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VALIDATION REPORT FOR THE ACUTE CHIRONOMID ASSAY

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IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

FOREWORD

This document presents the ring test report supporting the Test Guideline for the *Chironomus* SP. Acute Immobilisation Test. The ring test was performed in 2010-2011. The validation report was submitted by Germany. It was endorsed by the Working Group of National Coordinators of the Test Guidelines Programme at its meeting held on 12-14 April 2011. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (Joint Meeting) agreed to its declassification on 5 August 2011.

This document is published under the responsibility of the Joint Meeting.

VALIDATION REPORT SUPPORTING THE DRAFT TEST GUIDELINE FOR THE *CHIRONOMUS* SP., ACUTE IMMOBILISATION TEST

Lennart Weltje

BASF SE, Crop Protection – Ecotoxicology
Speyerer-Strasse 2
D-67117 Limburgerhof
Germany

March 2nd, 2011

INTRODUCTION

At the end of 2010 and beginning of 2011 a ring test with 14 participating laboratories was conducted on basis of the draft OECD TG "*Chironomus* sp., Acute Immobilisation Test" version of October 8, 2010. Participating laboratories came from The Netherlands, Germany, Switzerland, France and the UK.

Participating laboratories were provided with the draft Test Guideline, an Excel sheet to enter their raw data in and an approximate value for the 48-h EC50 of the two reference substances to guide their choice of test concentrations.

The selected test substances were potassium chloride (KCl) as an inorganic chemical and 3,5-dichlorophenol (3,5-DCP) as an organic chemical. Both chemicals have been used previously as reference toxicants.

In addition, laboratories were requested to provide analytical measurements of at least the lowest and highest test concentration of 3,5-DCP. Information on the analytical method used during the validation exercise of the ring test copepod reproduction test was provided, but laboratories were free to choose any available in-house method to quantify 3,5-DCP in water.
Laboratories used larvae from their own in-house chironomid cultures, their own test medium (culture water) and their own test chemicals. Therefore, the ring test described in this report is a real robust test of the protocol and not optimized to produce identical values by standardizing the organism strain, the analytical method, a single batch of chemicals etc.

Calculations

All data were entered into the software program ToxRat 2.10 (ToxRat Solutions GmbH, Naheweg 15, D-52477 Alsdorf, Germany. www.toxrat-solutions.de) and subjected to probit analysis to determine the EC50 values after 24 and 48 h. In case of control mortality Abbott's correction was performed. The NOEC was calculated with Fisher's exact test with Bonferroni correction and alpha = 0.05.

The 24-h and 48-h EC50 values were subjected to an outlier test according to Grubbs (significance level 0.05) by using the GraphPad online calculator (www.graphpad.com/quickcalcs/Grubbs1.cfm).

RESULTS

Table 1: Oxygen, pH and temperature data for the tests on 3,5-DCP

Lab No.	Physical-chemical parameters					
	pH		oxygen [mg/L]		temperature [°C]	
	t0	t48	t0	t48	t0	t48
1	7.9 - 8.0	8.0 - 8.1	8.3	8.3 - 8.4	20.5 - 20.9	20.4 - 20.7
2	7.7	8.1	8.4	9.02	19.8	204
3	7.7 - 7.8	7.9	9.1 - 9.2	8.9 – 9.1	18.6 - 18.9	19.5 - 20.0
4-1	7.7	7.9	8.3	8.6	21	--
4-2	7.7	7.9	8.3	8.6	21	--
5	7.8 - 8.0	8.2 - 8.3	9.1	9.0 - 9.3	20.0 - 20.6	18.5 - 19.0
6	7.78	7.99	8.5	9.1	21	21
7	7.2 - 8.2		8.7 - 8.9		19.9 - 21.4	
8	7.47 - 7.54	8.09 - 8.25	8.67 - 8.77	8.75 - 8.90	21.6 - 21.7	21.6 - 21.9
9	7.55 - 7.79	7.96 - 8.01	8.71 - 9.91	8.58 - 9.05	20.7 - 21.9	20.3 - 21.7
10	7.6 - 7.7	7.9	9.0 - 9.1	8.9 - 9.0	19.9 - 20.8	
11	7.69 - 7.80	7.89 - 7.98	8.23 - 8.44	7.88 - 8.19	21.1 - 21.2	19.6 - 20.2
12	7.8 - 7.9	8.2 - 8.3	8.3 - 8.4	#	20.1 - 20.3	18.4 - 19.0
13*						
14	7.88 - 7.98	7.92 - 7.99	8.6 - 8.7	8.8 - 8.9	21.2 - 21.3	20.3 - 20.4

