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THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY**

Report of the Validation of the Potamopyrgus Antipodarum Reproduction Toxicity Test

Series on Testing & Assessment

No. 235

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OECD Environment, Health and Safety Publications

Series on Testing and Assessment

No. 235

**REPORT OF THE VALIDATION OF THE POTAMOPYRGUS ANTIPODARUM REPRODUCTION
TOXICITY TEST**

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among **FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD**

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris 2016

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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

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FOREWORD

The project to develop mollusc reproduction tests was initiated by Denmark, Germany, France and the United Kingdom and included in the work plan of the Test Guidelines Programme in 2011.

Originally the project envisaged the development of a single Test Guideline (TG) on mollusc reproduction test, including both the mollusc species *Lymnaea stagnalis* and *Potamopyrgus antipodarum*. However, the Validation Management Group on Ecotoxicity (VMG-Eco) supported the development of two separate TGs for these two mollusc species after discussion at the 10th VMG-Eco meeting in December 2014.

The separate draft validation report for the Test Guideline using *Potamopyrgus antipodarum* was finalised in June 2015 and subsequently circulated to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) for review and commenting in July 2015 and again in December 2015.

No comments were received for the validation report in support of the Test Guideline using *Potamopyrgus antipodarum*, which was subsequently approved by the WNT at its 28th meeting in April 2016. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to declassification of the validation report on 8 July 2016.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

**REPORT OF THE VALIDATION OF THE POTAMOPYRGUS ANTIPODARUM
REPRODUCTION TOXICITY TEST**

Coordination:

Cornelia Geiß, Katharina Ruppert, Agnes Schimera, Jörg Oehlmann

Goethe University Frankfurt am Main

Aquatic Ecotoxicology

Max-von-Laue-Str. 13

60439 Frankfurt / Main

Germany

Tel. +49 (0) 69 798 42142

oehlmann@bio.uni-frankfurt.de

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SUMMARY

The aim of the project is to develop a standard test method with a selected mollusc species according to the specifications of the OECD. Goethe University designed the draft standard operating procedure (SOP) for the reproduction test with *Potamopyrgus antipodarum* based on literature and expert knowledge.

The validation studies were performed to validate the proposed test conditions and identify issues in performing the SOP:

1. In total, 17 partners participated in three validation exercises for the reproduction tests.
2. Snails, used for testing in validation I (2010) originated from the laboratory culture of Goethe University, which was built up with snails from Kalbach Frankfurt am Main (Germany) in August 2009. Snails used for validation II (2013) and III (2014) originated from a long term laboratory culture which was built up with snails from Lumda, Rabenau (Germany).
3. Before shipping, snails were checked for their ability to reproduce and for the absence of parasites with histological methods and gross measurements.
4. Prior to the reproduction test, snails were acclimated after shipping in the participating laboratories for at least five days.
5. In validation I and II, five nominal concentrations of cadmium (Cd: 1.56, 3.13, 6.25, 12.5, 25 µg/L) and a negative control were tested without the use of solvents. Tributyltin (TBT) was also tested in validation II in a nominal concentration range between 10 and 1000 ng Sn/L. Additionally a solvent control with glacial acetic acid (10 µL/L) was tested. In validation III prochloraz and trenbolone were chosen as test chemicals in a nominal concentration range from 3.2 to 320 µg/L and from 10 to 1000 ng/L, respectively. DMSO was used as a solvent (10 µL/L).
6. Exposure concentrations in validation exercises were analytically verified. All effect concentrations provided in this report refer to time weighted mean (TWM) concentrations because TWM concentrations deviated by more than 20% from nominal concentrations.
7. The reproduction tests with cadmium showed a good agreement among participating laboratories. Only laboratory 1B reported considerably lower effect concentrations (EC₁₀: 0.69 µg/L, EC₅₀: 4.59 µg/L). The higher sensitivity of snails in laboratory 1B is probably due to the use of a mixed cohort of snails from two batches. The originally shipped snails from batch 1 suffered from high mortality after arrival, indicating a poor health status. Therefore a second batch of snails was provided for laboratory 1B. Both batches were mixed in laboratory 1B and the mixed cohort was used for the testing with Cd. The mean effect concentrations (with coefficient of variation) for EC₁₀, EC₅₀, NOEC and LOEC of validation I and II from all laboratories (1B excluded) are 6.53 µg/L (35.5%), 14.2 µg/L (21.8%), 6.45 µg/L (50.5%) and 12.6 µg/L (42.2%), respectively with a minimum of a 1.7-fold difference (EC₅₀ values) and a maximum of a 3.9-fold difference (LOEC values).
8. The results of the reproduction tests with TBT in validation II also showed a good accordance among partners. Effect concentrations exhibit a minimum of a 4.8-fold difference (LOECs) and a maximum of a 13.5-fold difference (EC₁₀ values). The mean values (with coefficient of variation) for EC₁₀, EC₅₀, NOEC and LOEC values from these three laboratories are 35.6 ng Sn/L (76.9%), 127 ng Sn/L (39.3%), 39.2 ng Sn/L (68.3%) and 75.7 ng Sn/L (77.0%), respectively. Some

uncertainties concerning the measured concentrations might have influenced the results, because time weighted means have been calculated based on only two measuring intervals, due to the high costs of analytical measurements.

9. In validation III all laboratories found a decrease of the embryo numbers with increasing concentrations of prochloraz. The NOEC varies between 21.3 µg/L and 40.4 µg/L. The LOEC is in the range between 31.4 µg/L and 194 µg/L. The good match of results is also reflected by the EC₁₀ and EC₅₀ values (based on measured concentrations). The mean values (with coefficient of variation) are 24.1 µg/L (61.3%) and 336 µg/L (75.7%), respectively. The EC₁₀ values from all laboratories overlap with their 95%-confidence intervals. None of the participating laboratories found a concentration-dependent decrease of the embryo numbers in the brood pouch of *P. antipodarum* after exposure to trenbolone.
10. In validation IV four laboratories participated in this round robin and triclocarban and triclosan were chosen as test chemicals. In one of the participating laboratories the test was not valid (increased mortality in the solvent control). Two of the other three laboratories observed a concentration-dependent decrease of embryo numbers in *P. antipodarum* after 28 days exposure to triclocarban (NOEC 0.121 and 0.681 µg/L; LOEC 0.340 and 1.52 µg/L) while the third lab did not find any effects in the measured concentration range (<0.004 – 1.64 µg/L). For triclosan one laboratory reported a significantly reduced embryo number at the highest test concentration (0.964 µg/L as LOEC; NOEC = 0.480 µg/L) while the other two laboratories found no effects in the measured concentration range (<0.004 – 1.36 µg/L and 0.057 – 3.17 µg/L, respectively). In this validation study the food level was reduced to 62.5 µg/snail x day compared to 250 µg/snail x day in validations I – III to reduce the amount of unconsumed food and the resulting risk of fungus growth which may contribute to an increased mortality of the test organisms. The embryo numbers in the control snails were on the same level like in validations I – III without any statistically significant deviations in the dilution-water controls among validation studies.
11. Within the four validation studies, 43 reproduction tests have been performed, thereof one laboratory had to repeat the reproduction test with TBT due to very low concentrations of the test substance and five laboratories did not achieve the given validity criteria. One laboratory had technical issues to satisfy the temperature between 15°C and 17°C and the other four laboratories did not meet the biological criteria (maximum control mortality; or minimum embryo number in control groups in snails coming from a different culture).
12. For all tested chemicals the inter-laboratory reproducibility of the test has been shown as most of the laboratories detected comparable NOEC, LOEC, EC₁₀, EC₅₀ values with overlapping 95%-confidence intervals for the latter, even if difficult to handle substances were chosen as test compounds (e.g. TBT or trenbolone). In validation IV the actually measured test concentrations of triclocarban and triclosan were probably too low to induce significant effects on reproduction in all participating laboratories. Furthermore in validation I and II the repeatability/intra-laboratory reproducibility could be demonstrated as laboratory A repeated the reproduction test with TBT and cadmium. All in all, the reproduction test turned out to be a practical tool.

1. PARTICIPANTS

AstraZeneca - Brixham Lab. [UK]

Involved staff: G. Le Page, R. Brown

BASF SE - Limburgerhof [DE]

Involved staff: S. Hartmann, L. Weltje

Bayer Crop Science [DE]

Involved staff: K. Kuhl, D. Faber, E. Bruns

CEFAS – Centre for Environment, Fisheries & Aquaculture Science - Lowestoft Lab. [UK]

Involved staff: C. Askem, T. Hutchinson, A. Smith

ECT Oekotoxikologie GmbH Flörsheim [DE]

Involved staff: P. Egeler, J. Heinrich, T. Knacker, P. Lorenz

FERA - Department for Environment, Food & Rural Affairs - Sand Hutton [UK]

Involved staff: J. Turton, R. Benstead

Fraunhofer IME [DE]

Involved staff: M. Moenig, M. Lutter, M. Teigeler

Goethe University Frankfurt am Main [DE]

Involved staff: C. Geiß, K. Ruppert, A. Schimera, M. Behr, U. Schulte-Oehlmann

IBACON GmbH Rossdorf [DE]

Involved staff: A. Hengsberger, A. Seeland-Fremer

INRA - National Institute for Agricultural Research - Rennes [FRA]

Involved staff: M. Coke, M. Collinet, V. Ducrot

NIVA - Norwegian Institute for Water research - Oslo [NO]

Involved staff: M. Hultman, A. L. Macken

University of Antwerp [BE]

Involved staff: I. Planojevic, C. Schmitt

University of Aveiro [PT]

Involved staff: P. Sanchez Marin, C. Miguez

University of Helsinki [FI]

Involved staff: P. Kajankari, O.-P. Penttinen

University of Iowa [US]

Involved staff: C. Tucci, M. Neiman

University of Southern Denmark [DK]

Involved staff: B. Frost Holbech, K. L. Kinnberg, H. Holbech

University of Vigo [ES]

Involved staff: T. Tato, R. Beiras

Please note that the participating laboratories are provided in alphabetical order. This sequence is not identical with laboratory codes used in this report.

2. SCIENTIFIC AND FINANCIAL SUPPORT

1. T. Hutchinson, CEFAS [UK]

Responsibility: guideline project coordinator

2. P. Matthiessen, External consultant [UK]

Responsibility: scientific guidance for the development of OECD test guidelines

3. J. Bachmann, UBA [DE]

Responsibility: author of the SPSF; scientific advice from UBA; project officer for the UBA-funded projects

4. M. Roberts and D. Lovell, DEFRA [UK]

Responsibility: group secretary, scientific advice from DEFRA

5. International Graduate School Zittau (G. Kayser, H. Heidenreich) [DE]

Responsibility: chemical analyses of exposure media for Cd in validation I

6. Chemlab GmbH Bensheim (O. Wappelhorst) [DE]

Responsibility: chemical analyses of exposure media for Cd and TBT in validation II

7. University of Southern Denmark (H. Holbech) [DK]

Responsibility: chemical analyses of exposure media for TR and PCZ in validation III

The project is funded by the German Federal Environment Agency (UBA, project codes 3709 61 402 and 3711 65 417), the *Department for Environment, Food and Rural Affairs* of United Kingdom (DEFRA) and the Danish Ministry of the Environment.

3. BACKGROUND INFORMATION

1. The standard operating procedure was drafted using published and grey literature on toxicity tests with *Potamopyrgus antipodarum* and the experience of toxicity testing with this species for more than 15 years at Goethe University. Main scientific inputs were older SOP versions for tests with *P. antipodarum* at Goethe University, OECD (2010) and SIERATOWICZ ET AL. (2011). The draft for the SOP used in validation I was discussed and revised at an expert meeting at DEFRA, London, in August 2009. The SOP draft was adapted after every validation study taking into account the outcome of the studies and the feedback of partner laboratories.

4. VALIDATION I: CADMIUM

4.1 Organization of the validation test

4.1.1 Snail production, biological quality checking and shipping

2. *Potamopyrgus antipodarum* (Mollusca, Gastropoda, Neotaenioglossa, Hydrobiidae) specimens used for the experiments were taken from the long-term laboratory culture (haplotype *t*, morphotype “Warwick A”, STÄDLER ET AL. 2005) at Goethe University, which was last refreshed with animals collected from the Kalbach in August 2009. This slowly running stream in Frankfurt, Germany, does not receive any discharges except drainage ditches for rain water. The long-term laboratory culture is run under standardized conditions with a light/dark regime of 16:8 h and at a temperature of $16 \pm 1^\circ\text{C}$.

3. Before shipping to participants, the absence of external parasites and the snails’ ability to reproduce was examined. Table 1 summarizes snail shipping dates, acclimation duration, test period and snail shipping mortality in the different laboratories.

Table 1: Shipping, acclimation and test schedules for the partner laboratories.

Partner	Laboratory 1A	Laboratory 1B	Laboratory 1C	Laboratory 1D
Snail shipping date	03/05/2010	1 st batch: 11/01/2010 2 nd batch: 03/05/2010	21/05/2010	03/05/2010
Snails received	03/05/2010	1 st batch: 12/01/2010 2 nd batch: 04/05/2010	21/05/2010	04/05/2010
Number of snails sent	500	1 st batch: 550 2 nd batch: > 400	550	550
Post-shipment mortality [%]	0	1 st batch: > 50 2 nd batch: 0	0	0
Acclimation duration	24 d	At least 37 d	5 d	37 d
Test starting date	26/05/2010	10/06/2010	26/05/2010	10/06/2010
Test ending date	24/06/2010	07/07/2010	24/06/2010	07/07/2010

4.1.2 Snail acclimation in partners laboratories

4. Snails were acclimatized in the partner laboratories after arrival for at least 5 days as described in the draft SOP to ensure recovery from shipping stress. Post-shipping mortality only occurred in laboratory 1B for unknown reasons, hence, another batch of snails was sent in May 2010. Unfortunately the two batches of snails were mixed in laboratory 1B and the mixed cohort was used for the test. This is a possible explanation for the lower effect concentrations determined in this laboratory (cf. 4.4).

4.2 Implementation of the 28-day reproduction test

4.2.1 Principle of the test

5. Using animal originating from all female cultures, adult *Potamopyrgus antipodarum* of a defined size class are exposed in a 28-day-reproduction test to a concentration range of the test substance. The test substance is spiked into the water and adult snails are subsequently introduced into the test beakers. Survival is regularly determined and dead snails are removed from test vessels. During the test snails are fed three times per week after renewal of the exposure media. At the end of the test snails are anaesthetized with $MgCl_2$ as mentioned in the extended SOP but may also be quick-frozen in liquid nitrogen and stored at $-20^{\circ}C$. Shell length and number of embryos in the brood pouch are determined. The individual per female number of embryos is assessed separately after carefully cracking the shell of the parent specimen and opening of the brood pouch. The number of embryos with and without shell and the total embryo number per female are recorded.

4.2.2 Chemicals

6. Cadmium was used as cadmium sulphate hydrate (98% purity, CAS no. 7790-84-3, Merck KGaA Darmstadt, Germany) coming from the same batch and was provided to partners by Goethe University.

7. In the test with *P. antipodarum*, five concentrations of cadmium were used with a factor of 2 between concentrations. The nominal cadmium concentrations were chosen based on pre-tests at Goethe University:

1.56 $\mu g/L$, 3.13 $\mu g/L$, 6.25 $\mu g/L$, 12.5 $\mu g/L$, 25 $\mu g/L$.

