	92.8	26.094						
0.26	109.0	26.094	34	109.0	-22.4	1.32	-1.69	-
0.47	77.4	26.094	34	86.1	-23.4	-0.54	-1.77	-
0.83	93.9	26.094	34	86.1	-23.2	-0.56	-1.80	-
1.64	62.5	26.094	34	62.5	-38.8	-1.48	-1.77	-

+: significant; -: non-significant

The NOEC appears to be higher than or equal 1.64 mg TI/L.

The Cochran-Armitage trend-test revealed a significant trend in mortality. Thus, according to OECD 211 it is required to report the NOEC/EC for the cumulative offspring per introduced parent provided these values are lower than in the cumulative offspring per survivor.

A 2.4 Mobility of Daphnia magna

Mobility of Daphnia magna

%Immobility of Daphnia magna as caused by the test item.

Treatm.[µg/L]	Introduced	Mobile	Immobile	% Immobility
Control	10	9	1	10.0
0.26	10	9	1	10.0
0.47	10	9	1	10.0
0.83	10	10	0	0.0
1.64	10	2	8	80.0
2.98	10	0	10	100.0

The control response of 10.0% will be compensated using Abbott`s formula.

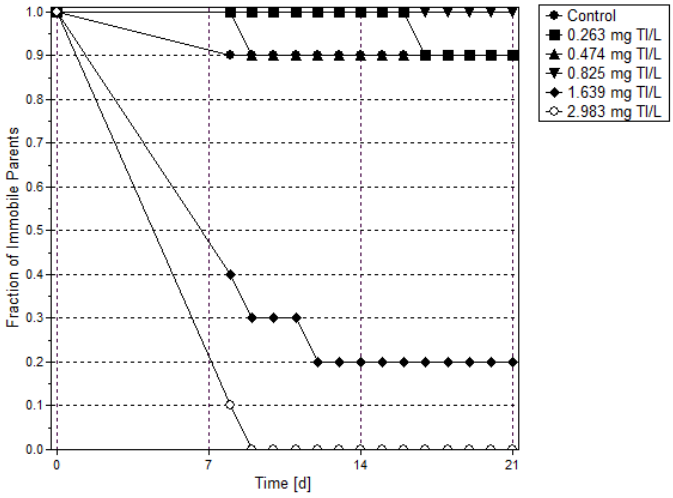


Figure 6: Mobility of the introduced *Daphnia magna* as observed under presence of the test item.

According to OECD 211 (2012) a trend test on mortality was performed as a decision criterion so that the user can choose the cumulative offspring either per survived or introduced parent as reproduction endpoint.

Cochran-Armitage test procedure

Cochran-Armitage test procedure with immobility at 21 d: Test procedure to detect an increasing trend in responses (Alpha is 0.050; one-sided greater); Chi²(tot): total (Pearson) Chi²; z(trend): standardized one-sided deviation due to the linear upward trend; Chi²(err): unexplained component of Chi²(tot); p(tot|trend|err): probabilities that the observed results could be due to chance; Ho (no trend) is accepted, if p(trend) > Alpha.

Treatm. [mg Tl/L]	Total Introduced	Immobile	% Immobility
Control	10	1	10.0
0.26	10	1	10.0
0.47	10	1	10.0
0.83	10	0	0.0
1.64	10	8	80.0
2.98	10	10	100.0

Chi²(tot): 41.099; p(tot): <0.001
z(trend): 5.151; p(trend): <0.001
Chi²(err): 14.568; p(err): 0.006
Since p(trend) <= Alpha, the observed trend is significant.

Effective Concentrations (ECx) with Mobility at 21 d

Results of the probit analysis

Results of the probit analysis: Selected effective concentrations (ECx) of the test item and their 95%-confidence limits

Toxicity Metric	EC10	EC20	EC50
Value [mg TI/L]	1.317	1.381	1.510
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

n.d.: not determined due to mathematical reasons

Slope function after Litchfield and Wilcoxon: 1.113 Inhibitions lower equal 0% or greater equal 100.0% were replaced by 0.100 and 99.9%, respectively.The treatment response was corrected by the control response (= 10.0%) using Abbott`s formula.
(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

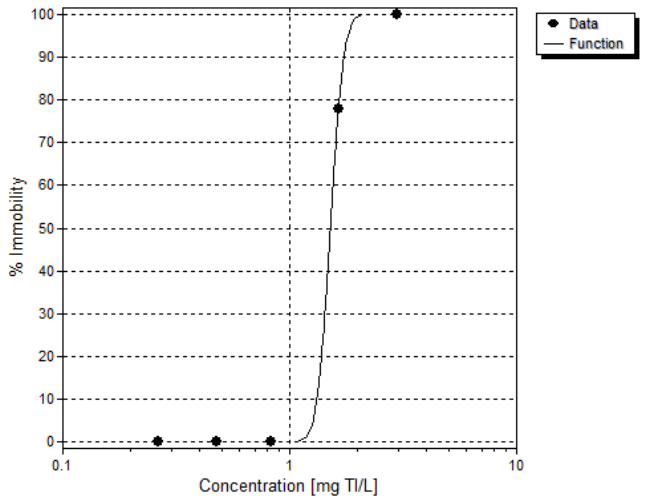


Figure 7: Mobility Concentration-effect curve showing the influence of the test item on mobility of the introduced *Daphnia magna* as observed after 21 d..

Threshold Concentrations (NOEC) with Mobility at 21 d

To justify the use of the Step-down Cochran-Armitage test at first a trend analysis by contrasts using proportions was performed.

Qualitative Trend Analysis by Contrasts (Monotonicity of Concentration/Response)

The linear trend is significant (p <= 0.05), thus the selected Step-down Cochran-Armitage test was performed.

Step-down Cochran-Armitage Test Procedure

Step-down Cochran-Armitage Test Procedure with immobility at 21 d: Step-down test to detect an increasing trend in responses (Alpha is 0.050; one-sided greater); Chi²(tot): total (Pearson) Chi²; z(trend): standardized one-sided deviation due to the linear upward trend; Chi²(err): unexplained component of Chi²(tot); p(tot|trend|err): probabilities that the observed results could be due to chance; Ho (no trend) is accepted, if p(trend) > Alpha.
Note that the step-down test terminates after the first non-significant treatment is encountered

Treatm. [mg TI/L]	Introduced	Immobile	%	Chi²(tot)	p(tot)	Chi²(err)	p(err)|z|(trend)		p(trend)	Sign.
Control	10	1	10.0							
0.26	10	1	10.0	0.000	1.000	0.000	<0.001	0.000	1.000	-
0.47	10	1	10.0	0.000	1.000	0.000	<0.001	0.000	1.000	-
0.83	10	0	0.0	1.081	0.782	0.432	0.806	0.805	0.790	-
1.64	10	8	80.0	24.942	<0.001	15.093	0.002	3.138	<0.001	+
2.98	10	10	100.0	41.099	<0.001	14.568	0.006	5.151	<0.001	+

+: significant; -: non-significant

A NOEC of 0.83 mg TI/L is suggested by the program.