* no test with 3,5-DCP was conducted

no measurement due to probe malfunction

Table 2: pH and temperature data for the tests on KCl

Lab No.	Physical-chemical parameters					
	pH		oxygen [mg/L]		temperature [°C]	
	t0	t48	t0	t48	t0	t48
1	7.9	7.9 - 8.0	8.3	8.3	20.5 - 20.9	20.6
2	7.7	8.1	8.8	9.1	19.7	20.5
3	7.7	7.8 - 7.9	9.3 - 9.4	8.6 - 9.2	18.2 - 18.6	19.8 - 20.1
4	7.6	7.8	8.5	8.7	20.2	20.6
5	7.9 - 8.0	8.2 - 8.3	9.0 - 9.1	8.8 - 8.84	20.0 - 20.2	19.0 - 19.2[1]
6	7.3	8.2	8.5	8.84	19.0	21.0
7-1	7.7 - 8.3	7.2 - 7.3	8.3 -8.4	8.2 -8.4	20 ± 2	
7-2	7.1 - 8.3	7.1 - 7.6	8.3 - 8.4	8.0 - 8.2	21 ± 2	
7-3	7.1 - 8.3	6.8 - 7.0	7.2 - 8.2	8.7 - 8.8	20.4 - 21.3	
8	7.61 - 8.14	7.90 - 8.19	8.46 - 8.88	8.47 - 8.88	21.4 - 21.9	20.9 - 21.8
9	7.46 - 7.58	8.01 - 8.04	8.73 - 9.88	8.42 - 8.65	21.4 - 21.9	21.3 - 21.9
10	7.5 - 7.7	7.8 - 8.1	9.0 - 9.3	8.9 - 9.0	19.9 - 21.0	
11	7.72 - 7.76	7.82 - 7.92	8.10 - 8.33	7.62 - 8.16	21.2 - 21.4	19.7 - 20.1
12	7.9	8.1 - 8.2	7.5	8.0 - 8.2	19.5	19.5
13	8.22 - 8.51	8.02 - 8.47	8.7 - 9.1	6.9 - 7.9	19.3 - 20.4	19.8 - 20.3

[1] original data mentioned 9.2°C but that was an obvious typo and corrected to 19.2°C

Table 3: Analytical measurements of 3,5-DCP

Lab No.	RECOVERY [%]			
	absolute minimum	absolute maximum	mean	sd
1	98,6	104,0	102,0	2,4
2	86,0	117,4	98,8	7,1
3	84,8	109,3	92,2	11,5
4-1	150,5	171,8	161,1[1]	9,8
4-2	92,7	105,7	100,3	5,5
5	85,0	122,1	102,3	9,5
6	97,0	128,0	114,0	12,7
7	81,6	108,6	97,6	10,1
8#	--	--	--	--
9	93,0	107,0	99,5	5,7
10	88,5	104,0	97,5	4,4
11	79,2	90,0	85,6	4,5
12#	--	--	--	--
13*				
14	100,3	107,2	103,0	2,0

* no test with 3,5-DCP was conducted

test conducted without analytical measurements [1] value used to correct the EC50

Table 4: 24-h NOEC and EC50 with 95% confidence interval for 3,5-DCP

Lab No.	EC50 [mg/L]	upper 95%-cl	lower 95%-cl	NOEC [mg/L]
1[2]	3,256	n.d.	n.d.	2,07
2	2,299	n.d.	n.d.	2
3	2,711	n.d.	n.d.	1,25
4-1#	0,77	1,079	0,478	< 0,419
4-2	0,863	1,264	0,55	0,26
5	1,98	2,665	1,484	0,34
6	2,198	n.d.	n.d.	0,91
7	2,31	2,609	2,043	1,34
8	5,739	n.d.	n.d.	4
9	2,196	2,664	1,817	1
10	4,424	n.d.	n.d.	2,2
11	1,37	n.d.	n.d.	1,25[3]
12	2,299[1]	n.d.	n.d.	2[1]
13*				
14	3,685	4,932	2,985	2,4

* no test with 3,5-DCP was conducted

results corrected for 161% recovery

[1] test not valid (control immobility 0% after 24 and 65% after 48 h)

[2] 10 instead of 5 larvae per replicate

[3] significant effect at the lowest concentration of 0.165 mg/L, but no significant effects up to and incl. 1.25 mg/L