4.2.3. Experimental conditions

8. Experimental conditions and instructions are given in the draft SOP. A semi-static test design is applied with medium renewals three times a week (Monday, Wednesday and Friday) for all exposure groups and controls.

9. Snails were exposed in closable 1 L beakers with aerated 800 mL test medium (see table 2). Each test concentration and the control were tested in four replicates with ten snails each. Snails were fed with fine grounded Tetraphyll® (0.25 mg per animal and day; Tetra GmbH, Melle, Germany).

10. Biological raw data per female (shell length, embryo numbers) as well as water parameters were summarised in a Microsoft Excel sheet for further data evaluation.

Table 2: Summary of main experimental conditions.

Test duration	28 days
Test water	Reconstituted water (with 0.3 g Tropic Marin® salt and 0.18 g NaHCO ₃ per 1 litre de-ionised water) water quality requirements: pH 7.5 – 8.5, conductivity 770 ± 100 µS/cm, oxygen concentration > 80% ASV (air saturation value)
Test vessels	1 L glass beakers with lids
Water renewal	3 times per week
Temperature	16 ± 1°C
Light intensity	300 – 500 lux
Water sampling	From all test concentrations and controls water was sampled over two renewal intervals: freshly prepared exposure media were sampled from vessels on days 3 and 25 of the test, old exposure media before renewal on days 5 and 28.
Photoperiod	16:8 h L:D
Food source	Fine grounded Tetraphyll®
Feeding	0.25 mg/animal and day
Snails origin	Laboratory culture, which was built up with snails from Kalbach Frankfurt, Germany (August 2009)
Test snails size	3.5 – 3.8 mm
Snails density	10 snails per 800 mL (4 replicates per tested concentration)
Core test endpoints	Mortality, reproduction

4.3 Chemical analysis and biological data analysis

11. In the first and in the last week of the test, samples were taken for chemical analysis. 25 mL of fresh contaminated test medium were sampled from the test vessels before (2 or 3 days old) and after (fresh) medium renewal for each test concentration, including controls, bottled into 50 mL tubes and acidified with 0.5 mL nitric acid (65%, suprapure®[®], Merck KgaA, Darmstadt, Germany). Samples for chemical analysis were sent to Goethe University according to a previously agreed time schedule. Chemical analysis of Cd was performed via inductively coupled plasma mass spectrometry (ICP-MS, ELAN DCR-e, Perkin Elmer, Überlingen) at the International Graduate School Zittau, Chair Environmental Technology, according to DIN 38406 E29 (1996).

12. Apparatus calibrations were as follows: 1150 W plasma, 6 bar argon primary pressure and 0.92 L atomising gas/min. The limit of determination was 0.01 µg/L, the limit of quantification was 0.025 µg/L. A certified reference material (SPS-SW2, Surface Water, Level 2 from Spectrapure Standards AS, Oslo) was measured simultaneously. Certified Cd concentration in the material is 0.50 ± 0.1 mg Cd/L and the measured concentration was 0.53 mg Cd/L.

13. Biological raw data were reported by the participating laboratories using an Excel spread sheet which had been provided by Goethe University. From the raw data means were calculated for biological parameters (shell height, embryo numbers). Effect concentrations were calculated by Dunnett's test (NOEC, LOEC) or by a non-linear regression using a four parameter logistic equation (EC₁₀, EC₅₀).

4.4 Results

4.4.1 Compliance with validity criteria

14. For a test to be valid the conditions were chosen based on available guidelines for freshwater invertebrates and as proposed in OECD (2010). For the purpose of validation I, the following validity criteria should be fulfilled:

- mortality in the controls should not exceed 20% and
- dissolved oxygen saturation must have been at least 60% of the air saturation value (ASV) throughout the test.

15. All participants complied with the validity criteria. No mortality was observed in controls of all participants and the mean ASV for oxygen ranged from 94.2% to 99.5%. Therefore the tests were valid.

4.4.2 Physico-chemical parameters

16. Physico-chemical parameters have been recorded for all laboratories. The means are presented in Table 3 and are similar among all partners. Only for laboratory 1 and 3 the mean temperature was 17.4°C and 14.9°C, respectively. Therefore the mean temperature was 0.4°C higher and 0.1°C lower than required. This might be due to equipment problems in the controlled temperature room but had not a significant effect on the health of the snails.

Table 3: Mean of the physico-chemical parameters of the four laboratories.

Laboratory	pH			Conductivity [$\mu\text{S}/\text{cm}$]			Temperature [$^{\circ}\text{C}$]			O ₂ saturation [%]		
	mean	SD	n	mean	SD	N	mean	SD	n	mean	SD	n
1A	8.15	0.196	120	840	16.0	120	15.3	0.362	120	94.3	1.75	120
1B	8.32	0.136	140	742	28.8	140	17.4	0.300	140	96.8	2.80	140
1C	8.11	0.090	120	718	23.8	120	16.0	0.597	120	98.5	4.50	120
1D	8.28	0.457	197	737	53.0	197	14.9	0.467	197	99.6	2.28	197

4.4.3 Results of validation I with cadmium

4.4.3.1 Actual exposure concentrations

17. The results of the analytical analyses (Tab. 4 – 7) show that measured concentrations were similar among laboratories although nominal concentrations could not always be reached. Initial concentrations varied between 70% and 110% of the nominal concentrations. After two days the values ranged from 68% to 75% of the nominal concentrations. This may be caused by adsorption of the substance to the vessel wall and to food particles and/or by direct uptake of Cd by the snails. PASCOE ET AL. (1990) demonstrated that Cd concentrations in water declined in tanks containing food (Tetramin®). In the mentioned study Cd was detected on the vessel walls and on the aeration apparatus but the greatest amount was found with the food and food-sediment mixture. Declining aqueous Cd concentrations in tests with *P. antipodarum* as a result of direct uptake of the test substance by the snails were also shown by JENSEN ET AL. (2001).

18. Time weighted mean concentrations were calculated according to Annex 6 of OECD guideline 211 (OECD, 2008). Values varied between 62% and 91% of the nominal concentrations and resulted in similar concentrations among all laboratories.

19. In the controls very low background concentrations occurred in the range of ng/L or were below the limit of determination (0.01 µg Cd/L). Only in laboratory 1B a concentration of 9.89 µg Cd/L was measured on day 5 in the 2 days old exposure medium during the first week of the test (table 5). The most probable explanation for this is a mix-up of sample tubes because the concentration in the fresh medium on day 3 was 0.04 µg Cd/L. It is very unlikely that a contamination could have occurred in between water renewals.

Table 4: Results of Cd analyses in exposure media from laboratory 1A [µg/L].

Nominal concentration	Measured concentrations				Time weighted mean	% of nominal concentration
	Day 3: after renewal	Day 5: before renewal	Day 25: after renewal	Day 28: before renewal		
Control	0.01	< LOD	< LOD	< LOD	n.c.	n.c.
1.56 µg/L	1.19	0.38	1.72	1.13	1.13	72.4
3.13 µg/L	5.03	0.79	2.99	2.19	2.46	78.6
6.25 µg/L	9.30	3.24	6.57	4.60	5.61	89.8
12.5 µg/L	13.6	4.61	12.7	12.7	10.9	87.2
25 µg/L	24.0	12.7	25.8	17.7	20.0	80.0

LOD = Limit of determination (0.01 µg/L) n.c. = not calculable

Table 5: Results of Cd analyses in exposure media from laboratory 1B [µg/L].

Nominal concentration	Measured concentrations				Time weighted mean	% of nominal concentration
	Day 3: after renewal	Day 5: before renewal	Day 25: after renewal	Day 28: before renewal		
Control	0.04	9.89	0.01	< LOD	0.72	n.c.
1.56 µg/L	1.35	1.07	1.12	1.20	1.18	75.6
3.13 µg/L	2.66	2.28	2.38	2.81	2.54	81.2
6.25 µg/L	5.22	4.54	4.72	4.87	4.83	77.3
12.5 µg/L	10.6	8.96	9.48	9.87	9.71	77.7
25 µg/L	21.3	18.7	18.6	18.5	19.1	76.4

LOD = Limit of determination (0.01 µg/L) n.c. = not calculable

Table 6: Results of Cd analyses in exposure media from laboratory 1C [µg/L].

Nominal concentration	Measured concentrations				Time weighted mean	% of nominal concentration
	Day 3: after renewal	Day 5: before renewal	Day 25: after renewal	Day 28: before renewal		
Control	< LOD	< LOD	< LOD	0.01	n.c.	n.c.
1.56 µg/L	1.18	0.99	1.18	1.07	1.12	71.8
3.13 µg/L	2.42	1.87	1.59	2.10	1.95	62.3
6.25 µg/L	4.56	4.06	8.95	4.55	5.62	90.0
12.5 µg/L	10.3	8.23	5.24	8.40	7.70	61.6
25 µg/L	19.4	16.3	36.9	17.7	22.8	91.2

LOD = Limit of determination (0.01 µg/L) n.c. = not calculable

Table 7: Results of Cd analyses in exposure media from laboratory 1D [µg/L].

Nominal concentration	Measured concentrations				Time weighted mean	% of nominal concentration
	Day 3: after renewal	Day 5: before renewal	Day 25: after renewal	Day 28: before renewal		
Control	< LOD	0.01	0.01	0.01	n.c.	n.c.
1.56 µg/L	1.09	1.11	1.68	0.95	1.21	77.6
3.13 µg/L	2.38	2.00	3.26	2.04	2.44	80.0
6.25 µg/L	4.56	4.28	6.40	3.61	4.69	75.0
12.5 µg/L	9.63	7.05	13.1	7.82	9.45	75.6
25 µg/L	18.6	12.4	26.1	17.3	19.0	76.0

LOD = Limit of determination (0.01 µg/L) n.c. = not calculable

4.4.3.2 Biological responses

Mortality

20. The mortality of snails was homogenous between the laboratories and did not exceed 5%, except in laboratory 1A. There a mortality of 32.5% was observed at the highest test concentration (time weighted mean of measured concentrations: 20 µg Cd/L) which is significant compared to control. Therefore only for this laboratory a graph is shown (Fig. 1).

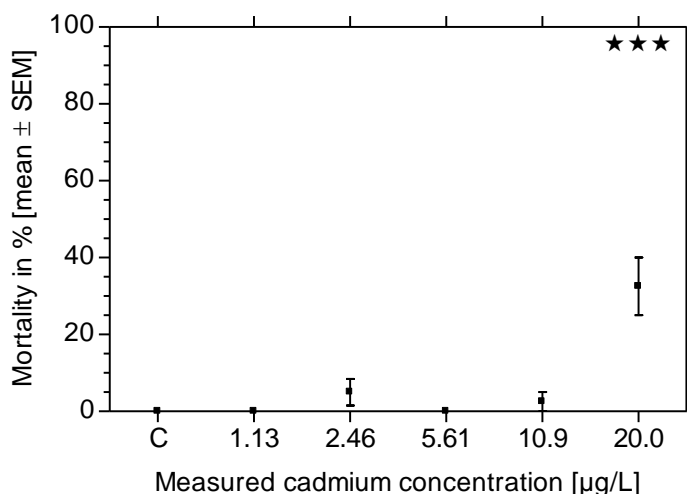


Figure 1: Mortality [in %] of *Potamopyrgus antipodarum* during 4 weeks of exposure to cadmium at laboratory 1A (time weighted mean of measured concentrations with standard error, 4 replicates with 10 snails each). Asterisks indicate significant differences compared to control (Fisher's exact test), *** = $p < 0.001$.

Reproduction

21. In this section only the results for the total embryo numbers are presented because this was the only reproductive parameter which has been used in following validation studies. Results for the numbers of embryos with and without shell were only assessed in validation study I and can be found in Annex 1. They provide a comparable pattern like the total number of embryos.

22. All four laboratories found a concentration-dependent decrease of embryo numbers in the brood pouch of *P. antipodarum* under exposure to Cd (Fig. 2), although with different effect concentrations. Laboratories 1A, 1C and 1D provided comparable NOECs (5.61, 1.95 and 4.69 µg Cd/L) and LOECs (10.9, 9.45 and 5.62 µg Cd/L). The results from laboratory 1B resulted in considerably lower effect concentrations with a significant reduced number of embryos a test concentration of 2.54 µg/L (LOEC value).

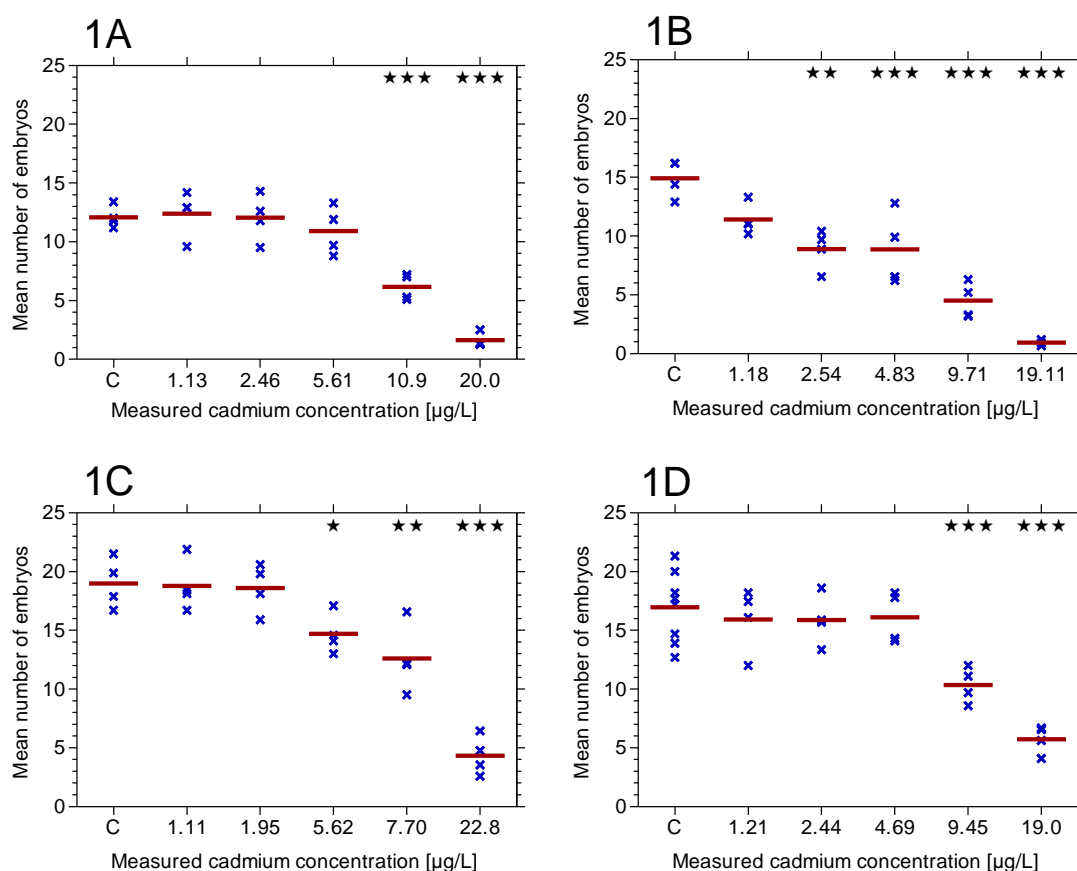


Figure 2: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks of exposure to cadmium at laboratory 1A, 1B, 1C and 1D (x: replicate mean, —: mean of the treatment group, 4 replicates with 3 - 10 surviving snails each). Asterisks indicate significant differences compared to control (Dunnett's test), $\star = p < 0.05$, $\star\star = p < 0.01$, $\star\star\star = p < 0.001$).