A 2.5 Age of first reproduction in Daphnia magna as Dependent on Concentration and Time

Age of first reproduction in Daphnia magna as Dependent on Concentration and Time

Age of first reproduction in Daphnia magna as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (calculated from InputRawData)

Treatm. [mg TI/L]	**Control**	**0.26**	**0.47**	**0.83**	**1.64**	**2.98**
	9.5	8.5	10.5	8.5	-	-
	8.5	9.5	8.5	9.5	-	-
	10.5	9.5	8.5	9.5	-	-
	-	9.5	9.5	10.5	-	-
	8.5	8.5	9.5	10.5	8.5	-
	11.5	9.5	11.5	9.5	-	-
	9.5	8.5	8.5	10.5	8.5	-
	8.5	8.5	-	9.5	-	-
	11.5	9.5	8.5	10.5	9.5	-
	9.5	8.5	9.5	9.5	-	-
Mean:	9.7	9.0	9.4	9.8	8.8	-
Std.Dev.:	1.20	0.53	1.05	0.67	0.58	-
n:	9	10	9	10	3	0
CV:	12.4	5.9	11.2	6.9	6.5	

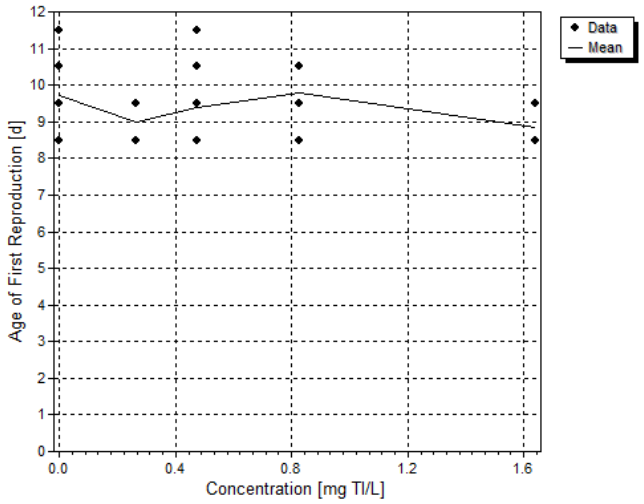


Figure 8: Age at first reproduction of *Daphnia magna* as observed under presence of the test item.

Effective Concentrations (ECx) for Age at First Reproduction

The age of first reproduction does not allow to calculate ECx-values because it is not possible to define the effect size x (maximum possible increase in age not known).

Threshold Concentrations (NOEC) with Age at First Reproduction

Statistical characteristics of the sample

Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [mg TI/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	9.7	9.5	8.5	11.5	9	1.20	12.4	0.40	4.1	8.8	10.6
0.26	9.0	9.0	8.5	9.5	10	0.53	5.9	0.17	1.9	8.6	9.4
0.47	9.4	9.5	8.5	11.5	9	1.05	11.2	0.35	3.7	8.6	10.2
0.83	9.8	9.5	8.5	10.5	10	0.67	6.9	0.21	2.2	9.3	10.3
1.64	8.8	8.5	8.5	9.5	3	0.58	6.5	0.33	3.8	7.4	10.3

Shapiro-Wilk´s Test on Normal Distribution

Normality check was passed (p > 0.01).

Levene´s Test on Variance Homogeneity (with Residuals)

Variance homogeneity check was passed (p > 0.01).

Normal-distribution and variance-homogeneity requirements are fulfilled.
A parametric multiple test is advisable. To justify the use of Williams test at first a trend analysis by contrasts is performed.

Trend analysis by Contrasts (Monotonicity of Concentration/Response)

The linear trend is not significant (p > 0.05), thus the selected Williams test was replaced by Dunnett test.

Dunnett`s Multiple t-test Procedure

Dunnett´s multiple t-test procedure with age of first reproduction at 21 d: Comparison of treatments with "Control". Significance was Alpha = 0.050, one-sided greater (multiple level); Mean: arithmetic mean; n: sample size; s: standard deviation; MDD: minimum detectable difference to Control (in percent of Control); t: sample t; t*: critical t for Ho: µ1 = µ2 = ... = µk; the differences are significant in case t > t* (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [mg TI/L]	Mean	s	df	%MDD	t	t*	Sign.
Control	9.7	0.877					
0.26	9.0	0.877	36	9.3	-1.79	2.24	-
0.47	9.4	0.877	36	9.5	-0.81	2.24	-
0.83	9.8	0.877	36	9.3	0.19	2.24	-
1.64	8.8	0.877	36	13.5	-1.52	2.24	-

+: significant; -: non-significant

The NOEC appears to be higher than or equal 1.64 mg TI/L.

A 2.6 Intrinsic rate of increase in Daphnia magna as Dependent on Concentration and Time

Intrinsic rate of increase in Daphnia magna as Dependent on Concentration and Time

Intrinsic rate of increase in Daphnia magna as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation (calculated from InputRawData)

Treatm. [mg TI/L]	**Control**	**0.26**	**0.47**	**0.83**	**1.64**	**2.98**
21 d	0.338	0.357	0.295	0.354		
	0.308	0.361	0.342	0.329		
	0.305	0.336		0.345		
		0.356	0.252	0.301		
	0.226	0.365	0.326	0.282	0.211	
	0.333	0.345	0.340	0.341		
	0.266		0.252	0.268	0.211	
	0.310	0.305	0.386	0.334		
	0.396	0.346	0.222	0.313		
	0.252	0.311	0.352	0.367		
IR r:	0.304	0.342	0.308	0.323	0.211	-
Std.Dev.:	0.0508	0.0217	0.0554	0.0319	0.0000	-
n:	9	9	9	10	2	0
CV:	16.7	6.3	18.0	9.9	0.0	

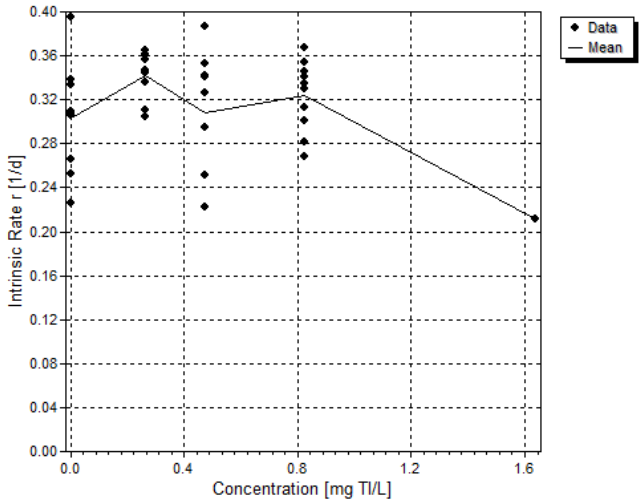


Figure 9: Intrinsic rate of *Daphnia magna* as observed under presence of the test item.