Table 5: 48-h NOEC and EC50 with 95% confidence interval for 3,5-DCP

Lab No.	EC50 [mg/L]	upper 95%-cl	lower 95%-cl	NOEC [mg/L]
1[2]	1,63	1,932	1,374	0,43
2	2,045	n.d.	n.d.	2
3	1,673	1,885	1,489	1,25
4-1#	0,578	0,823	0,320	< 0,419
4-2	0,517	0,723	0,326	0,26
5	0,627	0,797	0,478	0,34
6	0,925	2,735	0,254	0,91
7	1,77	n.d.	n.d.	1,34
8	3,857	n.d.	n.d.	1
9	1,794	2,134	1,507	2
10	3,128	3,53	2,779	2,2
11	0,935	n.d.	n.d.	1,25[3]
12	2,054[1]	n.d.	n.d.	2[1]
13*				
14	2,253	15,849	0,61	1,2

* no test with 3,5-DCP was conducted

results corrected for 161% recovery

[1] test not valid (control immobility 0% after 24 and 65% after 48 h)

[2] 10 instead of 5 larvae per replicate

[3] significant effect at the lowest concentration of 0.165 mg/L, but no significant effects up to and incl. 1.25 mg/L

Table 6: Outlier test after Grubbs on 48-h EC50 values for 3,5-DCP (the same results were obtained with 24-h EC50 values)

Descriptive Statistics

Mean:	1.69900
SD:	0.97100
# of values:	14
Outlier detected?	No
Significance level:	0.05 (two-sided)
Critical value of Z:	2.50732144944

Result outlier test

Lab No.	EC50	Z	Significant Outlier?
1	1.630	0.07106	
2	2.045	0.35633	
3	1.673	0.02678	
4-1	0.578	1.15448	
4-2	0.517	1.21730	
5	0.627	1.10402	
6	0.925	0.79712	
7	1.770	0.07312	
8	3.857	2.22246	Furthest from the rest, but no significant outlier (P > 0.05).
9	1.794	0.09784	
10	3.128	1.47168	
11	0.935	0.78682	
12	2.054[1]	0.36560	
14	2.253	0.57055	

[1] test not valid (control immobility 0% after 24 and 65% after 48 h)

Table 7: 24-h NOEC and EC50 with 95% confidence interval for KCl

Lab No.	EC50 [g/L]	upper 95%-cl	lower 95%-cl	NOEC [g/L]
1#	2,16	3,734	1,251	0,43
2	12,196*	106,215	5,031	2,2
3	3,149	n.d.	n.d.	0,625
4	4,934	n.d.	n.d.	0,26
5	1,529	n.d.	n.d.	0,47
6	2,816	n.d.	n.d.	0,68
7-1	4,107	4,676	3,608	0,878
7-2	3,802	4,278	3,416	4
7-3	2,554	3,55	2,107	2,14
8	5,938	n.d.	n.d.	3
9	2,842	3,487	2,362	2
10	2,209	5,182	0,961	2,2
11	1,548	6,699	0,476	0,63
12	3,511	n.d.	n.d.	3,2
13	3,204	4,57	2,548	2
14	2	n.d.	n.d.	1

* significant outlier

10 instead of 5 larvae per replicate

Table 8: 48-h NOEC and EC50 with 95% confidence interval for KCl

Lab No.	EC50 [g/L]	upper 95%-cl	lower 95%-cl	NOEC [g/L]
1#	1,099	1,279	0,943	0,43
2	4,664*	10,966	2,737	2,2
3	1,116	1,396	0,891	0,625
4	2,026	3,651	1,303	0,26
5	0,852	1,095	0,661	0,47
6	0,897	1,173	0,685	0,68
7-1	1,438	1,648	1,24	0,878
7-2	1,452	1,622	1,299	1,185
7-3	1,272	n.d.	n.d.	n.d.
8	1,87	2,314	1,513	0,75
9	2,185	n.d.	n.d.	2
10	1,851	4,096	0,827	1
11	0,33	1,047	n.d.	< 0,16
12	1,554	n.d.	n.d.	0,8
13	1,442	4,468	0,628	1
14	1,165	n.d.	n.d.	1

* significant outlier (see Table 9)

10 instead of 5 larvae per replicate

Table 9: Outlier test after Grubbs on 48-h EC50 values for KCl (the same results were obtained with 24-h EC50 values)

Descriptive Statistics

Mean:	1.57581
SD:	0.95087
# of values:	16
Outlier detected?	Yes
Significance level:	0.05 (two-sided)
Critical value of Z:	2.58567582497

Result outlier test

Lab No.	EC50	Z	Significant Outlier?
1	1.099	0.50145	
2	4.664	3.24776	Significant outlier. P < 0.05
3	1.116	0.48357	
4	2.026	0.47345	
5	0.852	0.76121	
6	0.897	0.71389	
7	1.438	0.14493	
8	1.452	0.13021	
9	1.272	0.31951	
10	1.870	0.30939	
11	2.185	0.64066	
12	1.851	0.28941	
13	0.330	1.31018	
14	1.554	0.02294	
15	1.442	0.14073	
16	1.165	0.43204	

DISCUSSION

Validity criteria

The participating labs had no problems in maintaining the oxygen validity criterion (minimum 3 mg/L) see Tables 1 and 2.