23. The good match of results for Cd between laboratories 1A, 1C and 1D are also reflected by the calculated EC_{10} and EC_{50} values (Tab. 8). The findings from laboratory 1B resulted in lower effect concentrations whose 95%-confidence intervals did not overlap with confidence intervals of the effect concentration from the other three laboratories.

Table 8: Effect concentrations (EC_{10} and EC_{50} with 95%-confidence intervals, NOEC, LOEC) for total embryo number based on time weighted means of measured concentrations in $\mu\text{g Cd/L}$. n.d.: not defined

	Laboratory 1A	Laboratory 1B	Laboratory 1C	Laboratory 1D
EC_{10}	4.52 (2.52 - 6.51)	0.689 (0.25 - 1.91)	3.46 (2.28 - 5.25)	4.49 (2.81 - 7.17)
EC_{50}	11.3 (9.48 - 13.2)	4.59 (3.00 - 7.02)	11.4 (9.30 - 13.9)	13.2 (10.8 - 16.0)
NOEC	5.61	1.18	1.95	4.69
LOEC	10.9	2.54	5.62	9.45

4.5 Comparison of present results to published and grey literature

24. The effect concentrations for Cd obtained in the ring tests are in good accordance with already published data. SIERATOWICZ ET AL. (2011) determined 28 days EC₁₀ and EC₅₀ values of 1.30 µg/L (95% CI: 0.662 – 2.55 µg/L) and 11.5 µg/L (95% CI: 8.58 – 15.4 µg/L), respectively. DORGELO ET AL. (1995) performed a ten weeks test with Cd and reported a significantly decreased number of released juveniles and a delayed production of young snails after five to six weeks at the lowest nominal test concentration of 25 µg Cd/L. The results are also consistent with determined “in-house data”. THEIS (2012) reported reduced embryo numbers after a 28 days exposure to cadmium with an EC₁₀ of 7.74 µg Cd/L (95% CI: 4.22 – 14.2) and an EC₅₀ of 10.1 µg Cd/L (95% CI: 7.58 – 13.4).

25. Compared to other invertebrates, e.g. *Daphnia magna*, *P. antipodarum* shows a comparable sensitivity. The LOEC for Cd (1.18 – 10.9 µg Cd/L) in *P. antipodarum* is in the same range as the reproduction LOEC for *D. magna* determined by BORGMANN ET AL. (1989) during chronic exposure.

4.6 Conclusions from validation I

26. In conclusion, the results of the reproduction tests with cadmium showed a good agreement among partners. Effect concentrations from laboratories 1A, 1C and 1D show a minimum of a 1.2-fold difference (EC₅₀ values) and a maximum of a 2.9-fold difference (NOECs). The mean values (with coefficient of variation) for EC₁₀, EC₅₀, NOEC and LOEC values from these three laboratories are 4.16 µg/L (14.5%), 12.0 µg/L (8.96%), 4.08 µg/L (46.6%) and 8.56 µg/L (33.8%), respectively. The variability of results among partners increases if also data from laboratory 1B are considered. The mean values (with coefficient of variation) for EC₁₀, EC₅₀, NOEC and LOEC values from all four laboratories are 3.29 µg/L (54.8%), 10.1 µg/L (37.4%), 3.36 µg/L (63.4%) and 7.05 µg/L (54.3%), respectively. Furthermore, effect data from validation I exercise is in good accordance with other studies (DORGELO ET AL. 1995; SIERATOWICZ ET AL. 2011; THEIS 2012).

27. The higher sensitivity of snails in laboratory 1B is probably due to a mixing of the originally shipped snail batch which suffered from high mortality after arrival, indicating a poor health status, with a second batch of snails shipped afterwards. These mixed cohorts were used for the testing with Cd in laboratory 1B. SIERATOWICZ ET AL. (2013) demonstrated in tests with *P. antipodarum* and Cd that a previous stress factor results in increased sensitivity of the snails to the test chemical.

28. The current results show that the four weeks reproduction test with *P. antipodarum* is a well suited tool for assessing reproductive effects of substances. However, as for regularly employed standard test species, the conditions for breeding and testing of *P. antipodarum*, like e.g. photoperiod and solvent effects, should be clearly studied and, if applicable, be redefined.

4.7 Proposed amendments of the draft standard operating procedure

Snail breeding

29. It was mentioned by the participating laboratories that during water changes in the culture tanks a high number of juveniles can be removed by accident. Breeding conditions should be further specified.

Test conduct

30. Participating laboratories reported that the time effort to culture snails, to prepare, perform and evaluate test is comparable to other invertebrate reproduction tests.

31. However, it should be considered to use smaller test vessels (e.g. 500 mL instead of 1000 mL) to reduce the consumption of chemicals and media as well as waste effluent.

32. Some snails were observed to crawl up and “hide” in the beaker cap. As a consequence they were not in the water and therefore not exposed constantly. The SOP should be amended accordingly: Snails found outside the exposure media during the daily inspections of vessels for mortality should be dropped back into the medium.

Counting of snail embryos

33. Most participating laboratories found it difficult to discriminate embryos with and without shell and proposed that the total embryo number should be assessed as sole parameter for reproduction. For the following validation studies it was decided to skip the discrimination between shelled and unshelled embryos because a re-analysis of historical data for the *Potamopyrgus* reproduction test showed that effect concentrations were comparable for the total number of embryos and for the number of unshelled embryos. Therefore, the additional effort to differentiate shelled and unshelled embryos seemed to be not justified.

34. Some newly hatched snails were found on the shells of the adults. The question was raised whether they were newly released and should be included in the total embryo number. Here, they have been excluded from the analysis.

4.8 Optimisation studies

4.8.1 Effect on the reproduction of *Potamopyrgus antipodarum* to different sizes of test vessels

35. A reproduction test with three different types of test vessels was conducted to investigate the influence of different volumes and densities on the reproduction of *P. antipodarum*. The use of smaller test vessels would reduce the consumption of medium, chemicals and space for testing.

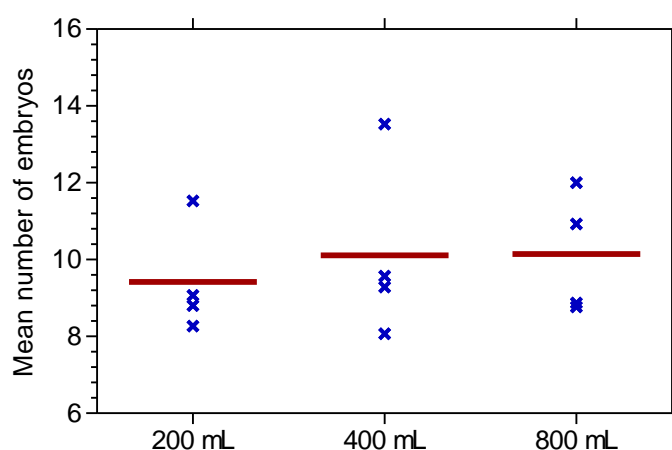


Figure 3: Total embryo numbers in *Potamopyrgus antipodarum* after 4 weeks in 200 mL, 400 mL and 800 mL media, (x: replicate mean, —: mean of the treatment group, 4 replicates with 10 - 15 snails each).

36. In addition to the recommended glass vessels containing 800 mL medium smaller vessels filled with 400 mL and 200 mL were tested according to the DRP (OECD, 2010). After 28 days the embryo numbers of the snails were assessed. The results are presented in figure 3. The mean embryo number did

not differ between treatments. These results indicate that the chosen test vessels and the resulting densities do not influence the reproduction in the test. These results confirm the outcome of a study conducted by SIERATOWICZ ET AL. (2013) suggesting the reduction of the medium volume in the reproduction test with *P. antipodarum* by half. However, smaller test vessels could adsorb more substances on the vessel walls due to a bigger surface-volume relationship. So the choice of the right test vessels depends on the physicochemical properties of the substance. In the test guideline a proposal for a volume-range between 200 mL and 800 mL would be an alternative for the user.

4.8.2 Study of reproductive effects of OECD recommended solvents

37. To assess the influence of solvents on the reproduction on *P. antipodarum*, additional tests have been performed for the following OECD recommended solvents: acetone, methanol, ethanol, dimethyl sulfoxide (DMSO) and triethylene glycol (TEG). Snails were exposed to concentrations of 0.02, 0.1, 0.5, 2.5 and 12.5 mL/L in a 28-days reproduction test. The reproductive effect of glacial acetic acid was also investigated, because this solvent was planned to be used for the reproduction tests with tributyltin in validation II. Glacial acetic acid was tested in a concentration range from 0.01 to 2.43 mL/L.

38. None of the tested solvents had a significant effect on mortality, except for glacial acetic acid. At the three highest test concentrations (0.27, 0.81, 2.43 mL/L) all exposed snails died due to a strong decrease of the pH in this exposure groups.

39. Figure 4 shows the influence of the different solvents on the number of total embryos after 28 days of exposure. While acetone had no effect on snail reproduction (Fig. 4A), the exposure of methanol caused a significant reduction at 0.1 mL/L (Fig. 4B). However, ethanol evoked a reproductive-increasing effect at 0.1 and 0.5 mL/L (Fig. 4C). This phenomenon was also observed for *Daphnia magna* by ZHANG & BAER (2000).

40. The exposure of *P. antipodarum* towards DMSO, TEG and glacial acetic acid had no significant effects on reproduction, whereas the embryo numbers of DMSO showed an increasing trend. Besides that, STANGE ET AL. (2012) demonstrated an influence of DMSO on the reproductive system of *P. antipodarum*. Because of this, DMSO should only be used in low concentrations with a maximum of 20 µL/L, as proposed by HUTCHINSON ET AL. (2006). The exposure of *P. antipodarum* towards glacial acetic acid had no effect on the reproductive output of the snails.

41. Table 9 summarizes the NOEC and LOEC values for the tested solvents. Only methanol and ethanol showed significant effects. For further studies acetone, DMSO, TEG and glacial acetic acid are recommended for the use of a solvent.

42. Based on the caused effects on reproduction of *P. antipodarum* at low concentrations of acetone, methanol and ethanol, these solvents are not suitable for the reproduction test with this snail. However, DMSO and glacial acetic acid seem to be relevant candidates for the studies with *P. antipodarum*.

Table 9: No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for total embryo numbers of *Potamopyrgus antipodarum* after exposure to solvents in µL/L.

	Acetone	Methanol	Ethanol	DMSO	Triethylene glycol	Glacial acetic acid
NOEC	-	0.02	0.02	-	-	-
LOEC	-	0.1	0.1	-	-	-

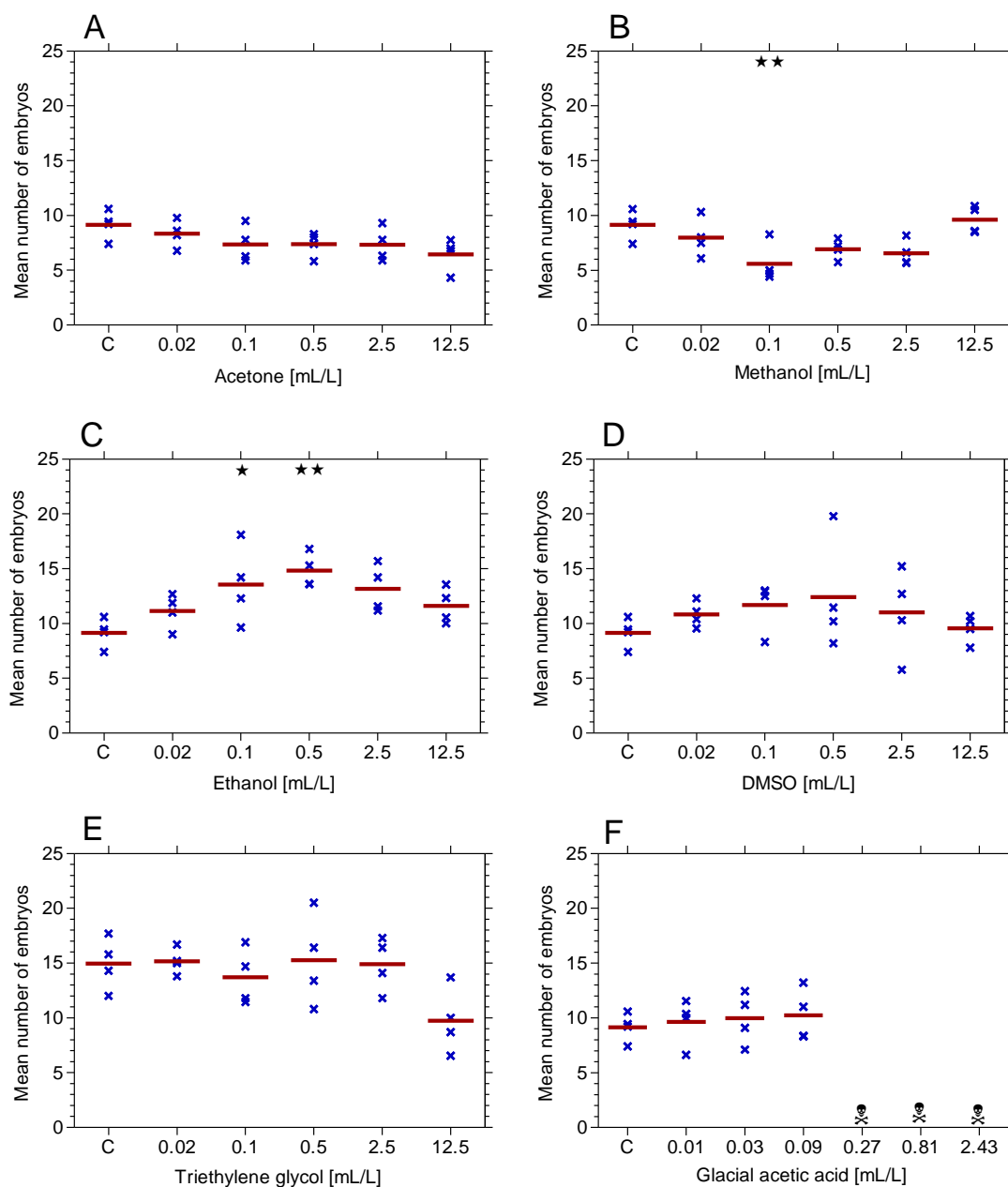


Figure 4: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks of exposure to acetone (A), methanol (B), ethanol (C), DMSO (D), triethylene glycol (E) and glacial acetic acid (F) (x: replicate mean, —: mean of the treatment group, 4 replicates with 10 snails each). Asterisks indicate significant differences compared to control (Dunnett's test), ★ = p < 0.05, ★★ = p < 0.01.

5. VALIDATION II: CADMIUM AND TRIBUTYLTIN

5.1 Organisation of the validation test

43. Eight laboratories participated in validation II, which was realized in 2013, coming from industries, government and academia. They were provided with snails for the test, salts for preparing the medium and also the test substances.

44. Cadmium and tributyltin (TBT) were chosen as test chemicals (see cf. 5.2.2), whereas five of the partners conducted the test with both substances and five laboratories only with TBT.