Effective Concentrations (ECx) for Intrinsic Rate r at 21 d

Results of the probit analysis

Results of the probit analysis: Selected effective concentrations (ECx) of the test item and their 95%-confidence limits

Toxicity Metric	EC10	EC20	EC50
Value [mg TI/L]	1.336	1.501	1.878
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

n.d.: not determined due to mathematical reasons

Slope function after Litchfield and Wilcoxon: 1.305 Inhibitions lower equal 0% or greater equal 100.0% were replaced by 0.100 and 99.9%, respectively.Computation was adjusted to metric data (Christensen & Nyholm 1984); variance and thus confidence limits were corrected by covariance with the control (Draper & Smith 1981)

(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

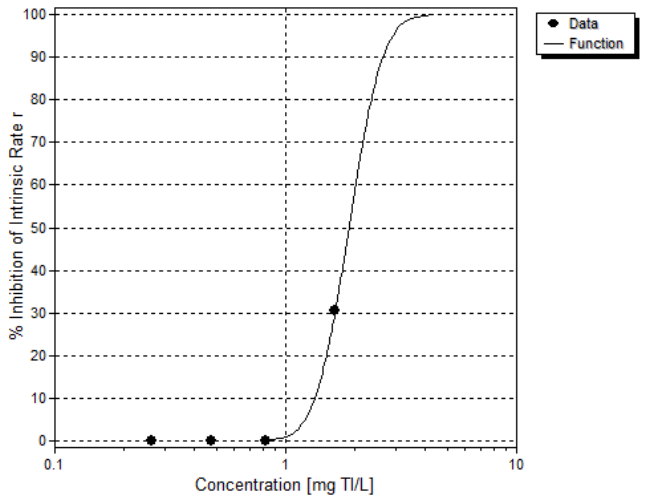


Figure 10: Concentration-effect curve showing the influence of the test item on intrinsic rate r of the introduced *Daphnia magna* as observed after 21 d.

Threshold Concentrations (NOEC) with Intrinsic rate of increase

Statistical characteristics of the sample

Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [mg TI/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	0.304	0.308	0.226	0.396	9	0.0508	16.7	0.0169	5.6	0.265	0.343
0.26	0.342	0.346	0.305	0.365	9	0.0217	6.3	0.0072	2.1	0.326	0.359
0.47	0.308	0.326	0.222	0.386	9	0.0554	18.0	0.0185	6.0	0.265	0.350
0.83	0.323	0.332	0.268	0.367	10	0.0319	9.9	0.0101	3.1	0.301	0.346
1.64	0.211	0.211	0.211	0.211	2	0.0000	0.0	0.0000	0.0	0.211	0.211

Shapiro-Wilk´s Test on Normal Distribution
Normality check was passed (p > 0.01).

Levene´s Test on Variance Homogeneity (with Residuals)
Variance homogeneity check was passed (p > 0.01).

Normal-distribution and variance-homogeneity requirements are fulfilled.
A parametric multiple test is advisable. To justify the use of Williams test at first a trend analysis by contrasts is performed.

Trend analysis by Contrasts (Monotonicity of Concentration/Response)
The linear trend is significant (p ≤ 0.05), thus the selected Williams test was performed.

Williams Multiple Sequential t-test Procedure
Comparison of treatments with "Control" by the t test procedure after Williams with intrinsic rate r at 21 d: Significance was Alpha = 0.050, one-sided smaller; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; MDD: minimum detectable difference to Control (in percent of Control); t: sample t; 't*: critical t for Ho: µ1 = µ2 = ... = µk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments). Note that the step-down test terminates after the first non-significant treatment is encountered

Treatm. [mg TI/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
Control	0.304	0.04133						
0.26	0.342	0.04133	34	0.342	-10.8	1.98	-1.69	-
0.47	0.308	0.04133	34	0.316	-11.3	0.62	-1.77	-
0.83	0.323	0.04133	34	0.316	-11.2	0.64	-1.80	-
1.64	0.211	0.04133	34	0.211	-18.8	-2.86	-1.77	+

+: significant; -: non-significant

A NOEC of 0.83 mg TI/L is suggested by the program.

A 2.7 Length at 21 d

Length of *Daphnia magna as* Dependent on Concentration and Time

Length of Daphnia magna as dependent on concentration of the test item and time; Mean: arithmetic mean; Std.Dev.: standard deviation; n: number of replicates; CV: coefficient of variation

Treatm. [mg TI/L]	**Control**	**0.263**	**0.474**	**0.825**	**1.639**	**2.983**
21 d	5.05	5.28	4.84	-	-	-
	5.06	4.96	4.72	4.96	-	-
	4.93	5.59	-	4.97	-	-
	-	5.23	5.09	5.39	-	-
	5.46	5.12	5.09	5.08	4.21	-
	4.97	4.98	5.30	5.25	-	-
	5.06	-	5.06	4.77	4.93	-
	5.21	5.17	2.91	5.10	-	-
	4.67	4.96	5.21	4.96	-	-
	4.84	5.11	4.52	5.53	-	-
Mean:	5.03	5.16	4.75	5.11	4.57	-
Std.Dev.:	0.223	0.200	0.732	0.239	0.509	-
n:	9	9	9	9	2	0
CV:	4.4	3.9	15.4	4.7	11.1	

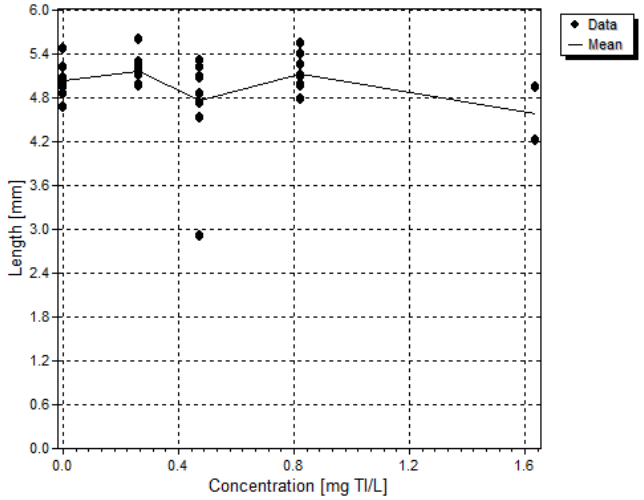


Figure 11: Length of *Daphnia magna* as observed under presence of the test item after 21 d.

Effective Concentrations (ECx) with Length in *Daphnia magna* after 21 d.

Length in *Daphnia magna* after 21 d.

%Inhibition of length caused by the test item after 21 d.

Treatm.[mg TI/L]	Mean	Std. Dev.	n	%Decrease
Control	5.03	0.223	9	
0.26	5.16	0.200	9	-2.5
0.47	4.75	0.732	9	5.5
0.83	5.11	0.239	9	-1.7
1.64	4.57	0.509	2	9.1

Results of the probit analysis

Results of the probit analysis: Selected effective concentrations (ECx) of the test item and their 95%-confidence limits (according to Fieller`s theorem).

Toxicity Metric	EC10	EC20	EC50
Value [mg TI/L]	1.915	> 1.64	n.d.
lower 95%-cl	n.d.	n.d.	n.d.
upper 95%-cl	n.d.	n.d.	n.d.

n.d.: not determined due to mathematical reasons or inappropriate data

Slope function after Litchfield and Wilcoxon: 4.842 Inhibitions lower equal 0% or greater equal 100.0% were replaced by 0.100 and 99.9%, respectively.Computation was adjusted to metric data (Christensen & Nyholm 1984); variance and thus confidence limits were corrected by covariance with the control (Draper & Smith 1981).
The probability p(F) is greater than 0.05; i.e. the slope was not significantly different from zero. The shown toxic metrics could be meaningless.
(The slope function is derived from the slope, b, of the linearized probit function and computes as S = 10^(1/b); please note that small values refer to a steep concentration/response relation and large ones to a flat relation.)