There were however, a few problems with the control immobility. Only one of the submitted data sets was invalid (Table 5), but a few participants communicated that they had submitted only the repeat studies as the first studies were invalid. In any case, based on the data of the ring test and the experience of the participating labs, it seems appropriate to lower the validity criterion from the originally proposed 20% to 15%.

Temperature and pH

The pH values were well within the limits proposed in the draft TG and did not appear to be an issue (see Tables 1 and 2).

The only temperature deviation from the draft TG recommendations to stay within ± 1°C and between 18 and 22°C for each individual test was for KCl in tests 7-1 and 7-2 where the data mentioned 20 ± 2°C and 21 ± 2°C, respectively (Table 2).

Analytical measurements

The chemical analysis of 3,5-DCP in the test medium at $t = 0$ and $t = 48$ h showed a very good reproducibility and an excellent spiking by the participating laboratories, with mean recovery values between 85.6% and 114% of nominal concentrations (Table 3) with one exception (see below). These excellent recovery values were achieved with largely independent methods, since it was explicitely decided not to validate the analytical part of the test protocol and also not to perform the chemical analysis in one central laboratory. Information on an analytical method used in the OECD copepod ring test was provided, but laboratories were free to use this methods or any available in-house method to quantify 3,5-DCP.

Due to the high recoveries close to the target concentrations, the toxicity data were evaluated based on nominal concentration values. Only in one case the mean recovery was 161% of nominal concentrations (test 4-1, Table 5) and it was decided to correct the obtained EC50 for this higher recovery. Thereafter, the results were in excellent agreement with the repeat test (4-2) from that same laboratory.

For KCl no analytical support was required, as it is a soluble and stable compound. As a consequence, a correction of nominal values for analytical recoveries is not possible.

Toxicity results

The mean, minimum and maximum 24-h and 48-h EC50 values, excluding the outlier for KCl (lab 2) and the invalid test with 3,5-DCP (lab 12) are summarized in Table 10.

It should be noted that the invalid 3,5-DCP test produced 24-h and 48-h EC50 values that fitted well within the range and were clearly no outliers (see Table 6), but the EC50 calculation program ToxRat did not take the control response into account. Therefore, these values were excluded from Table 10.

Table 10: Summary results of the obtained EC50 values for 3,5-DCP (in mg/L) and KCl (in g/L)

Test substance (exposure duration)	EC50mean	EC50min	EC50max	SD (n)
3,5-DCP (24 h)	2.600	0.770	5.739	1.401 (13)
3,5-DCP (48 h)	1.672	0.517	3.857	1.005 (13)
KCl (24 h)	3.087	1.529	5.938	1.234 (15)
KCl (48 h)	1.370	0.330	2.185	0.492 (15)

The span of the valid EC50 values (i.e. the EC50max divided by the EC50min) was 7.5 for 3,5-DCP for both 24 and 48 h, and for KCl, 3.9 and 6.6 for 24 and 48 h, respectively. Thus the 24-h EC50 for KCl had the narrowest range. On the other hand, the KCl 48-h EC50 value had the lowest standard deviation with 0.492 (implying there were only a few extreme values).

In determining an appropriate EC50 range for 3,5-DCP and/or KCl for the acute chironomid test it should be borne in mind that for *Daphnia magna* the 24-h EC50 for $K_2Cr_2O_7$ is used to test the sensitivity of the animals periodically. The validity range for $K_2Cr_2O_7$ according to the TG 202 is 0.6 to 2.1 mg/L.

In any case, based on the obtained data in the ring test an EC50 range can be derived and proposed for one or both of the reference compounds for either 24 or 48 h.