5.1.1 Snail production, biological quality checking and shipping

45. Snails used for validation II were taken from a laboratory culture (haplotype *t*, morphotype “Warwick A”, STÄDLER ET AL. 2005) at Goethe University which was built up with specimens from the river Lumda in Hesse, Germany. The laboratory culture runs under standardized conditions at a temperature between 15°C and 17°C with a light/dark regime of 16:8 h. Before shipping to participants, size of the snails was determined (3.5 – 4.5 mm) and the reproductive output was checked.

46. Table 10 summarizes the shipping and acclimation duration of the animals and gives an overview of the test schedules. Post-shipping mortality did not appear in partner laboratories.

Table 10: Shipping, acclimation and test schedules for the partner laboratories. Laboratories 2A and 2J performed the tests twice (as indicated by the letters a and b at the end of the codes).

Partner	2Aa	2Ab	2E	2F	2G	2H	2I	2Ja	2Jb	2K
Snail shipping date	-	-	19/6/13	16/7/13	7/8/13	20/8/13	20/8/13	20/8/13	5/2/14	5/8/13
Snails received	-	-	20/6/13	18/7/13	7/8/13	21/8/13	21/8/13	21/8/13	6/2/14	5/8/13
Number of snails sent	-	-	550	550	550	550	350	350	350	350
Acclimation duration	-	-	18 d	46 d	19 d	68 d	28 d	26 d	18 d	14 d
Test starting date	7/8/13	18/3/14	8/7/13	2/9/13	26/8/13	28/10/13	18/9/13	16/9/13	24/2/14	19/8/13
Test ending date	5/8/13	15/4/14	5/8/13	30/9/13	23/9/13	25/11/13	16/10/13	14/10/13	24/3/14	16/9/13

5.2 Implementation of the 28-day reproduction test

5.2.1 Principle of the test

47. Adult females of *Potamopyrgus antipodarum* are exposed in a 28-day-reproduction test to a test chemical with different concentrations. The test substance is spiked into the water and the snails are subsequently introduced into the test beakers. Survival is regularly determined and dead snails are removed from beakers. Renewal of exposure water and controls takes place three times per week. Animals are fed with fine grounded Tetraphyll after every water renewal. After 28 days snails are quick-frozen in liquid nitrogen and stored at -20°C until evaluation. The total number of embryos in the brood pouch per female is determined.

5.2.2 Chemicals

48. Cadmium was used as cadmium chloride (CAS-No.: 10108-64-2, Sigma-Aldrich®, Germany) and was provided from a single batch to partners by Goethe University. Five of the partners conducted the reproduction test with cadmium.

49. In validation II the same nominal cadmium concentrations were chosen as for validation I:

1.56 µg/L, 3.13 µg/L, 6.25 µg/L, 12.5 µg/L, 25 µg/L.

50. TBT was used as tributyltin chloride (CAS-No.: 1461-22-9, Sigma-Aldrich®, Germany) and was provided from a single batch to partners by Goethe-University. For this test chemical glacial acetic acid was used as solvent at a concentration of 10 µL/L. An additional solvent control was considered in the test. The following nominal concentrations were used for the test, whereby the concentrations refer to the tin of TBT (TBT-Sn):

10 ng/L, 25 ng/L 65 ng/L 160 ng/L 400 ng/L 1000 ng/L

51. All laboratories tested in a concentration range from 10 to 400 ng TBT-Sn/L, except for the two repeat studies in laboratories 2A and 2J (tests 2Ab and 2Jb). Laboratory 2J had problems with preparing of the TBT-stock solutions for the first test run (2Ja) which resulted in 500-fold lower concentrations than required. Because a statistically significant reduction of embryo numbers occurred only at the highest tested concentration in most of the reproduction tests, laboratories 2A and 2J repeated the reproduction test with *P. antipodarum* in a concentration range from 25 up to 1000 ng TBT-Sn/L.

5.2.3 Experimental conditions

52. Experimental conditions and instructions are given in the draft SOP. A semi-static test design is applied with medium renewals three times a week for all exposure groups and controls.

53. Snails were exposed in closable 500 mL beakers with aerated 400 mL test medium (see table 11). Each test concentration and the control were tested in four replicates with ten snails each. Snails were fed with fine grounded Tetraphyll® (0.25 mg per animal and day; Tetra GmbH, Melle, Germany).

54. Biological raw data per female (shell length, embryo numbers) as well as water parameters were summarised in a Microsoft Excel sheet for further data evaluation.

Table 11: Summary of main experimental conditions.

Test duration	28 days
Test water	Reconstituted water (with 0.3 g Tropic Marin® salt and 0.18 g NaHCO ₃ per 1 litre de-ionised water) water quality requirements: pH 7.5 – 8.5, conductivity 770 ± 100 µS/cm, oxygen concentration > 80% ASV (air saturation value)
Test vessels	500 mL glass beakers with lids
Water renewal	3 times per week
Temperature	16 ± 1°C
Light intensity	500 ± 100 lux
Water sampling	From all test concentrations and controls water was sampled over two renewal intervals for TBT and over four renewal intervals for cadmium
Photoperiod	16:8 h L:D
Food source	Fine grounded Tetraphyll®
Feeding	0.25 mg/animal and day
Snails origin	Laboratory culture, which was built up with snails from Lumda Hesse, Germany
Test snails size	3.5 – 4.5 mm
Snails density	10 snails per 400 mL medium (4 replicates per tested concentration)
Core test endpoints	Mortality, reproduction

5.3 Chemical analysis and biological data analysis

55. In the first and in the last week of the test, samples from TBT exposure groups and solvent control were taken for chemical analysis. Therefore, on day 0 and day 25 water samples were taken from freshly prepared exposure media and on day 2 and day 28 from old water which was pooled from every replicate of one treatment group. Samples were stored in HDPE-bottles at 4°C until analysis.

56. Chemical analysis of TBT was performed via gas chromatography according to DIN EN ISO 17353 – F13 (2005) at chemlab GmbH in Bensheim, Germany. The limit of quantification (LOQ) and the limit of determination (LOD) for this method are 2.05 and 0.82 ng TBT-Sn/L, respectively.

57. For the chemical analysis of cadmium, samples of exposure groups and control were taken every week. 25 mL of fresh contaminated test medium were sampled from the test vessels before (2 or 3 days old) and after (fresh) medium renewal for each test concentration, including controls, bottled into 50 mL tubes and acidified with 125 µL nitric acid (65%, suprapure®, Merck KgaA, Darmstadt, Germany). Chemical analysis of Cd was performed via inductively coupled plasma mass spectrometry according to DIN EN ISO 1729-2 (2005) at chemlab GmbH Bensheim, Germany.

The limit of quantification (LOQ) and the limit of determination (LOD) for this method are 0.1 and 0.03 µg Cd/L, respectively.

58. Time weighted mean (TWM) concentrations of the chemicals were calculated according to Annex 6 of OECD guideline 211 (OECD, 2012).

59. Biological raw data were reported by the participating laboratories using an Excel® spread sheet which had been provided by Goethe University. From the raw data means were calculated for biological parameters (shell height, embryo numbers). Effect concentrations were calculated by Dunnett's test (NOEC, LOEC) or by a non-linear regression using a four parameter logistic equation (EC₁₀, EC₅₀).

5.4 Results

5.4.1 Compliance with validity criteria

60. For a test to be valid the conditions were chosen based on available guidelines for freshwater invertebrates and as proposed in OECD (2010). For the purpose of validation II, the following validity criteria should be fulfilled:

- mortality in the controls should not exceed 20%,
- dissolved oxygen saturation must have been at least 60% of the air saturation value (ASV) and
- water temperature should be $16 \pm 1^\circ\text{C}$ throughout the test.

61. All laboratories achieved adequate oxygen saturation values. The mean ASV for oxygen ranged from 86.9% (laboratory 2K) to 102% (laboratory 2I). Laboratory 2Jb did observe mortality in the solvent control. 30% of the snails died throughout the test. Except for laboratory 2K, all of the participants achieved the defined temperature scale and the mean temperature was between 15.4°C and 17.0°C . The mean temperature of laboratory 2K was 19.0°C . Therefore, the tests from laboratories 2Jb and 2K were not valid and test results from these laboratories are not considered in the following evaluation of reproduction data.

5.4.2 Physico-chemical parameters

62. Table 12 summarizes the means of the physico-chemical parameters for all laboratories. The pH values ranged between 7.95 (laboratory 2E) to 8.34 (laboratory 2F) which is in the determined range of 7.5 - 8.5. Also the measured conductivity was similar among all partners.

Table 12: Mean physico-chemical parameters of all laboratories. n.r.: not received.

Laboratory	pH			Conductivity [$\mu\text{S}/\text{cm}$]			Temperature [$^\circ\text{C}$]			O ₂ saturation [%]		
	mean	SD	n	mean	SD	n	mean	SD	n	mean	SD	n
2Aa	8.26	0.700	144	811	58.8	144	15.4	0.386	144	95.0	5.99	144
2Ab	8.13	0.900	84	788	31.3	84	16.7	0.311	84	93.8	7.01	84
2E	7.95	0.707	144	774	22.5	24	15.4	0.344	24	101	1.26	24
2F	8.39	0.680	155	819	31.3	108	16.1	0.709	156	98.5	4.99	155
2G	8.34	0.670	156	755	44.5	156	16.2	0.405	156	95.0	4.28	156
2H	8.06	0.660	156	711	64.4	156	15.8	0.559	156	90.6	6.34	156
2I	8.24	0.890	85	668	37.5	85	17.0	0.498	85	102	5.05	85
2Ja	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
2Jb	8.24	0.863	91	762	34.3	91	15.6	0.279	91	93.4	7.90	70
2K	8.31	0.870	91	719	128	91	19.0	1.05	91	86.9	10.4	91

5.4.3 Results of validation II with cadmium

5.4.3.1 Actual exposure concentrations

63. The results of the analytical analyses of cadmium for all laboratories are shown in tables 13 - 17. The measured concentrations are similar among partners although nominal concentrations could not always be reached. In total, 76.9% of the nominal concentrations were achieved. Because of this, measured

TWM concentrations were used to calculate effect concentrations. Initial concentrations varied between 69.6% and 304% of nominal concentrations. After two or three days values ranged between 3.27% and 88.4%. The degradation of cadmium might be due to adsorption on test vessel walls, food particles or direct uptake of cadmium by animals (JENSEN ET AL. 2001, PASCOE ET AL. 1990).

64. The measured concentrations of cadmium in controls were below the limit of determination (0.3 µg/L), except for one sample from laboratory 2E. Here, at day 7 the measured concentration was 0.9 µg/L, which could not be rediscovered two days later (day 9).

65. Noticeable are the concentrations of laboratory 2G on day 0 and day 7, where up to 664% of nominal concentrations were achieved. An explanation for this could be a mistake in the preparation of spiking solutions. Nevertheless, these abnormalities in concentrations could not be observed on the other sampling days.

Table 13: Results of Cd analyses in exposure media from laboratory 2Aa [µg/L]. *Italic: not included in TWM calculations.*

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
1.56 µg/L	1.3	0.7	1.0	0.7	1.2	0.8	1.1	1.6	1.07	68.6
3.13 µg/L	2.6	1.6	2.4	1.6	2.3	1.8	2.6	1.9	2.09	66.8
6.25 µg/L	5.8	3.7	5.5	3.8	5.1	3.8	5.4	4.1	4.62	73.9
12.5 µg/L	12.0	7.7	11.3	8.2	10.8	8.8	11.6	16.5	11.1	88.8
25.0 µg/L	No sample	17.1	23.0	17.9	20.8	18.9	24.2	19.4	20.8	83.2

LOD = Limit of determination (0.03 µg/L) n.c. = not calculable

Table 14: Results of Cd analyses in exposure media from laboratory 2E [µg/L].

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	< LOD	< LOD	0.9	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
1.56 µg/L	1.4	< LOD	1.4	0.9	1.4	1.0	1.5	0.9	1.06	67.9
3.13 µg/L	2.7	< LOD	3.0	1.9	2.7	2.2	3.1	2.0	2.14	68.3
6.25 µg/L	5.7	< LOD	5.6	4.1	6.2	4.1	6.2	3.9	4.24	67.8

12.5 µg/L	11.0	1.1	11.3	7.5	12.6	10.2	13.3	8.1	9.03	72.2
25.0 µg/L	22.4	1.8	22.9	15.4	25.5	20.5	26.1	15.2	17.8	71.3

LOD = Limit of determination (0.03 µg/L) n.c. = not calculable

Table 15: Results of Cd analyses in exposure media from laboratory 2F [µg/L]. *Italic: not included in TWM calculations.*

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
1.56 µg/L	1.3	0.7	1.3	0.9	1.3	0.7	1.5	0.9	1.06	67.9
3.13 µg/L	2.9	1.5	2.8	2.4	3.0	1.9	2.9	1.9	2.37	75.7
6.25 µg/L	5.9	3.4	5.9	4.3	6.1	3.6	5.9	3.6	4.74	75.8
12.5 µg/L	12.1	7.1	12.0	9.4	12.1	No sample	12.0	10.6	10.6	84.8
25.0 µg/L	23.6	16.4	23.4	23.9	24.0	16.9	24.0	17.6	21.0	84.0

LOD = Limit of determination (0.03 µg/L) n.c. = not calculable

Table 16: Results of Cd analyses in exposure media from laboratory 2G [µg/L].

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
1.56 µg/L	7.5	0.6	1.3	0.9	< LOD	0.8	1.8	1.0	1.41	90.4
3.13 µg/L	3.4	1.6	3.1	2.2	2.2	1.9	3.8	1.9	2.48	79.2
6.25 µg/L	41.5	2.8	26.4	3.7	5.5	3.8	5.4	4.5	8.42	135
12.5 µg/L	26.5	7.1	8.4	8.7	13.8	7.4	6.5	9.6	10.1	80.9
25.0 µg/L	14.1	15.9	43.0	16.9	15.8	17.5	25.9	19.0	15.9	63.5

LOD = Limit of determination (0.03 µg/L) n.c. = not calculable

Table 17: Results of Cd analyses in exposure media from laboratory 2H [$\mu\text{g/L}$].

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
1.56 $\mu\text{g/L}$	1.2	< LOD	1.5	0.9	1.5	0.6	1.4	0.7	0.95	60.9
3.13 $\mu\text{g/L}$	2.3	1.0	3.0	2.7	2.4	1.2	3.3	1.9	2.21	70.6
6.25 $\mu\text{g/L}$	4.8	1.5	7.4	6.1	6.2	2.8	6.0	4.3	4.78	76.5
12.5 $\mu\text{g/L}$	9.7	4.4	13.4	10.0	11.5	6.0	14.0	6.4	9.19	73.5
25.0 $\mu\text{g/L}$	20.3	10.6	25.8	19.5	24.1	14.0	25.2	13.1	18.6	74.4

LOD = Limit of determination (0.03 $\mu\text{g/L}$) n.c. = not calculable

5.4.3.2 Biological responses

Mortality

66. In laboratories 2Aa, 2E, 2G and 2H the maximal observed mortality was 5.1% throughout the test. In laboratory 2F a mortality of 37.5% occurred at the highest test concentration (21.0 $\mu\text{g/L}$), which was significant compared to control group (Fisher's exact test, $p < 0.0001$). The maximal mortality at the other cadmium concentrations was 7.5%.