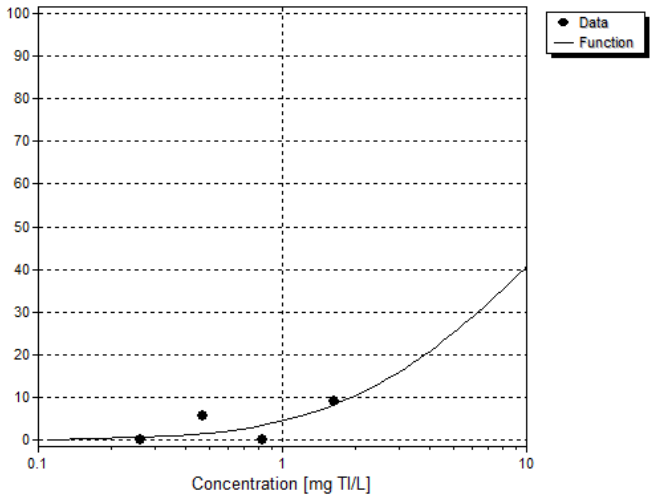


Figure 12: Concentration-effect curve showing the influence of the test item on length of the introduced *Daphnia magna* as observed after 21 d.

Threshold Concentrations (NOEC) with Length at 21 d
Statistical characteristics of the sample

Statistical characteristics: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [mg TI/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	5.03	5.05	4.67	5.46	9	0.223	4.4	0.074	1.5	4.86	5.20
0.26	5.16	5.12	4.96	5.59	9	0.200	3.9	0.067	1.3	5.00	5.31
0.47	4.75	5.06	2.91	5.30	9	0.732	15.4	0.244	5.1	4.19	5.31
0.83	5.11	5.08	4.77	5.53	9	0.239	4.7	0.080	1.6	4.93	5.30
1.64	4.57	4.57	4.21	4.93	2	0.509	11.1	0.360	7.9	0.00	9.14

Shapiro-Wilk´s Test on Normal Distribution

Normality check failed ($p \leq 0.01$).

Levene´s Test on Variance Homogeneity (with Residuals)

Variance homogeneity check was passed ($p > 0.01$)

Normal distribution is poore, but variance homogeneity requirements may be seen as fulfilled. A parametric multiple test is yet possible. To justify the use of the Step-down Jonckheere-Terpstra test at first a non-parametric trend analysis by contrasts is performed.

Trend analysis by Contrasts (Monotonicity of Concentration/Response)

The linear trend is not significant ($p > 0.05$), thus the selected SD Jonckheere-Terpstra test was replaced by the Bonferroni U test.

Multiple sequentially rejective U-test after Bonferroni-Holm

Multiple sequentially rejective U-test between treatments and control, each (alpha is 0,05; one-sided greater); Mean: arithmetic mean, n: sample size; RsC: sum of ranks in the reference treatment; RsT: sum of ranks in the treatment; U: test statistic; p(U): probability of the test statistic; Alpha(i): adjusted significance level; Ho is accepted, if p(U) > Alpha(i).

Treatm. [mg TI/L]	Mean	n	RsC	RsT	U	p(U)	Alpha(i)	Sign.
Control	5.03	9						
0.263	5.16	9	70.0	101.0	25.0	0.911	-	
0.474	4.75	9	88.0	83.0	38.0	0.412	-	
0.825	5.11	9	76.5	94.5	31.5	0.777	-	
1.639	4.57	2	60.5	5.5	2.5	0.091	-	

+: significant; -: non-significant

There is no statistically significant difference between Control and any treatment. The NOEC appears to be higher than 1.64 mg/L.

A 3 Annex 3: Chemical analyses

A 3.1 Preface and Scope

The purpose of the analytical part of the study was to develop quantitative residue analytical methods for the determination of the test item 1-Oxa-4,5-dithiepane in water.
The analytical methods for the determination of 1-Oxa-4,5-dithiepane in water using GC-MS were developed in experiments described here.
The LOQ was set at 0.1 mg/L. The quantitative measurements were carried out by gas chromatography (GC) coupled to a triple quadrupole mass spectrometer (MS) using electrospray ionization positive (ESI); the mass spectrometer (MS) was operated in the single ion monitoring mode (SIM).1,4-Oxathian was used as internal standard.

A 3.2 Chemicals, reagents and analytical equipment

- Analytical standard (= test item), 1-Oxa-4,5-dithiepane. Purity > 99.8 %, Batch/Lot-No EN1502b (see CoA of the test item)
- Internal standard, 1,4-Oxathian Purity 98% (Sigma Aldrich)
- Cyclohexane (JT. Baker)
- Aceton, min. 99.95 % (ChemSolute)
- Copper free water (from Fraunhofer institute IME)
- GC-MS Agilent inert 5793
- Balance Mettler AT 201
  Mettler PM 2000
- Pipet Microman 0-25 µL
- Pipet Microman 0-50 µL
- Pipet Microman 0-250 µL
- Pipet Gilson 0-1000 µL
- Pipet Gilson 0-5000 µL

A 3.3 GC measurement (water analysis)

Details of instrumental analysis

GC-MS-System:	GC 6890N with MSD 5973 inert (Agilent)
Autosampler:	MPS 2 with 10µL-liquid injection unit (Gerstel)
Column:	Rtx-1701; 30 m, 0.25 mm ID, 0.5 µm film (Agilent)
Oven:	1.0 min 50°C, then 15°C/min -> 140°C, then 25°C/min 230°C hold for 2 min
Carrier gas:	Helium, constant flow, 1.0 mL/min

Inlet:	Splitless Inlet -> 250°C
Acquisition mode:	SIM-Mode
SIM masses:	1-Oxathian
	(internal Standard; Target ion m/z 46.0)
	1-Oxa-4,5-dithiepane
	(analyte; Target ion m/z 136.0)
MS source:	250°C
MS Quadrupol:	150°C
Solvent delay:	4 min
Run time:	12.6 min

A 3.4 Sample preparation

A 3.4.1 Water samples

Samples with nominal concentrations of 0.29 mg/L, 0.51 mg/L, 0.93 mg/L, 1.67 mg/L and 3.00 mg/L were analysed for their true concentrations. 20 µL of the internal standard spike solution were added to the samples before analysis via GC-MS.

A 3.5 Calibration, Quantification and Calculation of the analytical results

A 3.5.1 Preparation of stock solutions of the analyte and internal standard

The stock solution of the test item was prepared by weighing 19.85 mg of the test item (purity = 99.8 %) directly into a 10 mL volumetric flask and by subsequently filling it up to the ring mark with acetone. Therefore the analyte concentration of the stock solution was 1.981 g/L. The stock solution of the internal standard was prepared by weighing 17.7 mg of the internal standard (purity= 98 %) directly into a 10 mL volumetric flask and subsequently filling it up to the ring mark with acetone. Therefore the internal standard concentration of the stock solution was 1.735 mg/L. The prepared stock solutions were stored in a refrigerator at 4 °C.