Appendix - raw data

1. Studies on potassium-chloride (KCl)

Laboratory 1

number of immobile animals

	24 h replicate *			
treatment	A	B	C	D
water control	0	0	0	0
0.43 g /L	0	0	0	0
0.94 g /L	0	1	0	0
2.07 g /L	6	4	7	8
4.55 g /L	8	9	8	8
10.0 g /L	10	10	10	10

* deviating from all other laboratories 10 larvae were inserted per replicate

number of immobile animals

	48 h replicate *			
treatment	A	B	C	D
water control	0	1	0	0
0.43 g /L	1	0	0	1
0.94 g /L	4	6	3	4
2.07 g /L	9	7	9	10
4.55 g /L	10	10	10	10
10.0 g /L	10	10	10	10

* deviating from all other laboratories 10 larvae were inserted per replicate

Laboratory 2

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.1 g /L	0	0	0	0
0.22 g /L	0	0	0	0
0.46 g /L	2	2	2	0
1.0 g /L	0	1	1	2
2.2 g /L	1	1	1	2
4.6 g /L	2	2	2	2
10.0 g /L	2	3	1	2

	48 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.1 g /L	0	0	0	0
0.22 g /L	0	0	0	0
0.46 g /L	2	2	2	0
1.0 g /L	0	1	1	2
2.2 g /L	1	1	1	2
4.6 g /L	2	2	2	2
10.0 g /L	5	4	3	3

Laboratory 3

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
water control	1	0	0	0
0.313 g /L	0	0	0	1
0.625 g /L	0	0	0	0
1.25 g /L	0	1	0	0
2.5 g /L	0	1	3	1
5.0 g /L	4	5	5	5

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	1	0	0	1
0.313 g /L	0	1	0	1
0.625 g /L	3	0	1	2
1.25 g /L	3	3	3	3
2.5 g /L	5	3	5	5
5.0 g /L	5	5	5	5

Laboratory 4

number of immobile animals

	24 h			
	replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.26 g/L	0	0	1	0
0.56 g/L	0	1	2	1
1.24 g/L	1	1	1	0
2.73 g/L	1	1	0	0
6.0 g/L	3	4	3	5

number of immobile animals

	48 h			
	replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.26 g/L	0	0	1	2
0.56 g/L	1	1	2	1
1.24 g/L	2	1	2	1
2.73 g/L	2	3	2	1
6.0 g/L	4	5	4	5

Laboratory 5

number of immobile animals

	24 h			
	replicate			
treatment	A	B	C	D
Control	0	0	0	0
0.21 g/L	0	0	0	1
0.47 g/L	0	1	0	2
1.03 g/L	0	0	0	1
2.27 g/L	4	3	5	2
5.0 g/L	5	5	5	5

	number of immobile animals			
	48 h			
	replicate			
treatment	A	B	C	D
Control	0	0	0	0
0.21 g/L	0	0	1	0
0.47 g/L	0	1	1	2
1.03 g/L	1	3	2	3
2.27 g/L	5	5	5	5
5.0 g/L	5	5	5	5

Laboratory 6

	number of immobile animals			
	24 h			
	replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.14 g/L	0	0	0	0
0.31 g/L	0	0	0	0
0.68 g/L	0	0	0	0
1.5 g/L	0	0	0	0
3.3 g/L	4	4	5	5
7.3 g/L	5	5	5	5

	number of immobile animals			
	48 h			
	replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.14 g/L	0	1	0	1
0.31 g/L	0	1	1	0
0.68 g/L	2	1	1	0
1.5 g/L	4	3	3	5
3.3 g/L	5	5	5	5
7.3 g/L	5	5	5	5

Laboratory 7-1

number of immobile animals

	24 h			
	replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.878 g/L	0	0	0	0
1.317 g/L	0	0	0	0
1.975 g/L	0	1	0	0
2.963 g/L	1	1	2	0
4.444 g/L	5	0	0	3
6.667 g/L	5	5	5	5
10 g/L	5	5	5	5

number of immobile animals

	48 h			
	replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.878 g/L	0	1	2	0
1.317 g/L	2	2	1	2
1.975 g/L	3	5	3	4
2.963 g/L	5	5	5	5
4.444 g/L	5	5	5	5
6.667 g/L	5	5	5	5
10 g/L	5	5	5	5

Laboratory 7-2

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
water control	1	0	1	1
0.527 g/L	1	0	0	0
0.790 g/L	0	1	0	0
1.185 g/L	1	0	0	1
1.778 g/L	1	0	0	1
2.667 g/L	2	0	0	3
4.0 g/L	3	2	4	2
6.0 g/L	5	5	5	5

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	1	0	1	1
0.527 g/L	1	0	1	1
0.790 g/L	1	1	1	0
1.185 g/L	2	2	0	3
1.778 g/L	5	4	3	4
2.667 g/L	5	5	5	5
4.0 g/L	5	5	5	5
6.0 g/L	5	5	5	5