Reproduction

67. In figure 6 the total embryo numbers of *P. antipodarum* after four weeks of exposure from all participating laboratories are shown. All of them observed a significant reduction in embryo numbers with increasing cadmium concentrations. Laboratory 2Aa, 2G and 2H found comparable NOEC (11.1 $\mu\text{g/L}$, 10.1 $\mu\text{g/L}$, 9.19 $\mu\text{g/L}$) and LOEC (20.8 $\mu\text{g/L}$, 15.9 $\mu\text{g/L}$, 18.6 $\mu\text{g/L}$) values, whereas laboratory 2E and 2F showed slightly lower values (NOEC: 4.24 $\mu\text{g/L}$, 4.74 $\mu\text{g/L}$; LOEC: 9.03 $\mu\text{g/L}$, 10.6 $\mu\text{g/L}$). Snails thus showed the highest sensitivity towards cadmium in these two laboratories. Indeed, the animals of laboratory 2F might have been in a bad condition, because only in this laboratory a significant mortality at the highest test concentration (21.0 $\mu\text{g/L}$) was observed.

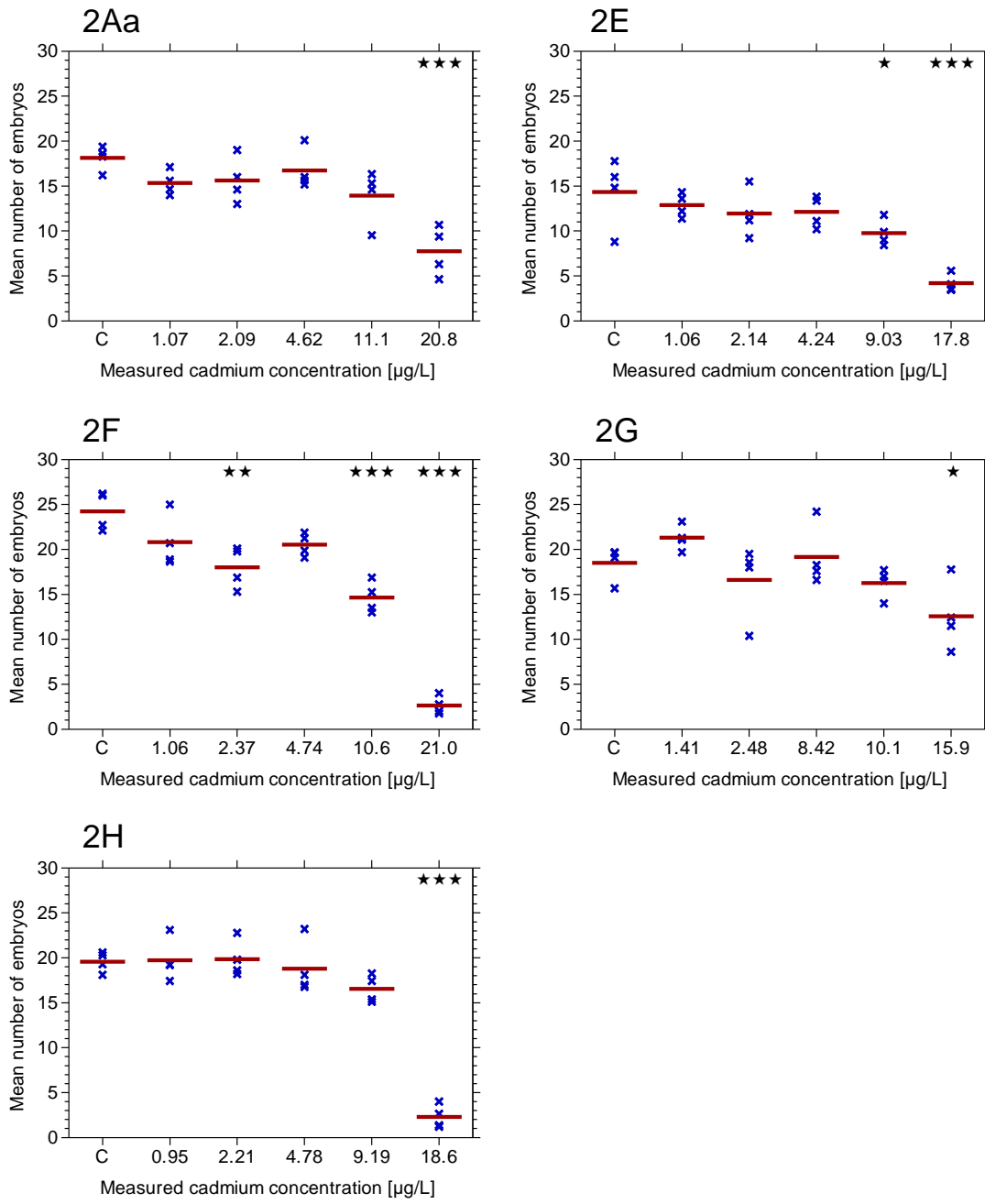


Figure 6: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks of exposure to cadmium at laboratories 2Aa, 2E, 2F, 2G and 2H (x: replicate mean, —: mean of the treatment group, 4 replicates with 10 snails each).

68. The good inter-laboratory reproducibility becomes apparent in the calculated effect concentrations (see table 18 and figure 7). The EC₁₀ values ranged from 6.10 µg/L to 10.3 µg/L and the EC₅₀ from 12.8 µg/L to 18.5 µg/L.

69. In figure 7 the effect concentrations including the 95%-confidence intervals for cadmium from each laboratory of validation I and validation II are shown. Taken into account that laboratory 1B might be an outlier, most of the partners overlap with their 95%-confidence intervals which demonstrates the robustness of the test.

Table 18: Effect concentrations (EC₁₀ and EC₅₀ with 95%-confidence intervals, NOEC, LOEC) for total embryo number based on time weighted means of measured concentrations in µg Cd/L.

	Laboratory 2Aa	Laboratory 2E	Laboratory 2F	Laboratory 2G	Laboratory 2H
EC ₁₀	7.19 (4.37 - 11.8)	6.10 (3.88 - 9.59)	7.74 (6.3 - 9.78)	10.3 (6.61 - 16.2)	8.47 (7.15 - 10.0)
EC ₅₀	18.5 (15.1 - 22.5)	13.5 (11.3 - 16.0)	13.1 (11.6 - 14.8)	19.4 (13.5 - 27.9)	12.8 (11.4 - 14.3)
NOEC	11.1	4.24	4.74	10.1	9.19
LOEC	20.8	9.03	10.6	15.9	18.6

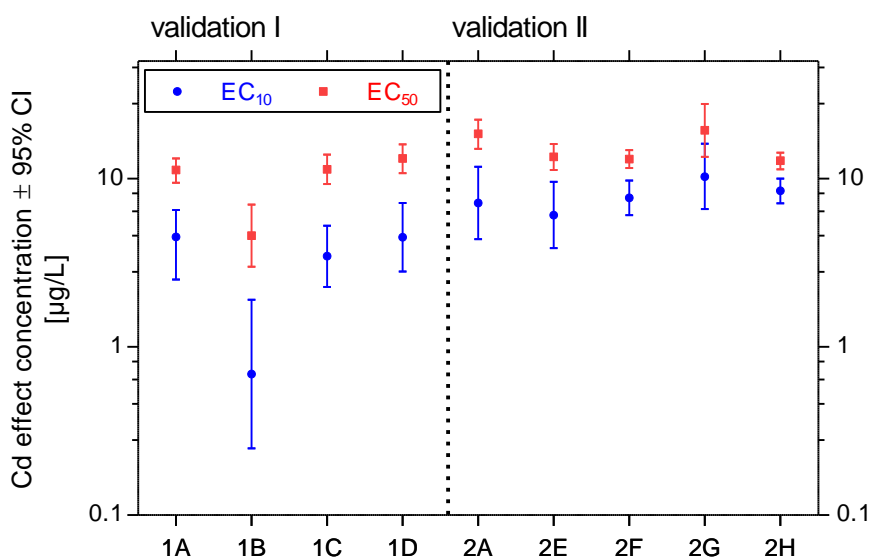


Figure 7: Calculated effect concentration (EC₁₀ in blue, EC₅₀ in red) values (µg/L) including 95% confidence intervals of the reproduction test with *Potamopyrgus antipodarum* with cadmium from all participating laboratories of validation I and validation II.

5.4.4 Results of validation II with tributyltin

5.4.4.1 Actual exposure concentrations

70. The results for chemical analyses (Tab. 19 - 26) show that measured TBT concentrations were below nominal concentrations in most of the laboratories. Initial concentrations varied between 6.31% and 285% of nominal concentrations. Calculated time weighted mean values varied between 10.1% and 121% of the nominal concentrations. Average time weighted mean for all laboratories was 44.2%. An explanation for the low exposure concentrations could be the degradation of TBT to monobutyltin and dibutyltin during the exposure. Another reason might be the adsorption of the substance to the vessel wall and to food particles due to the low solubility of TBT and/or by direct uptake by the snails.

71. In the test 2Ia, no TBT could be found because of a mistake during stock solution preparation. In all laboratories no TBT could be detected in the solvent controls.

Table 19: Results of TBT analyses in exposure media from laboratory 2Aa [ng TBT-Sn/L].

Nominal concentration [TBT-Sn]	Measured concentration				Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
10 ng/L	3.69	2.87	5.33	4.10	4.11	41.1
25 ng/L	27.5	25.4	71.3	11.9	30.5	121
65 ng/L	41.8	31.1	21.3	33.6	30.7	47.2
160 ng/L	58.6	52.5	50.8	62.7	56.1	35.1
400 ng/L	229	122	123	57.8	120	29.9

LOD = Limit of detection (0.82 ng Sn/L) n.c. = not calculable

Table 20: Results of TBT analyses in exposure media from laboratory 2Ab [ng TBT-Sn/L].

Nominal concentration [TBT-Sn]	Measured concentration				Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	n.b.	n.b.
25 ng/L	33.6	11.5	26.2	10.7	18.6	74.4
65 ng/L	67.2	19.3	37.7	9.84	27.8	42.7
160 ng/L	109	19.7	118	14.8	50.8	31.8
400 ng/L	455	326	185	77.0	229	57.2
1000 ng/L	885	889	459	1291	838	83.8

LOD = Limit of detection (0.82 ng Sn/L) n.c. = not calculable

Table 21: Results of TBT analyses in exposure media from laboratory 2E [ng TBT-Sn/L]. *Italics: not included in TWM calculations.*

Nominal concentration [TBT-Sn]	Measured concentration				Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	n.c..	n.c.
10 ng/L	<i>No sample</i>	29.9	4.10	6.97	5.40	50.4
25 ng/L	<i>No sample</i>	41.8	9.43	65.2	28.8	115
65 ng/L	11.1	109	51.2	19.3	36.8	56.6
160 ng/L	54.1	220	103	63.9	96.3	60.2
400 ng/L	107	541	219	99.6	198	49.6

LOD = Limit of detection (0.82 ng Sn/L) n.c. = not calculable

Table 22: Results of TBT analyses in exposure media from laboratory 2F [ng TBT-Sn/L].

Nominal concentration [TBT-Sn]	Measured concentration				Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	n.c..	n.c.
10 ng/L	2.46	2.05	2.46	3.28	2.61	26.1
25 ng/L	5.36	4.10	3.67	5.74	4.66	18.6
65 ng/L	4.10	6.56	13.5	10.7	9.30	14.3
160 ng/L	11.1	7.79	26.2	16.0	16.1	10.1
400 ng/L	44.7	85.7	20.9	36.5	41.8	10.5

LOD = Limit of detection (0.82 ng Sn/L) n.c. = not calculable

Table 23: Results of TBT analyses in exposure media from laboratory 2G [ng TBT-Sn/L].

Nominal concentration [TBT-Sn]	Measured concentration				Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	n.c..	n.c.
10 ng/L	6.15	2.46	4.51	4.51	4.30	43.0
25 ng/L	10.7	21.3	10.2	11.1	12.5	50.1
65 ng/L	21.3	7.79	24.6	108	39.2	60.3
160 ng/L	44.3	25.0	61.9	34.4	41.4	25.8
400 ng/L	118	84.4	158	147	132	32.9

LOD = Limit of detection (0.82 ng Sn/L) n.c. = not calculable

Table 24: Results of TBT analyses in exposure media from laboratory 2H [ng TBT-Sn/L].

Nominal concentration [TBT-Sn]	Measured concentration				Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	n.c..	n.c.
10 ng/L	2.05	< LOD	4.51	3.69	3.04	30.4
25 ng/L	6.15	2.87	2	11.1	7.75	31.0
65 ng/L	20.9	16.8	32.0	7.38	17.6	27.0
160 ng/L	32.0	2.87	95.1	23.8	35.7	22.3
400 ng/L	88.5	11.5	216	74.6	94.9	23.7

LOD = Limit of detection (0.82 ng Sn/L) n.c. = not calculable

Table 25: Results of TBT analyses in exposure media from laboratory 2I [ng TBT-Sn/L].

Nominal concentration [TBT-Sn]	Measured concentration				Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	n.c..	n.c.
10 ng/L	6.15	6.56	9.43	4.10	6.39	63.9
25 ng/L	6.15	12.7	5.33	16.0	9.43	37.7
65 ng/L	29.1	13.9	13.52	13.1	16.2	25.0
160 ng/L	121	22.5	34.4	16.4	38.0	23.7
400 ng/L	180	37.3	97.5	28.3	69.7	17.4

LOD = Limit of detection (0.82 ng Sn/L) n.c. = not calculable

Table 26: Results of TBT analyses in exposure media from laboratory 2K [ng TBT-Sn/L].

Nominal concentration [TBT-Sn]	Measured concentration				Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	n.c..	n.c.
10 ng/L	14.8	5.74	10.2	4.10	7.84	78.4
25 ng/L	6.56	7.38	5.74	4.92	5.97	23.9
65 ng/L	79.1	11.5	108	25.0	48.0	73.8
160 ng/L	136	75.0	256	76.2	130	81.3
400 ng/L	296	170	340	154	232	58.0

LOD = Limit of detection (0.82 ng Sn/L) n.c. = not calculable

5.4.4.2 Biological responses

Mortality

72. The mortality of control snails was homogenous between the laboratories and did not exceed 5.0%, except in laboratory 2Jb. There a mortality of 30.0% was observed in the solvent control which is significant compared to the negative control (Fig. 8). Therefore laboratory 2Jb did not meet the validity criterion for mortality (max. 20%) and was excluded from the evaluation of reproduction data.

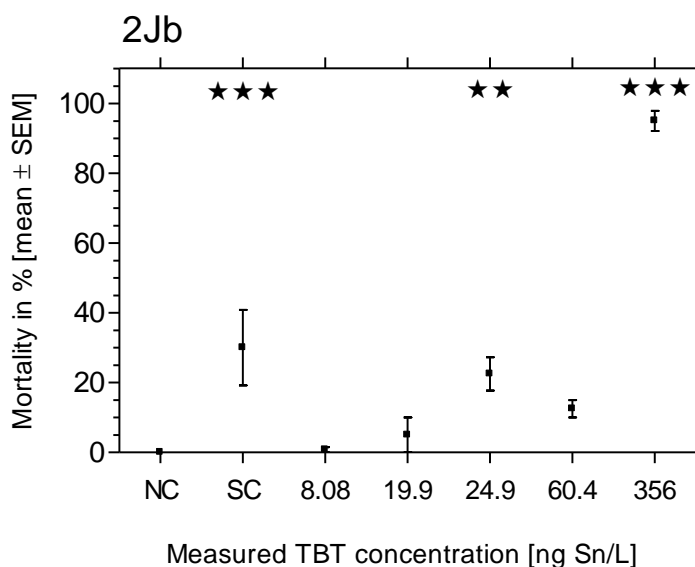


Figure 8: Mortality [in %] of *Potamopyrgus antipodarum* during 4 weeks of exposure to TBT at laboratory 2Jb (time weighted mean of measured concentrations with standard error, 4 replicates with 10 snails each). Asterisks indicate significant differences compared to negative control (NC) (Fisher's exact test), ** = $p < 0.01$, *** = $p < 0.001$).