A 3.5.2 Calibration of the GC-MS system

Preparation of the calibration solutions (water analysis)

Seven ‘calibration standards', including a zero value with just the internal standard, were produced in the concentration range from 0.05 to 1.24 mg/L by diluting the stock solutions with Acetone in volumetric flasks (Microman pipettes were used for this dilution step).

For preparation of the ‘calibration samples' 4 mL of copper free water were pipetted into a 8 mL vial and 2 mL of Cyclohexane added. This solution was spiked with 20 µL of the respective spike solution. The prepared solution was shook vigorously for 2 min (Vortex). 1 mL of the organic phase was transferred to a GC micro vial and measured via GC-MS.

Creating the calibration functions

The GC-MS system was calibrated by measuring the prepared matrix calibration samples. The calibration function was created afterwards by processing (integration) the chromatographic raw data and by plotting the response of the detector against the injected analyte concentrations. With the received data quadratic regression calculations were performed.

A 3.5.3 Quantification and calculation of the analytical results

The quantification data was generated by processing the chromatographic raw data of the measured samples and by subsequent calculation of the quantification results using the respective basic calibration function.

A 3.5.4 Preparation of the LOQ and 10xLOQ samples

4 mL of copper free water were spiked with 20 µL of the respective spike solution (LOQ: 0.1 mg/L, 10 x LOQ: 1.0 mg/L). 2 mL of Cyclohexane were added and the solution shaken vigorously (Vortex) for 2min. 1 mL of the organic phase was transferred to a GC vial and measured via GC-MS.

A 3.5.5 Quality control standards

Two quality control standards were prepared and analyzed additionally to the calibration standards to insure the validity of the measurements.

A 3.6 Analytical results

A 3.6.1 Water analysis

Calibration

The respective calibration function for the analyte 1-Oxa-4,5-dithiepane was determined by processing (integration) the chromatographic raw data and by plotting the response of the detector against the analyte concentration. With the received data quadratic regression calculations were performed. The function was calculated with the Mass Hunter quantification software using quadratic regression model.

Calibration function: $y = 0.087732x^2 + 0.941277x - 0.014892$

All derived coefficients of determination were $r^2 > 0.999$

Linearity/quadratic curve fit

Using the quadratic regression model the coefficient of determination for 1-Oxa-4,5-dithiepane was calculated to be greater than 0.999. As the calculated r^2-value was close to 1, the quadratic curve fit of the calibration function ($0.087732x^2 + 0.941277x - 0.014892$) was accepted.

Validation

For the validation of the method according to SANCO 3029/99 [6] two stock solutions with concentrations of 0.200 mg/L and 0.798 mg/L were prepared. Five samples with the concentration of the LOQ and five samples with a concentration of ten times the LOQ (0.10 and 1.00 mg/L) were prepared by spiking 4 mL of copper free water with 20 µL of the standards in Cyclohexane, adding 2 mL of Cyclohexane, shaking vigorously (Vortex), pipetting 1 mL of the organic phase into GC micro vial and measuring via GC-MS.

Results of the analyzed water samples

Five nominal concentrations of 0.29 mg/L, 0.51 mg/L, 0.93 mg/L, 1.67 mg/L and 3.00 mg/L were applied and one additional test system served as an untreated control. The results of the analysed samples for 1-Oxa-4,5-dithiepane are listed in Table 12 to Table 17. Starting with day nine only the four lower treatment levels were prepared and measured since no test specimens survived.

Table 12: Measured concentration and recovery of 1-Oxa-4,5-dithiepane in fresh media on the 2nd day of the study

Sample	Nominal conc. 1-Oxa-4,5-dithiepane (mg/L water)	Measured conc. 1-Oxa-4,5-dithiepane (mg/L water)	Recovery (%)
Control Day 2, fresh	< LOQ	< LOQ	-
Conc.1 Day 2, fresh	0.29	0.29	101.45
Conc.2 Day 2, fresh	0.51	0.55	107.73
Conc.3 Day 2, fresh	0.93	0.97	103.88
Conc.4 Day 2, fresh	1.67	1.76	105.11
Conc.5 Day 2, fresh	3.00	3.22	107.36

Table 13: Measured concentration and recovery of 1-Oxa-4,5-dithiepane in aged media on the fifth day of the study.

Sample	Mean conc. 1-Oxa-4,5-dithiepane (mg/L water)	Measured conc. 1-Oxa-4,5-dithiepane (mg/L water)	Recovery (%)
Control Day 5, aged	< LOQ	< LOQ	-
Conc.1 Day 5, aged	0.29	0.25	85.03
Conc.2 Day 5, aged	0.51	0.46	90.76
Conc.3 Day 5, aged	0.93	0.78	83.59
Conc.4 Day 5, aged	1.67	1.50	90.07
Conc.5 Day 5, aged	3.00	2.76	91.88

Table 14: Measured concentration and recovery of 1-Oxa-4,5-dithiepane in fresh media on the 9th day of the study

Sample	Mean conc. 1-Oxa-4,5-dithiepane (mg/L water)	Final conc. 1-Oxa-4,5-dithiepane (mg/L water)	Recovery (%)
Control Day 9, fresh	< LOQ	< LOQ	-
Conc.1 Day 9, fresh	0.29	0.26	89.41
Conc.2 Day 9, fresh	0.51	0.51	99.76
Conc.3 Day 9, fresh	0.93	0.91	97.81
Conc.4 Day 9, fresh	1.67	1.83	109.65

Table 15: Measured concentration and recovery of 1-Oxa-4,5-dithiepane in aged media on the 12th day of the study

Sample	Mean conc. 1-Oxa-4,5-dithiepane (mg/L water)	Final conc. 1-Oxa-4,5-dithiepane (mg/L water)	Recovery (%)
Control Day 12, aged	< LOQ	< LOQ	-
Conc.1 Day 12, aged	0.29	0.21	71.28
Conc.2 Day 12, aged	0.51	0.40	78.43
Conc.3 Day 12, aged	0.93	0.68	73.26
Conc.4 Day 12, aged	1.67	1.61	96.53

Table 16: Measured concentration and recovery of 1-Oxa-4,5-dithiepane in fresh media on the 16th day of the study

Sample	Mean conc. 1-Oxa-4,5-dithiepane (mg/L water)	Final conc. 1-Oxa-4,5-dithiepane (mg/L water)	Recovery (%)
Control Day 16, fresh	< LOQ	< LOQ	-
Conc.1 Day 16, fresh	0.29	0.28	96.62
Conc.2 Day 16, fresh	0.51	0.52	101.65
Conc.3 Day 16, fresh	0.93	0.90	96.51
Conc.4 Day 16, fresh	1.67	1.77	105.83

Table 17: Measured concentration and recovery of 1-Oxa-4,5-dithiepane in aged media on the 19th day of the study

Sample	Mean conc. 1-Oxa-4,5-dithiepane (mg/L water)	Final conc. 1-Oxa-4,5-dithiepane (mg/L water)	Recovery (%)
Control Day 19, old	< LOQ	< LOQ	-
Conc.1 Day 19, old	0.29	0.29	101.24
Conc.2 Day 19, old	0.51	0.42	81.45
Conc.3 Day 19, old	0.93	0.74	79.85
Conc.4 Day 19, old	1.67	1.39	83.20

A 3.7 Representative GC-MS chromatograms

Representative chromatograms of calibration samples, LOQ and 10 x LOQ samples, a blank, samples taken at the test start and on day 3 (treatments and controls) are shown in Figure Figure 13 to Figure 19; the retention times (tR) for 1-Oxa-4,5-dithiepane was 9.28 min and 5.99 min for the internal standard.