Laboratory 7-3

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.56 g/L	0	0	0	0
0.78 g/L	0	1	0	0
1.09 g/L	0	1	1	1
1.53 g/L	1	2	0	0
2.14 g/L	1	1	2	0
3.00 g/L	4	3	5	3

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	0	0	0	1
0.56 g/L	2	2	2	1
0.78 g/L	3	1	1	3
1.09 g/L	0	1	3	1
1.53 g/L	3	4	1	1
2.14 g/L	2	4	5	3
3.00 g/L	5	5	5	5

Laboratory 8

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.38 g/L	0	0	0	0
0.75 g/L	0	1	0	0
1.5 g/L	0	0	0	0
3 g/L	0	0	0	0
6 g/L	4	1	4	4

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.38 g/L	0	0	0	0
0.75 g/L	0	0	0	0
1.5 g/L	2	3	4	1
3 g/L	4	4	4	2
6 g/L	5	5	5	5

Laboratory 9

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.25 g/L	0	0	0	0
0.5 g/L	0	0	0	0
1 g/L	0	0	0	0
2 g/L	1	2	1	0
4 g/L	3	5	4	4

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.25 g/L	0	0	0	0
0.5 g/L	0	0	1	0
1 g/L	1	0	0	1
2 g/L	1	2	1	1
4 g/L	4	5	5	5

Laboratory 10

number of immobile animals (loading 5 per vessel)

	24 h replicate			
treatment	A	B	C	D
water control	0	0	0	0 (1)
0.22 g/L	0	0	0	0
0.46 g/L	0	0 (1)	1	0
1.0 g/L	0 (1)	1	1	2
2.2 g/L	0	1	2	2
4.6 g/L	3 (2)	3 (2)	4 (1)	4
10 g/L	5	4 (1)	5	5

( ): Between brackets the number of missing larvae; added to immobile larvae for evaluation.

number of immobile animals (loading 5 per vessel)

	48 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.22 g/L	0	0	0	0
0.46 g/L	0	0	1	1
1.0 g/L	1	0	0	1
2.2 g/L	0	3	3 (1)	2
4.6 g/L	5	5	5	5
10 g/L	4 (1)	5	5	5

( ): Between brackets the number of missing larvae; added to immobile larvae for evaluation.

Laboratory 11

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.16 g/L	0	0	0	0
0.31 g/L	0	1	0	1
0.63 g/L	0	0	0	0
1.25 g/L	4	1	1	0
2.50 g/L	4	5	2	4
5.00 g/L	5	5	5	5

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.16 g/L	3	2	1	2
0.31 g/L	3	4	2	3
0.63 g/L	4	1	2	0
1.25 g/L	5	2	5	4
2.50 g/L	5	5	5	5
5.00 g/L	5	5	5	5

Laboratory 12

number of immobile animals

	24 h			
	replicate			
treatment	A	B	C	D
water control	0	0	0	1
0.4 g/L	0	0	0	0
0.8 g/L	0	0	0	1
1.6 g/L	1	2	0	0
3.2 g/L	2	2	1	0
6.4 g/L	5	5	5	5

number of immobile animals

	48 h			
	replicate			
treatment	A	B	C	D
water control	0	0	0	1
0.4 g/L	2	0	0	2
0.8 g/L	0	0	0	1
1.6 g/L	2	3	1	2
3.2 g/L	5	5	5	4
6.4 g/L	5	5	5	5

Laboratory 13

number of immobile animals

	24 h			
	replicate			
treatment	A	B	C	D
water control	1	0	1	0
0.25 g/L	0	1	0	0
0.5 g/L	1	0	1	0
1 g/L	0	0	1	0
2 g/L	3	1	2	1
4 g/L	5	1	3	4

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	1	0	1	0
0.25 g/L	0	2	0	1
0.5 g/L	2	1	1	0
1 g/L	1	2	2	1
2 g/L	3	3	3	3
4 g/L	5	5	5	5

Laboratory 14

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.5 g/L	0	0	0	0
1 g/L	0	0	0	0
2 g/L	3	2	2	3
4 g/L	5	5	5	5
8 g/L	5	5	5	5

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.5 g/L	0	0	0	0
1 g/L	0	1	0	0
2 g/L	5	5	5	5
4 g/L	5	5	5	5
8 g/L	5	5	5	5