73. In experiments with a maximum nominal concentration of 400 ng TBT-Sn/L mortality in TBT exposed snails did not exceed 5.0% in laboratories 2Aa, 2E, 2F, 2G, 2H and 2I. In laboratory 2Ab (Figure 9) with a maximum tested nominal concentration of 1000 ng TBT-Sn/L mortality increased up to 87.5% at the highest tested concentration ($p < 0.0001$).

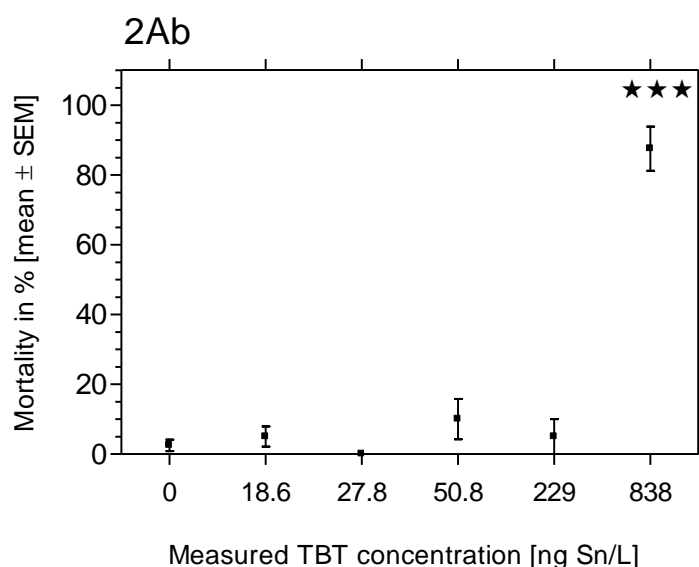


Figure 9: Mortality [in %] of *Potamopyrgus antipodarum* during 4 weeks of exposure to TBT at laboratory 2Ab (time weighted mean of measured concentrations with standard error, 4 replicates with 10 snails each). Asterisks indicate significant differences compared to control (Fisher's exact test), *** = $p < 0.0001$).

Reproduction

74. Figure 10 shows the results of the reproduction tests with *Potamopyrgus antipodarum* of all participating labs which met the validity criteria. All laboratories found a concentration-dependent decrease of embryo numbers in the brood pouch of *P. antipodarum* under exposure to TBT, although with slightly different effect concentrations. Laboratories 2Aa, 2Ab, 2F, 2G, 2H and 2I provided comparable NOECs (30.7, 18.6, 16.1, 39.2, 35.7 and 38.0 ng Sn/L) and LOECs (56.1, 27.8, 41.8, 41.4, 94.9 and 69.7/L). The results from laboratory 2E resulted in considerably higher effect concentrations with a significant reduced number of embryos at 198 ng TBT-Sn/L (LOEC). Results from laboratory 2Ja with no effects of TBT on reproduction (due to a low dosing of TBT) are provided in Annex 2.

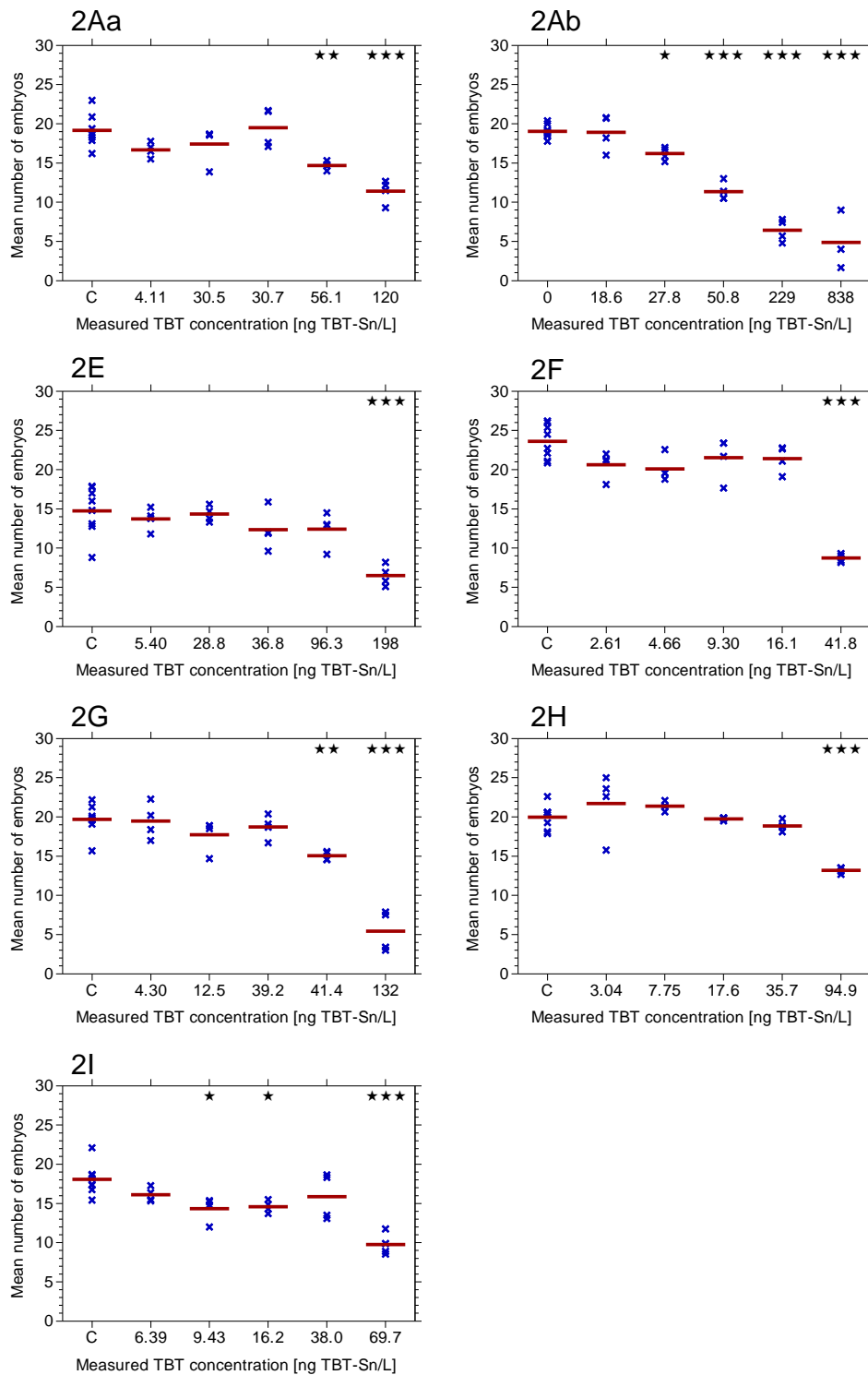


Figure 10: Total embryo numbers of *Potamopyrgus antipodarum* after 4 weeks of exposure to TBT at laboratory 2Aa, 2Ab, 2E, 2F, 2G, 2H, and 2I (x: replicate mean, —: mean of the treatment group, 4 replicates with 8 - 10 snails each, in 2Ab at 838 ng/L: 3 replicates with 1-3 snails each). Asterisks indicate significant differences compared to control (Dunnett's test), * = p<0.05, ** = p<0.01, *** = p < 0.001.

75. The good match of results for TBT between laboratories is also reflected by the calculated EC_{10} and EC_{50} values (Tab. 27, Figure 11). The findings from laboratory 2E resulted in higher EC_{10} values whose 95%-confidence intervals only overlapped with the EC_{10} confidence interval of laboratory 2H. Laboratory 2F showed lowest effect concentrations with an EC_{50} of 37.2 ng Sn/L.

Table 27: Effect concentrations (EC_{10} and EC_{50} with 95%-confidence intervals, NOEC, LOEC) for total embryo number based on time weighted means of measured concentrations in ng TBT-Sn/L. n.d.: not defined. *Italics:* data from laboratories with non-valid test results.

	2Aa	2Ab	2E	2F	2G	2H	2I	2Jb	2K
EC_{10}	45.0	12.7	89.1	22.4	36.8	36.5	6.62	-	-
95%-CI	28.4 – 71.2	5.73 – 28.3	53.2 - 149	15.6 – 32.3	26.1 – 51.7	20.4 – 65.0	0.95 – 45.9	-	-
EC_{50}	153	124	188	37.9	88.8	137	159	-	-
95%-CI	109 - 213	77 - 200	157 - 226	28.5 – 50.4	73.5 - 107	93.2 - 200	48.8 - 519	-	-
NOEC	30.7	18.6	96.3	16.1	39.2	35.7	38.0	8.08	130
LOEC	56.1	27.8	198	41.8	41.4	94.9	69.7	19.9	232

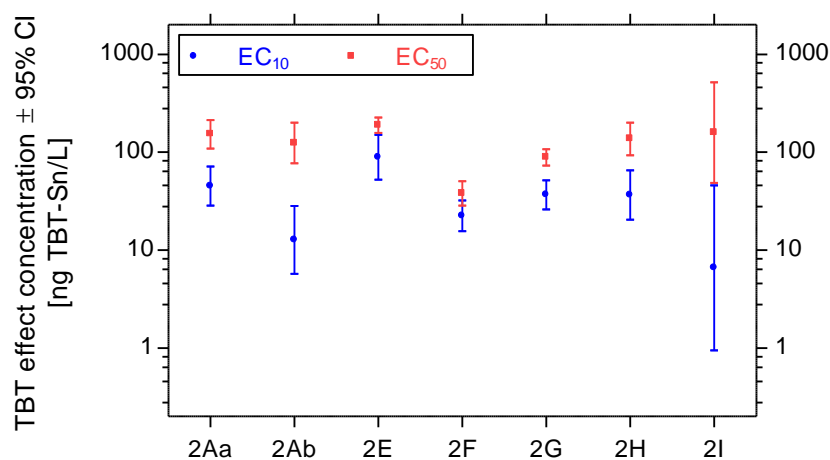


Figure 11: Calculated effect concentration (EC_{10} in blue, EC_{50} in red) values (ng Sn/L) including 95% confidence intervals of the reproduction test with *Potamopyrgus antipodarum* with TBT from all valid tests conducted in the participating laboratories.

5.5 Comparison of present results to published and grey literature

76. The effect concentrations for TBT obtained in the ring tests are in good accordance with published data. Exposure to TBT, conducted *inter alia* in experiments during the EU-project COMPRENDO (project code: EVK1-CT-2002-00129) resulted in an identical response pattern. The total embryo number significantly decreased resulting in an EC₅₀ of 115 ng Sn/L, an EC₁₀ of 37.8 ng Sn/L, and a LOEC of 50 to 100 ng Sn/L after 8 weeks exposure (SCHULTE-OEHLMANN 1997; DUFT et al. 2007).

77. Compared to other invertebrates, e.g. *Daphnia magna*, *P. antipodarum* shows a higher sensitivity for TBT. The NOEC for TBT in *D. magna* reproduction is 456 ng Sn/L (OBERDÖRSTER et al. 1997); a LOEC could not be assessed due to high mortality at the highest test concentration of 912 ng Sn/L (LOEC_{mortality}). MATHIJSEN-SPIEKMAN et al. (1989) also assessed higher effect concentrations in a 21 day reproduction test with TBT-oxide and *D. magna*. The detected LOEC for reproduction was 1.8 µg TBTO/L (\cong 716 ng TBT-Sn/L), the NOEC 1.0 µg TBTO/L (\cong 398 ng TBT-Sn/L).

5.6 Conclusions from validation II

78. In summary, it can be stated that the results of the reproduction tests with cadmium showed a good agreement among participating laboratories. The mean values (with coefficient of variation) for EC₁₀, EC₅₀, NOEC and LOEC from these five laboratories are 7.96 µg/L (19.7%), 15.5 µg/L (20.8%), 7.87 µg/L (40.2%) and 15.0 µg/L (33.8%), respectively. If results of validation I (1B excluded) are also considered, than the mean values for EC₁₀, EC₅₀, NOEC and LOEC are 6.53 µg/L (35.5%), 14.2 µg/L (21.8%), 6.45 µg/L (50.5%) and 12.6 µg/L (42.2%), respectively with a minimum of a 1.72-fold difference (EC₅₀ values) and a maximum of a 3.91-fold difference (LOEC values).

79. Furthermore, also the results of the reproduction tests with TBT showed a good accordance among partners. Effect concentrations show a minimum of a 4.78-fold difference (LOECs) and a maximum of a 13.5-fold difference (EC₁₀ values). The mean values (with coefficient of variation) for EC₁₀, EC₅₀, NOEC and LOEC values from these laboratories are 35.6 ng Sn/L (76.9%), 127 ng Sn/L (39.3%), 39.2 ng Sn/L (68.3%) and 75.7 ng Sn/L (77.0%), respectively. In addition no significant mortality occurred during the experiments except for the highest additionally tested concentration of 838 ng Sn/L at laboratory 2Ab.

80. Some uncertainties concerning the measured concentrations might have influenced the results, because time weighted means have been calculated based on only two measuring intervals, due to the high costs of analytical measurements.

81. All in all it can be concluded, that the reproduction test with *P. antipodarum* turned out to be a well-suited tool for the investigation of reproductive toxicants and also for endocrine disrupting chemicals such as TBT.

6. VALIDATION III: TRENBOLONE AND PROCHLORAZ

6.1 Organisation of the validation test

82. Eight laboratories participated in the third ring test with *Potamopyrgus antipodarum* which started in June 2014. The partners were provided with snails, salts for preparing the medium and also the test substances, except laboratory 3P. Trenbolone and prochloraz were chosen as test chemicals, which had been used as reference chemicals in other recent OECD validation tests.

6.1.1 Snail production, biological quality checking and shipping

83. Snails used for the experiments came from the same laboratory culture which has been run under the same conditions as described in section 5.1.1 for validation II. Only laboratory 3P used animals from their own laboratory culture. These snails were acclimatized for 28 days to the proposed medium. Normally these snails are cultured in a different medium.

84. Table 28 summarizes the shipping and acclimation duration and gives an overview of the test schedules. Post shipping mortality did not occur in the partner laboratories.

Table 28: Shipping, acclimation and test schedules for the partner laboratories.

Partner	3A	3D	3H	3L	3M	3N	3O	3P
Snail shipping date	-	25/06/14	30/07/14	16/07/14	16/06/14	29/07/14	11/08/14	-
Snails received	-	27/06/14	31/07/14	17/07/14	17/06/14	30/07/14	12/08/14	-
Number of snails sent	-	500	500	500	500	500	500	-
Acclimation duration	-	23 d	18 d	18 d	13 d	14 d	20 d	28 d
Test starting date	07/07/14	21/07/14	18/08/14	04/08/14	30/06/14	13/08/14	01/09/14	16/06/14
Test ending date	04/08/14	28/08/14	15/09/14	01/09/14	28/07/14	10/09/14	29/09/14	14/07/14

6.2 Implementation of the 28-day reproduction test

6.2.1 Principle of the test

85. The principle of the reproduction test with *P. antipodarum* and the test procedure is the same as in validation II (see section 5.2.1). Only the number of replicates and the number of introduced snails per replicate are modified (see section 6.2.3).