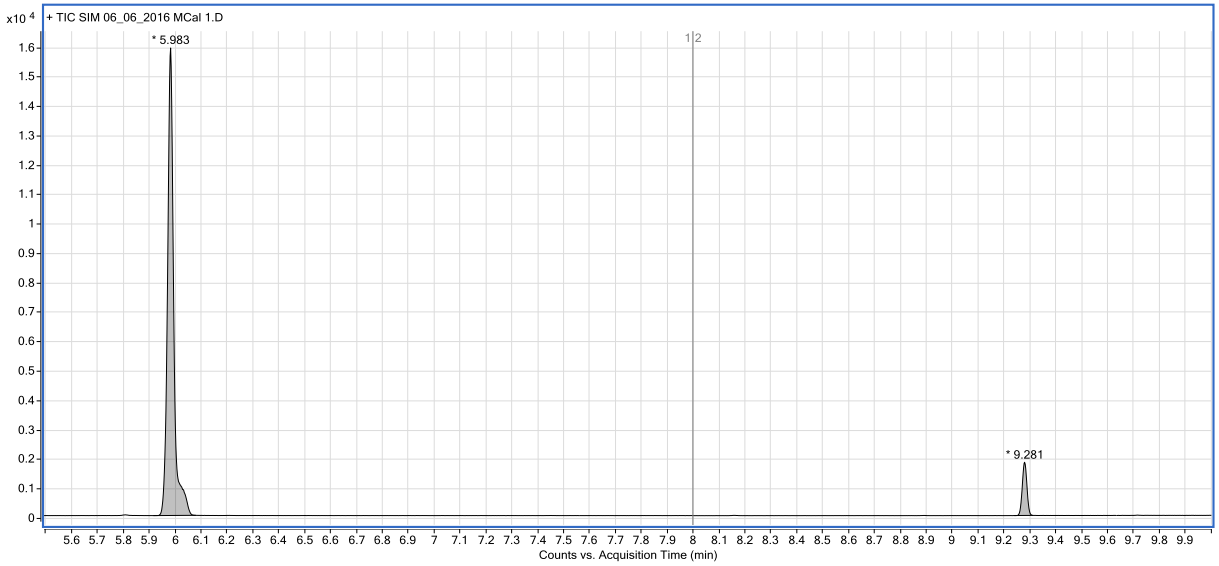


Figure 13: Calibration level 1 (0.05 mg/L) measured June 6, 2016

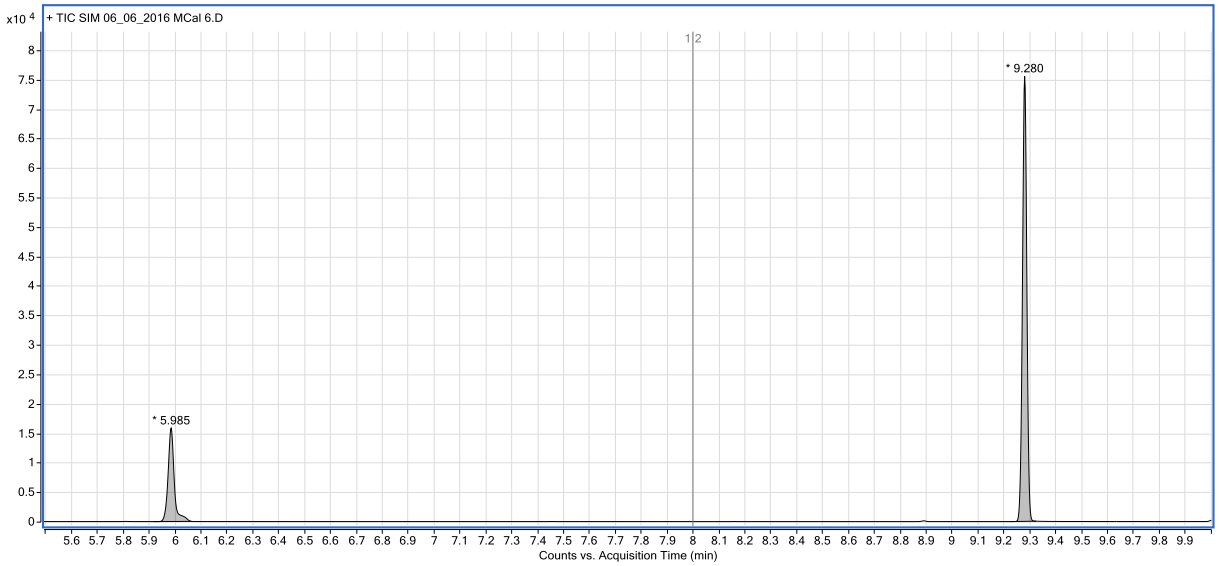


Figure 14: Calibration level 6 (1.238 mg/L) measured June 6, 2016

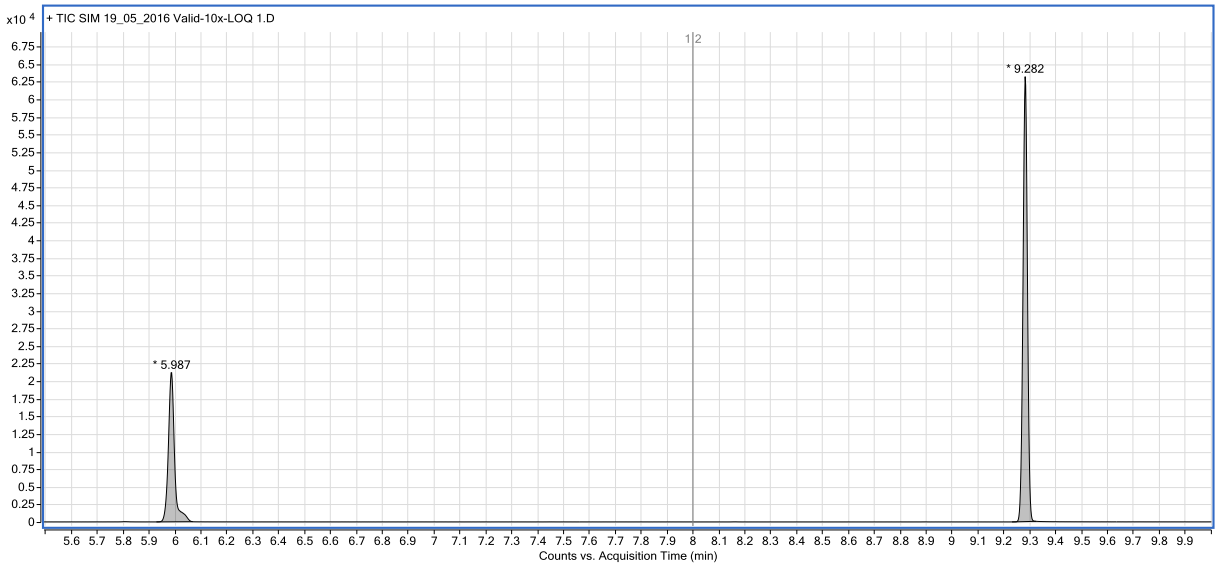


Figure 15: 10 x LOQ sample (0.1 mg/L) measured May 19, 2016

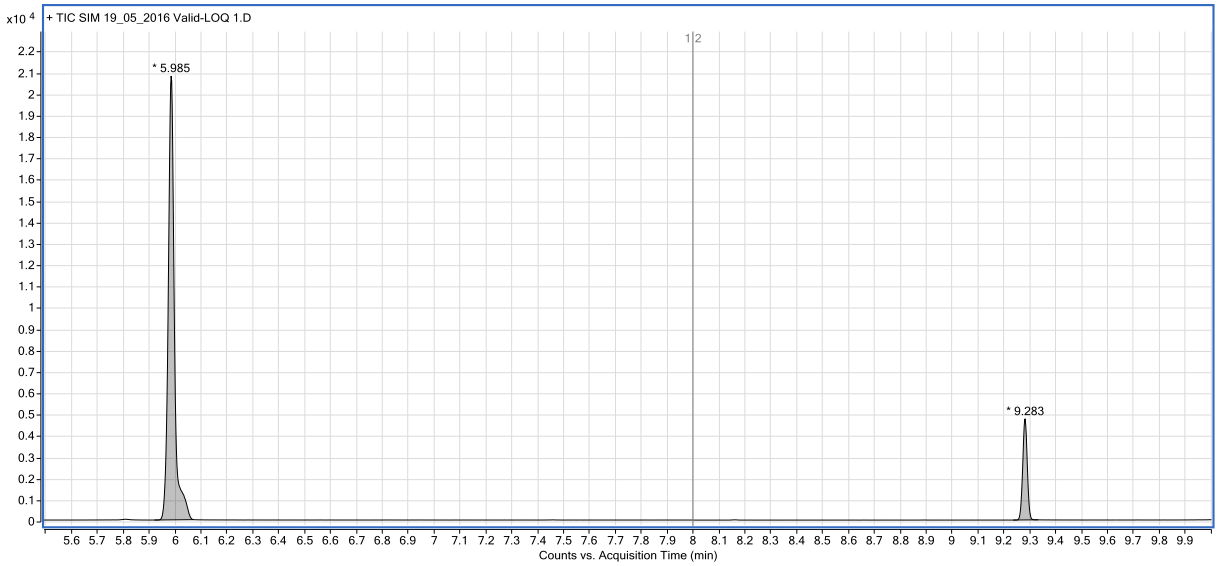


Figure 16: LOQ sample (1.0 mg/L) measured May 19, 2016

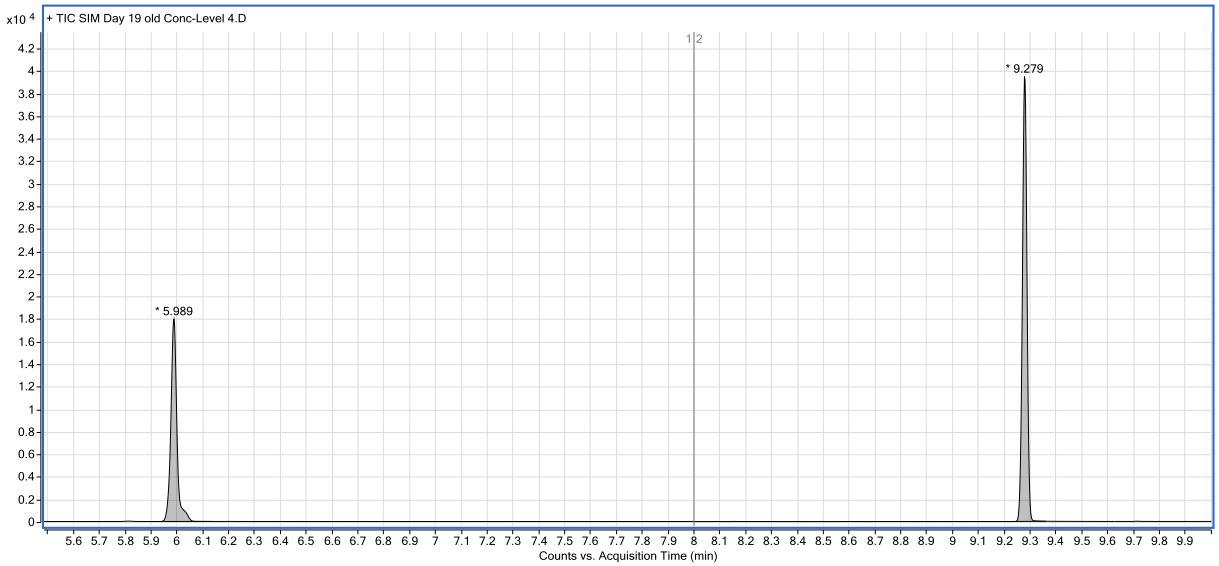


Figure 17: Concentration Level 4 (3.00 mg/L) day 19 measured June 6, 2016

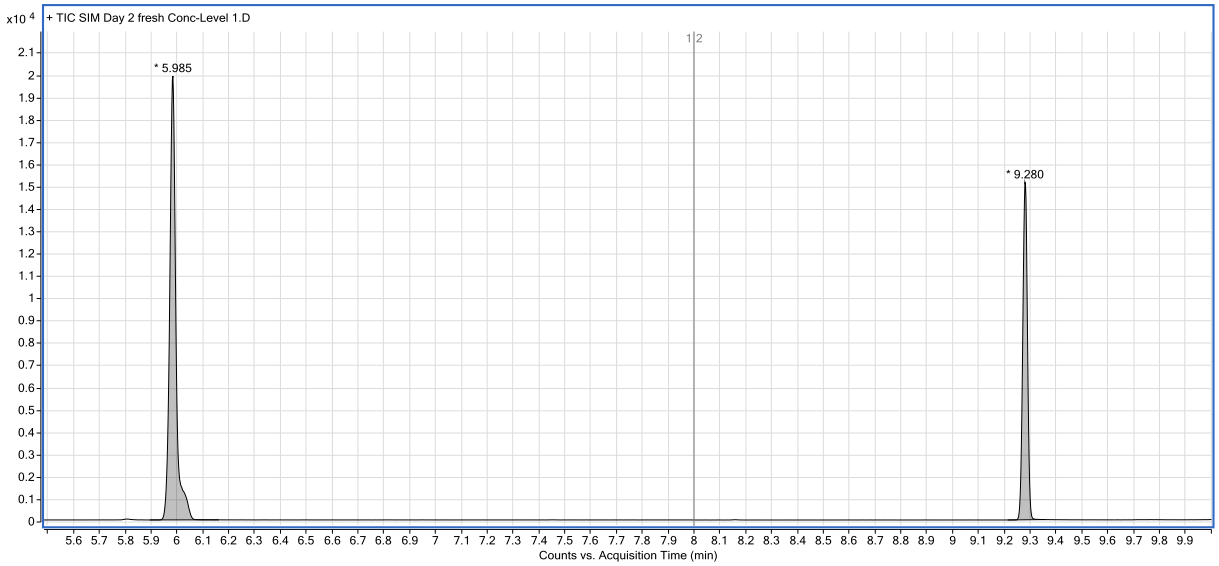