2. Studies on 3,5-Dichlorophenol (3,5-DCP)

Laboratory 1

number of immobile animals

	24 h replicate *			
treatment	A	B	C	D
water control	0	0	0	0
solvent control	0	1	0	0
0.43 mg /L	1	0	0	0
0.94 mg /L	1	1	1	0
2.07 mg /L	0	0	1	1
4.55 mg /L	6	9	6	8
10.0 mg /L	10	10	10	10

* deviating from all other laboratories 10 larvae were inserted per replicate

number of immobile animals

	48 h replicate *			
treatment	A	B	C	D
water control	1	0	0	0
solvent control	0	1	0	0
0.43 mg /L	1	0	1	0
0.94 mg /L	1	2	2	4
2.07 mg /L	8	6	6	5
4.55 mg /L	10	10	9	9
10.0 mg /L	10	10	10	10

* deviating from all other laboratories 10 larvae were inserted per replicate

Laboratory 2

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.10 mg /L	0	0	0	0
0.22 mg /L	0	0	0	0
0.46 mg /L	0	0	0	0
1.0 mg /L	0	0	0	0
2.2 mg /L	0	1	0	0
4.6 mg /L	5	2	2	4
10 mg /L	4	5	5	5

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.10 mg /L	0	0	0	0
0.22 mg /L	0	0	0	0
0.46 mg /L	0	0	2	1
1.0 mg /L	1	0	1	1
2.2 mg /L	2	3	3	4
4.6 mg /L	5	4	4	5
10 mg /L	5	5	5	5

Laboratory 3

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
solvent control*	-	-	-	-
0.32 mg /L	1	0	0	0
0.63 mg /L	0	0	0	0
1.25 mg /L	0	0	0	1
2.5 mg /L	2	2	1	0
5 mg /L	5	5	5	5

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	0	0	1	1
solvent control*	-	-	-	-
0.32 mg /L	1	0	0	1
0.63 mg /L	0	0	1	1
1.25 mg /L	0	1	1	1
2.5 mg /L	5	5	5	5
5 mg /L	5	5	5	5

Laboratory 4-1

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
solvent control	-	-	-	-
0.26 mg /L	1	2	0	2
0.56 mg /L	4	3	3	2
1.24 mg /L	4	5	5	3
2.73 mg /L	5	5	4	4
6.0 mg /L	5	5	5	5

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
solvent control	-	-	-	-
0.26 mg /L	2	2	1	2
0.56 mg /L	4	4	3	3
1.24 mg /L	4	5	5	4
2.73 mg /L	5	5	5	4
6.0 mg /L	5	5	5	5

Laboratory 4-2

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
solvent control	-	-	-	-
0.26 mg /L	0	1	0	2
0.56 mg /L	1	3	3	2
1.24 mg /L	3	2	3	3
2.73 mg /L	4	3	5	5
6.0 mg /L	4	4	5	5

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	1	0	1	0
solvent control	-	-	-	-
0.26 mg /L	1	2	2	2
0.56 mg /L	2	4	4	2
1.24 mg /L	4	4	3	4
2.73 mg /L	5	4	5	5
6.0 mg /L	5	5	5	5

Laboratory 5

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
Control	1	0	0	0
0.34 mg /L	0	0	0	0
0.75 mg /L	0	5	0	0
1.65 mg /L	3	0	4	2
3.64 mg /L	4	5	2	2
8.0 mg /L	5	5	5	5

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
Control	1	0	0	0
0.34 mg /L	0	1	1	1
0.75 mg /L	4	5	3	3
1.65 mg /L	5	3	5	5
3.64 mg /L	5	5	5	5
8.0 mg /L	5	5	5	5

Laboratory 6

number of immobile animals

	24 h			
	replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.19 mg/L	2	0	0	0
0.41 mg/L	0	1	0	0
0.91 mg/L	0	0	1	1
2.0 mg/L	0	1	1	0
4.4 mg/L	5	5	4	5
9.7 mg/L	5	5	5	5

number of immobile animals

	48 h			
	replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.19 mg/L	2	1	0	1
0.41 mg/L	0	2	0	1
0.91 mg/L	1	1	2	1
2.0 mg/L	4	3	4	5
4.4 mg/L	5	5	5	5
9.7 mg/L	5	5	5	5

Laboratory 7

number of immobile animals

	24 h			
	replicate			
treatment	A	B	C	D
water control	0	0	0	0
0.36 mg/L	0	0	0	0
0.56 mg/L	0	0	0	0
0.87 mg/L	0	0	0	0
1.34 mg/L	0	1	0	0
2.1 mg/L	2	1	1	2
3.2 mg/L	5	5	3	5
5 mg/L	5	5	5	5

number of immobile animals

	48 h **replicate**			
treatment	A	B	C	D
water control	0	0	1	1
0.36 mg/L	1	1	1	1
0.56 mg/L	0	0	0	0
0.87 mg/L	0	1	1	2
1.34 mg/L	0	2	0	0
2.1 mg/L	3	2	3	4
3.2 mg/L	5	5	5	5
5 mg/L	5	5	5	5