6.2.2 Chemicals

86. As test chemicals trenbolone (CAS-No.: 10161-33-8, Sigma-Aldrich®, Germany) and prochloraz (CAS-No.: 67747-09-5, Sigma-Aldrich®, Germany) were chosen. For both substances DMSO was used as a solvent at a concentration of 10 µL/L. Therefore, an additional solvent control group was considered. Trenbolone is a synthetic mammalian androgen and was tested at the following nominal concentrations:

0.01 µg/L, 0.03 µg/L, 0.1 µg/L, 0.3 µg/L, 1 µg/L.

87. Prochloraz belongs to the imidazole fungicides. This chemical was tested at the following nominal concentrations:

3.2 µg/L, 10 µg/L, 32 µg/L, 100 µg/L, 320 µg/L.

6.2.3 Experimental conditions

88. The experimental conditions were the same as in validation II. Only the number of replicates was enhanced to six and the number of snails per replicate was six to increase the statistical power of the test. Experimental conditions are summarized in table 29.

Table 29: Summary of main experimental conditions.

Test duration	28 days
Test water	Reconstituted water (with 0.3 g Tropic Marin® salt and 0.18 g NaHCO ₃ per 1 litre de-ionised water) water quality requirements: pH 7.5 – 8.5, conductivity 770 ± 100 µS/cm, oxygen concentration > 80% ASV (air saturation value)
Test vessels	500 mL glass beakers with lids
Water renewal	3 times per week
Temperature	16 ± 1°C
Light intensity	500 ± 100 lux
Water sampling	From all test concentrations and solvent control water was sampled over four renewal intervals for trenbolone and prochloraz
Photoperiod	16:8 h L:D
Food source	Fine grounded Tetraphyll®
Feeding	0.25 mg/animal and day
Snails origin	Laboratory culture, which was built up with snails from Lumda Hesse, Germany
Test snails size	3.5 – 4.5 mm
Snails density	6 snails per 400 mL medium (6 replicates per tested concentration)
Core test endpoints	Mortality, reproduction

6.3 Chemical analysis and biological data analysis

89. Every week of the test, samples from prochloraz and trenbolone exposure groups and solvent control were taken before (2 or 3 days old) and after (fresh) medium renewal for chemical analysis. Samples from old medium were pooled from every replicate of one treatment group. Samples were stored in HDPE-bottles at -20°C until analysis.

90. Trenbolone and prochloraz samples were analysed by LC-MS-MS (Agilent 1200 series triples quadrupole) with detection limits of 0.39 ng/L and 1.56 µg/L, respectively. Trenbolone samples were

extracted on solid-phase columns with methyl-testosterone as internal standard before analysis whereas prochloraz was analysed directly from filtered samples.

91. Time weighted mean (TWM) concentrations of the chemicals were calculated according to Annex 6 of OECD guideline 211 (OECD, 2012).

92. Biological raw data were reported by the participating laboratories using an Excel® spread sheet which had been provided by Goethe University. From the raw data means were calculated for biological parameters (shell height, embryo numbers). Effect concentrations were calculated by Dunnett's test (NOEC, LOEC) or by a non-linear regression using a four parameter logistic equation (EC₁₀, EC₅₀).

6.4 Results

6.4.1 Compliance with validity criteria

93. For a test to be valid the conditions were chosen based on available guidelines for freshwater invertebrates and as proposed in OECD (2010). The validity criteria were amended by a biological criterion requiring a minimum mean embryo number in the controls:

- mortality in the controls should not exceed 20%,
- mean embryo number per snail in the controls should be ≥ 5 ,
- dissolved oxygen saturation must have been at least 60% of the air saturation value (ASV) and
- water temperature should be $16 \pm 1^\circ\text{C}$ throughout the test.

94. All laboratories achieved adequate oxygen saturation values. The mean ASV for oxygen ranged from 93.6% (laboratory 3N) to 99.6% (laboratory 3O). All participants achieved the required temperature with a mean temperature between 15.7°C and 16.4°C. Laboratory 3H exceeded the validity criterion for the maximum control mortality in the negative control (22.2%) and the solvent control (30.6%) (Figure 12). In the exposure groups with trenbolone in laboratory 3H mortality of snails was in the same range. In prochloraz exposed snails no mortality was observed. The high mortality in control groups and trenbolone exposed snails was probably caused by fungus growth in the vessels during the test. Hence, the fungicide prochloraz prevented fungal growth and therefore reduced snail mortality. In laboratory 3P the mean embryo number in the controls was 1.08 and thus below the validity criterion of ≥ 5 . Hence, the tests from laboratories 3H and 3P were not valid and for this reason reproduction data are not considered here.

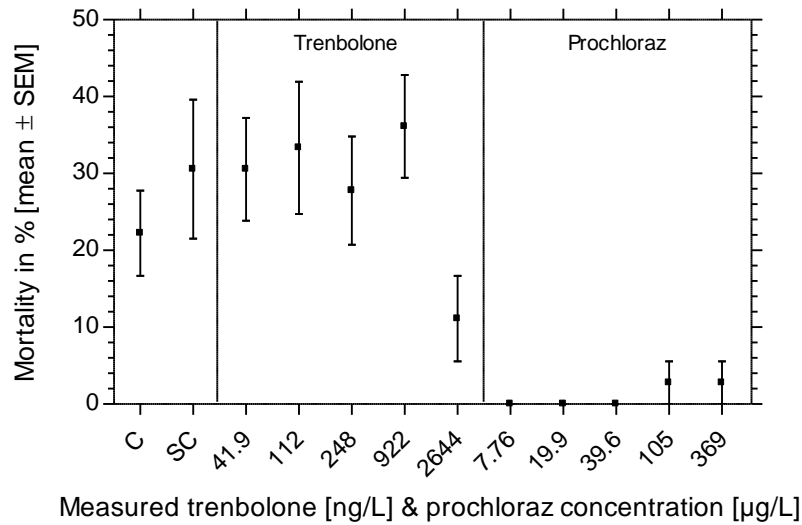


Figure 12: Mortality [in %] of *Potamopyrgus antipodarum* during four weeks of exposure in the control (C), solvent control (SC) and in trenbolone (TR) and prochloraz (PCZ) exposure groups at laboratory 3H (6 replicates with 6 snails each).

6.4.2 Physico-chemical parameters

95. Table 30 summarizes the means of the physico-chemical parameters for all laboratories. The pH values ranged between 7.58 (laboratory 3P) to 8.44 (laboratory 3L) which is in the required range of 7.5 - 8.5. The same applies to the values for conductivity which is similar among all partners. Mean temperatures in all laboratories were also within the validity criterion of $16 \pm 1^\circ\text{C}$.

Table 30: Mean physico-chemical parameters of all laboratories. n.r.: not received

Laboratory	pH			Conductivity [$\mu\text{S}/\text{cm}$]			Temperature [$^\circ\text{C}$]			O ₂ saturation [%]		
	mean	SD	n	mean	SD	N	mean	SD	n	mean	SD	n
3A	8.28	0.69	145	791	39.9	145	16.2	0.61	145	96.1	3.06	145
3D	8.26	0.68	156	750	23.2	156	15.9	0.44	156	99.3	1.41	156
3H	8.33	0.68	156	725	37.3	156	15.9	0.21	156	94.4	4.20	144
3L	8.44	0.67	156	718	23.6	156	16.5	0.34	156	98.4	2.31	156
3M	8.11	0.65	156	751	30.2	156	16.4	0.85	157	99.4	4.07	156
3N	8.24	0.66	156	722	18.6	156	15.8	0.34	156	93.6	6.95	156
3O	8.16	0.68	144	818	15.1	145	16.3	0.52	140	99.6	1.75	145
3P	7.58	0.74	156	n.r.	n.r.	n.r.	15.7	0.57	157	n.r.	n.r.	n.r.

6.4.3 Results of validation III with trenbolone

6.4.3.1 Actual exposure concentrations

96. The results of the analytical analyses of trenbolone for all laboratories are shown in tables 31 - 36. In total, 152% of the nominal concentrations were achieved. Because of this, measured TWM concentrations were used to calculate effect concentrations. Initial concentrations varied between 41.6% and 704% of nominal concentrations. After two or three days values ranged between 8.98% and 398%. In all laboratories, with the exception of laboratory 3N, the measured concentrations were higher than nominal concentrations. Especially the highest exposure concentrations were considerably higher compared to nominals. In laboratory 3N the measured concentrations varied between 50% and 105% of nominal concentrations.

97. The measured concentrations of trenbolone in controls were below the limit of determination (0.39 ng/L), except for samples from laboratory 3M and 3L. In laboratory 3M trenbolone was detected in all control samples indicating a continuous exposure of control snails to the test compound. The calculated TWM was 14 ng/L. In laboratory 3L trenbolone was detected in five out of eight samples. The measured concentrations varied between 1.25 – 33.2 ng/L; a TWM could not be calculated.

Table 31: Results of trenbolone analyses in exposure media from laboratory 3A [$\mu\text{g/L}$].

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
10 ng/L	11.8	12.6	16.0	14.9	9.46	14.3	13.4	14.5	13.3	133
30 ng/L	31.0	33.3	36.1	48.2	28.3	32.5	32.9	34.4	34.4	115
100 ng/L	134	151	150	163	115	118	130	109	132	132
300 ng/L	327	417	335	387	385	406	355	376	372	124
1000 ng/L	1743	1148	1154	1206	1534	1429	1398	1395	1373	137

LOD = Limit of determination (0.39 ng/L) n.c. = not calculable

Table 32: Results of trenbolone analyses in exposure media from laboratory 3D [ng/L]. *Italics: not included in TWM calculations.*

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
10 ng/L	20.3	15.4	19.5	16.8	23.6	15.0	25.4	19.9	19.7	197
30 ng/L	71.5	42.7	69.2	44.1	61.0	43.1	70.1	44.7	55.1	184
100 ng/L	493	23.0	690	428	429	<i>No sample</i>	759	122	350	350
300 ng/L	2159	1161	2433	1217	2406	1678	2573	1612	1882	627
1000 ng/L	5123	3600	5698	3952	4832	3406	7043	4218	4763	476

LOD = Limit of determination (0.39 ng/L) n.c. = not calculable

Table 33: Results of trenbolone analyses in exposure media from laboratory 3L [ng/L].

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	22.8	< LOD	< LOD	1.25	14.8	33.2	2.42	< LOD	n.c.	n.c.
10 ng/L	40.9	38.1	36.6	27.6	43.5	25.1	36.4	16.2	31.6	316
30 ng/L	123	54.2	115	70.4	114	41.2	80.8	46.2	75.5	252
100 ng/L	309	94	259	185	397	296	250	89.2	217	217
300 ng/L	569	238	688	529	587	530	695	178	469	156
1000 ng/L	4663	1635	5204	3985	5468	3688	2252	1136	3205	321

LOD = Limit of determination (0.39 ng/L) n.c. = not calculable

Table 34: Results of trenbolone analyses in exposure media from laboratory 3M [ng/L].

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	2.93	2.71	23.5	12.1	17.8	37.4	12.2	11.3	14.0	n.c.
10 ng/L	22.3	25.8	30.1	25.7	41.7	24.6	29.3	23.1	27.4	274
30 ng/L	64.9	34.0	54.9	59.5	57.5	39.6	59.7	38.9	50.2	167
100 ng/L	223	185	179	164	201	168	164	131	173	173
300 ng/L	523	344	426	378	501	390	360	321	396	132
1000 ng/L	1899	1049	1918	1271	1374	1297	1283	960	1335	134

LOD = Limit of determination (0.39 ng/L) n.c. = not calculable

Table 35: Results of trenbolone analyses in exposure media from laboratory 3N [ng/L].

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
10 ng/L	9.45	7.63	9.03	5.65	9.35	3.23	10.3	14.9	8.92	89.2
30 ng/L	23.4	19.1	21.1	14.5	26.6	5.13	19.4	4.48	14.9	49.7
100 ng/L	91.5	76.8	142	46.3	41.6	27.8	101	17.0	60.9	60.9
300 ng/L	304	246	377	85.9	388	44	290	26.9	177	59.0
1000 ng/L	2266	1583	1899	680	1397	355	1242	201	1046	105

LOD = Limit of determination (0.39 ng/L) n.c. = not calculable

Table 36: Results of trenbolone analyses in exposure media from laboratory 3O [ng/L].

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
10 ng/L	16.0	15.0	9.0	17.0	22.0	17.0	17.0	20.0	16.7	167
30 ng/L	41.0	39.0	41.0	35.0	44.0	35.0	35.0	39.0	38.4	128
100 ng/L	156	152	175	136	213	183	196	153	170	170
300 ng/L	617	542	533	388	592	485	418	465	496	165
1000 ng/L	2345	1921	1778	1975	1709	1669	2123	2563	2043	204

LOD = Limit of determination (0.39 ng/L) n.c. = not calculable

6.4.3.2 Biological responses

Mortality

98. In laboratories 3A, 3D, 3M and 3O no mortality was observed in any of the exposure groups. In laboratory 3L the maximal observed mortality was 8.34% in the solvent control. In laboratory 3N a mortality of 2.78% occurred at the test concentration of 15 ng/L.

Reproduction

99. None of the participating laboratories found a concentration-dependent decrease of the embryo numbers in the brood pouch of *P. antipodarum* (Fig. 13). Only laboratory 3A did observe significant reduced embryo numbers at the two lowest concentrations, which were not detected at higher test concentrations. Laboratory 3L found a significant effect on reproduction of the solvent DMSO with a decreased number of embryos.