Figure 18: Concentration Level 1 (0.29 mg/L) day 2 measured May 20, 2016

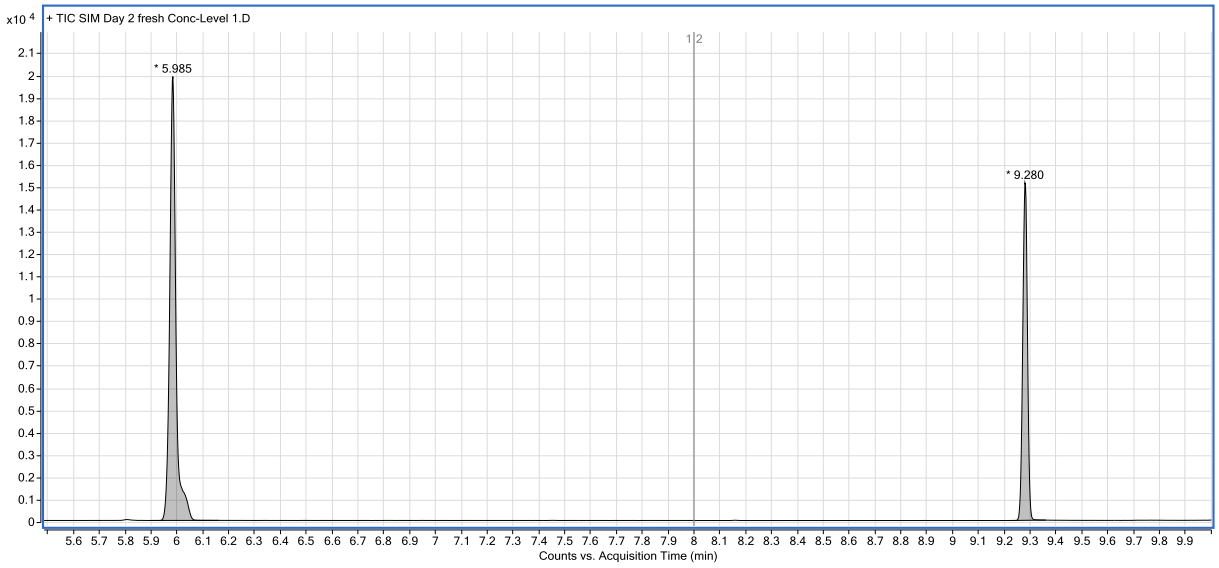


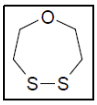
Figure 19: Control Sample day 2 measured May 20, 2016

A 4 Annex 4: Certificate of analyses

Envilytix GmbH

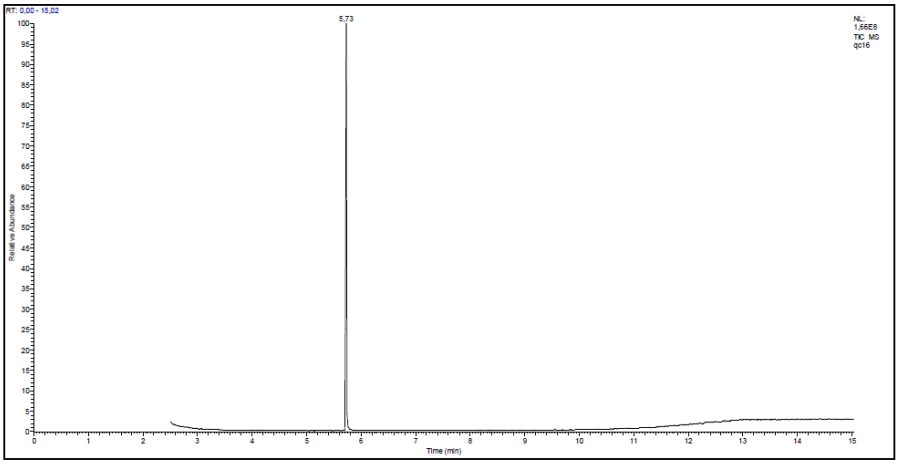
Certificate of analysis

Envilytix GmbH – Rheingaustrasse 190-196 – D-65174 Wiesbaden

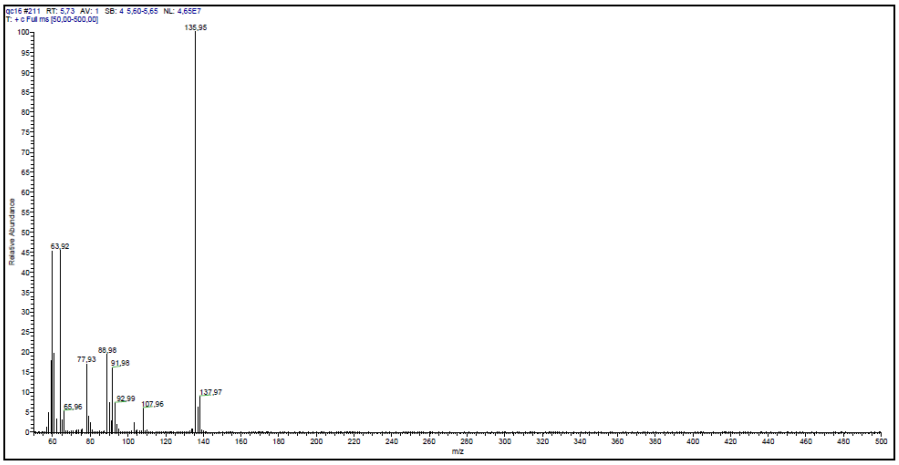


Product:	**1-Oxa-4,5-dithiepane**
CAS-No.:	3886-40-6
Empirical formula:	$C_4H_8OS_2$
Molecular weight:	136,24 g/Mol
Lot-No.:	EN1502b
Storage:	Store at -20°C
Appearance:	Very faint yellow oil
Purity (EI-GC/MS):	99,8%
Identity:	EI-MS conforms to structure

Total ion current chromatogram



Mass spectrum



Wiesbaden, 09.01.2015

PROSPECTIVE ADDED ENVIRONMENTAL RISK ASSESSMENT FROM RE-SUSPENSION OF CHEMICAL WARFARE AGENTS FOLLOWING THE INSTALLATION OF THE NORD STREAM 2 PIPELINES

The report reviews the prospective risk associated the resuspension of chemical warfare agent residues flowing the construction and placement of the Nord Stream 2 gas pipeline in the Baltic Sea.

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