Laboratory 8

number of immobile animals

	24 h **replicate**			
treatment	A	B	C	D
water control	0	0	0	0
acetone control	0	0	0	0
0.5 mg/L	0	1	0	0
1 mg/L	0	0	1	1
2 mg/L	0	0	0	0
4 mg/L	0	0	0	0
8 mg/L	5	5	5	4

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
acetone control	1	0	0	0
0.5 mg/L	1	2	0	1
1 mg/L	1	1	1	1
2 mg/L	0	2	0	0
4 mg/L	2	2	0	2
8 mg/L	5	5	5	4

Laboratory 9

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
solvent control	-	-	-	-
0.25 mg/L	0	0	0	0
0.5 mg/L	0	0	0	0
1 mg/L	0	0	1	0
2 mg/L	1	1	2	3
4 mg/L	5	4	5	5

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
solvent control	-	-	-	-
0.25 mg/L	0	0	0	0
0.5 mg/L	0	0	0	0
1 mg/L	0	0	1	0
2 mg/L	5	2	2	3
4 mg/L	5	5	5	5

Laboratory 10

number of immobile animals

	24 h **replicate**			
treatment	A	B	C	D
water control	0	1	0 (1)	0
solvent control	-	-	-	-
0.46 mg/l	1	0	0	0
1.0 mg/l	1 (1)	0 (1)	0	0
2.2 mg/l	0	1	0	0
4.6 mg/l	2	2 (1)	1 (2)	3 (2)
10 mg/l	5	4 (1)	5	5

( ): Between brackets the number of missing larvae; added to immobile larvae for evaluation.

number of immobile animals

	48 h **replicate**			
treatment	A	B	C	D
water control	0	1	0 (1)	0
solvent control				
0.46 mg/l	1	1	0	0
1.0 mg/l	1	1	0	0
2.2 mg/l	1	1	0	0
4.6 mg/l	5	4 (1)	4 (1)	5
10 mg/l	5	4 (1)	5	5

( ): Between brackets the number of missing larvae; added to immobile larvae for evaluation.

Laboratory 11

number of immobile animals

	24 h **replicate**			
treatment	A	B	C	D
water control	0	0	0	0
solvent control	0	0	0	0
0.16 mg/L	0	0	3	4
0.31 mg/L	2	0	0	0
0.63 mg/L	0	0	0	0
1.25 mg/L	0	1	1	1
2.50 mg/L	3	5	4	4
5.00 mg/L	5	5	5	5

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
solvent control	0	0	0	0
0.16 mg/L	0	0	5	5
0.31 mg/L	2	0	0	0
0.63 mg/L	0	0	0	0
1.25 mg/L	1	1	0	3
2.50 mg/L	5	5	5	5
5.00 mg/L	5	5	5	5

Laboratory 12

number of immobile animals

	24 h replicate			
treatment	A	B	C	D
water control	0	0	0	0
solvent control	0	0	0	0
0.5 mg/L	0	0	0	0
1 mg/L	0	0	0	1
2 mg/L	1	0	2	1
4 mg/L	5	5	5	5
8 mg/L	5	5	5	5

number of immobile animals

	48 h replicate			
treatment	A	B	C	D
water control	4	1	5	3
solvent control	3	4	3	3
0.5 mg/L	0	2	3	1
1 mg/L	1	0	2	2
2 mg/L	3	4	4	5
4 mg/L	5	5	5	5
8 mg/L	5	5	5	5

Laboratory 14

number of immobile animals

	24 h			
	replicate			
treatment	A	B	C	D
water control	0	0	0	0
solvent control	-	-	-	-
0.3 mg/L	0	0	0	0
0.6 mg/L	0	0	0	0
1.2 mg/L	0	1	0	0
2.4 mg/L	1	1	0	0
4.8 mg/L	4	5	3	3

number of immobile animals

	48 h			
	replicate			
treatment	A	B	C	D
water control	0	0	0	0
solvent control	-	-	-	-
0.3 mg/L	0	0	0	0
0.6 mg/L	0	1	0	0
1.2 mg/L	0	1	0	1
2.4 mg/L	1	2	3	2
4.8 mg/L	5	5	5	5