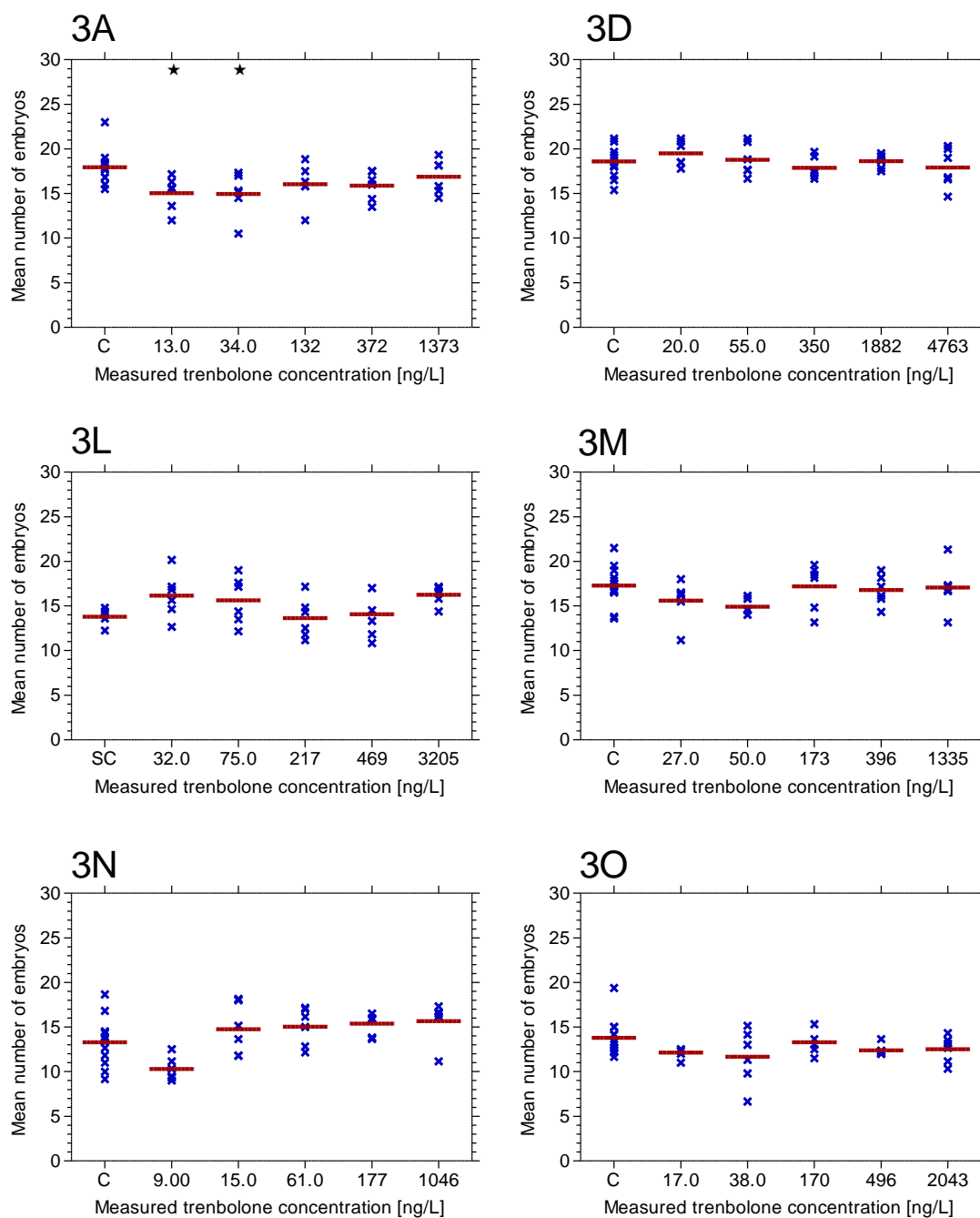


Figure 13: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks of exposure to trenbolone at laboratories 3A, 3D, 3L, 3M, 3N and 3O (x: replicate mean, —: mean of the treatment group, 6 replicates with 6 snails each). Asterisks at laboratory 3A indicate significant differences compared to control (Dunnett's test), ★ = $p < 0.05$.

6.4.4 Results of validation III with prochloraz

6.4.4.1 Actual exposure concentrations

100. The results of the analytical measurement of prochloraz for all laboratories are shown in tables 37 - 42. In total, 273% of the nominal concentrations were achieved. Therefore, TWM concentrations were used to calculate effect concentrations. Initial concentrations varied between 153% and 617% and after two or three days values varied between 169% and 506%. Measured concentrations of prochloraz in controls were below the LOD which was 1.56 µg/L except for laboratories 3D, 3L and 3N. Prochloraz was measured at a maximum concentration of 9.63 µg/L during the last two water renewal intervals in the controls of laboratory 3D (table 38). In laboratory 3L prochloraz was measured in every solvent control sample, indicating a continuous exposure to the test compound (table 39). Here, a TWM of 9.98 µg/L was calculated. Therefore, laboratory 3L was excluded from the evaluation of the ring test. For laboratory 3N only on day 2 a positive finding with a measured concentration of 1.20 µg/L was found in the control.

Table 37: Results of prochloraz analyses in exposure media from laboratory 3A [µg/L].

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
3.20 µg/L	11.7	13.6	9.47	11.0	8.39	11.8	7.97	10.1	10.3	322
10.0 µg/L	29.5	26.9	25.8	24.5	21.3	28.0	25.9	34.5	27.3	273
32.0 µg/L	50.0	54.5	51.1	47.4	47.4	51.7	55.2	51.7	51.4	161
100 µg/L	221	254	214	221	225	259	227	219	229	229
320 µg/L	551	567	513	493	629	599	675	602	585	183

LOD = Limit of determination (1.56 µg/L) n.c. = not calculable

Table 38: Results of prochloraz analyses in exposure media from laboratory 3D [$\mu\text{g/L}$].

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	< LOD	< LOD	< LOD	< LOD	4.00	9.34	9.63	9.32	n.c.	n.c.
3.20 $\mu\text{g/L}$	25.2	25.7	32.8	23.3	49.4	34.5	31.6	30.7	31.4	981
10.0 $\mu\text{g/L}$	51.9	49.1	53.1	78.9	73.9	64.6	53.4	49.5	58.2	582
32.0 $\mu\text{g/L}$	47.8	55.8	54.4	66.4	71.6	65.9	40.1	35.9	52.8	165
100 $\mu\text{g/L}$	237	240	257.0	245	337	392	277	186	266	266
320 $\mu\text{g/L}$	537	516	561	718	781	659	379	291	529	165

LOD = Limit of determination (1.56 $\mu\text{g/L}$) n.c. = not calculable

Table 39: Results of prochloraz analyses in exposure media from laboratory 3L [$\mu\text{g/L}$].

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	0.290	10.3	6.58	15.1	14.6	14.9	10.4	12.6	9.98	n.c.
3.20 $\mu\text{g/L}$	7.57	13.3	8.90	19.2	15.2	11.6	17.8	14.1	13.5	422
10.0 $\mu\text{g/L}$	21.3	29.9	28.2	31.0	23.9	21.2	20.9	23.5	24.6	256
32.0 $\mu\text{g/L}$	44.8	48.4	46.2	45.0	39.3	38.4	40.3	39.7	42.5	133
100 $\mu\text{g/L}$	195	176	171	178	140	145	149	143	160	160
320 $\mu\text{g/L}$	468	443	455	402	308	344	341	322	379	118

LOD = Limit of determination (1.56 $\mu\text{g/L}$) n.c. = not calculable

Table 40: Results of prochloraz analyses in exposure media from laboratory 3M [$\mu\text{g/L}$].

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
3.20 $\mu\text{g/L}$	15.8	19.3	22.8	22.7	27.3	23.2	22.5	26.4	22.7	709
10.0 $\mu\text{g/L}$	28.8	32.9	33.5	35.4	35.0	33.7	30.1	34.5	32.9	329
32.0 $\mu\text{g/L}$	55.2	60.1	55.6	57.7	71.7	57.0	55.2	57.0	58.3	182
100 $\mu\text{g/L}$	302	330	314	325	307	303	284	290	305	305
320 $\mu\text{g/L}$	702	733	702	713	593	620	503	548	626	196

LOD = Limit of determination (1.56 $\mu\text{g/L}$) n.c. = not calculable

Table 41: Results of prochloraz analyses in exposure media from laboratory 3N [$\mu\text{g/L}$].

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	< LOD	1.20	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
3.20 $\mu\text{g/L}$	8.74	8.79	11.4	10.7	10.6	10.9	10.5	11.3	10.4	325
10.0 $\mu\text{g/L}$	18.6	17.9	26.7	24.9	24.8	22.4	23.4	24.7	23.0	230
32.0 $\mu\text{g/L}$	40.5	37.7	51.7	45.2	23.1	24.5	48.7	45.7	40.4	126
100 $\mu\text{g/L}$	160	154	235	208	209	197	184	203	194	194
320 $\mu\text{g/L}$	375	375	561	466	502	498	491	471	468	146

LOD = Limit of determination (1.56 $\mu\text{g/L}$) n.c. = not calculable

Table 42: Results of prochloraz analyses in exposure media from laboratory 3O [$\mu\text{g/L}$].

Nominal concentration	Measured concentrations								Time weighted mean	% of nominal concentration
	Day 0: fresh medium	Day 2: old medium	Day 7: fresh medium	Day 9: old medium	Day 14: fresh medium	Day 16: old medium	Day 25: fresh medium	Day 28: old medium		
Control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
3.20 $\mu\text{g/L}$	13.9	19.4	14.6	12.1	11.3	13.2	6.69	8.47	11.9	372
10.0 $\mu\text{g/L}$	23.3	26.7	26.8	19.9	18.1	22.4	17.3	19.6	21.3	213
32.0 $\mu\text{g/L}$	41.4	43.7	35.1	35.3	33.2	43.3	29.4	38.5	40.0	125
100 $\mu\text{g/L}$	169	214	155	197	156	217	147	222	183	183
320 $\mu\text{g/L}$	488	522	475	491	449	513	460	518	489	153

LOD = Limit of determination (1.56 $\mu\text{g/L}$) n.c. = not calculable

6.4.4.2 Biological responses

Mortality

101. None of the participating laboratories did observe mortalities in the medium control group. The maximum observed mortality in solvent control was 8.34%. Only laboratory 3N observed a significant mortality compared to control groups at a concentration of 22.7 $\mu\text{g/L}$ (8.33%) and 626 $\mu\text{g/L}$ (11.1%).

Reproduction

102. Figure 14 shows the test results for prochloraz of all partners which met the validity criteria. The results from laboratory 3L were also not considered because the control was continuously exposed to the test compound with a TWM of 9.98 $\mu\text{g/L}$ (cf. section 6.4.4.1). All laboratories found a decrease of embryo numbers with increasing concentrations of prochloraz. Laboratories 3A, 3M, 3N and 3O found comparable NOEC (27.3 $\mu\text{g/L}$, 32.9 $\mu\text{g/L}$, 40.4 $\mu\text{g/L}$ and 21.3 $\mu\text{g/L}$) and LOEC (51.4 $\mu\text{g/L}$, 58.3 $\mu\text{g/L}$, 194 $\mu\text{g/L}$ and 40 $\mu\text{g/L}$) values.

103. The animals used for the reproduction test in laboratory 3D showed the highest sensitivity for prochloraz. Already at the lowest test concentration (31.4 $\mu\text{g/L}$) the number of embryos was significantly reduced.

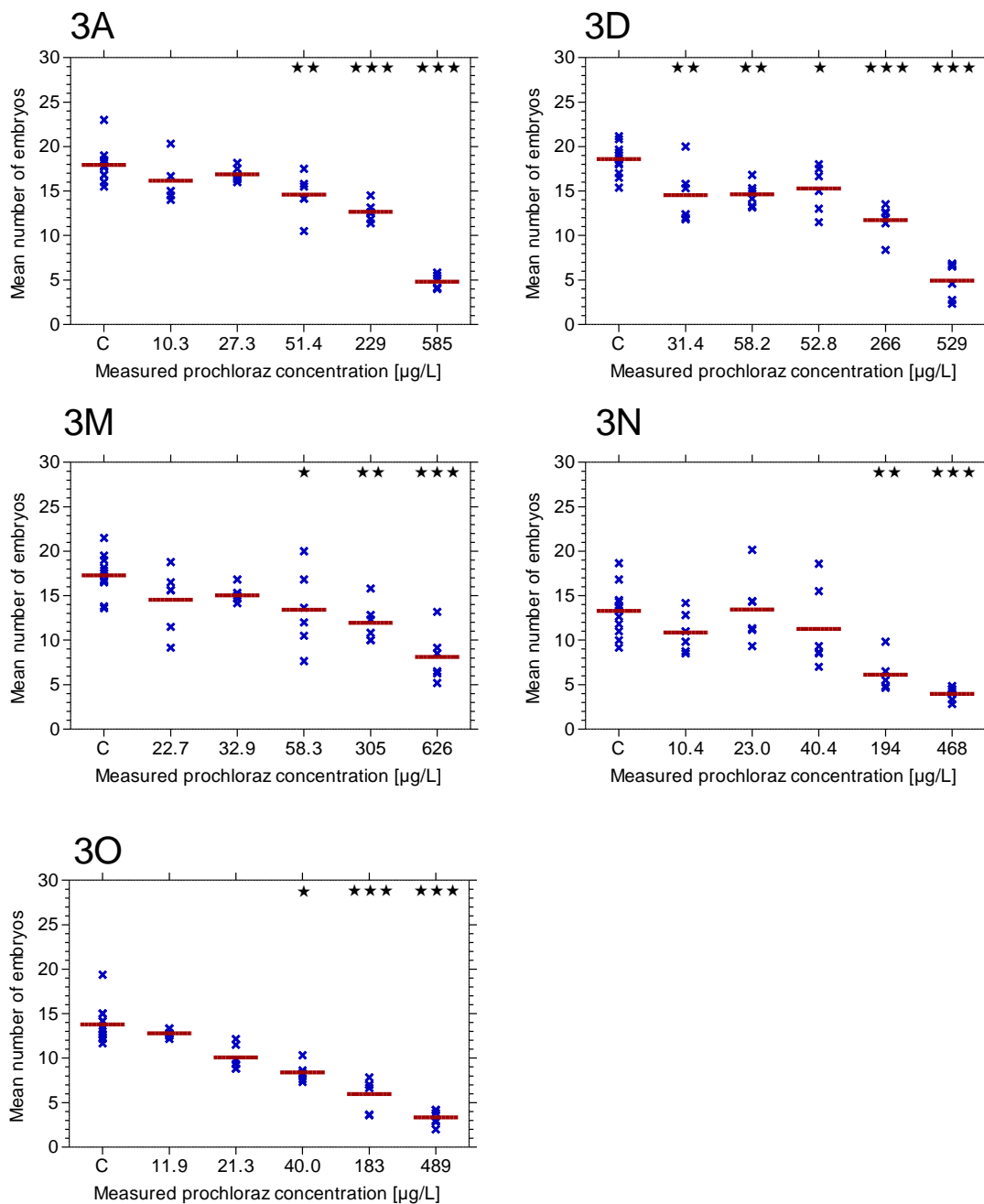


Figure 14: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks of exposure to prochloraz at laboratories 3A, 3D, 3M, 3N and 3O (x: replicate mean, —: mean of the treatment group, 6 replicates with 6 snails each). Asterisks indicate significant differences compared to control (Dunnett’s test), ★ = p<0.05, ★★ = p<0.01, ★★★ = 0.001.

104. Table 43 and figure 15 summarize the calculated effect concentrations based on measured concentrations from all participating laboratories providing valid data. The good match of results is reflected by the EC₁₀ and EC₅₀ values. The EC₁₀ values from all laboratories overlap with their 95%-confidence intervals. The EC₁₀ ranges from 15.6 µg/L (laboratory 3M) to 45.4 µg/L (laboratory 3A) and the EC₅₀ from 103 µg/L (laboratory 3O) to 763 µg/L (laboratory 3M).

Table 43: Effect concentrations (EC₁₀ and EC₅₀ with 95%-confidence intervals, NOEC, LOEC) for total embryo number based on measured concentrations of prochloraz in µg/L. *Italics: data from laboratories with non-valid test results.*

	3A	3D	3M	3N	3O	3L	3H	3P
EC ₁₀	45.4	24.9	15.6	28.3	6.04		-	-
95%-CI	(25.3 - 81.5)	(9.11 - 67.8)	(2.27 - 107)	(6.50 - 123)	(3.20 - 11.4)		-	-
EC ₅₀	327	285	763	200	103		-	-
95%-CI	(257 - 416)	(190 - 429)	(289 - 2015)	(116 - 346)	(75.3 - 140)		-	-
NOEC	27.3	-	32.9	40.4	21.3	160	39.6	47.8
LOEC	51.4	31.4	58.3	194	40.0	379	105	80.9

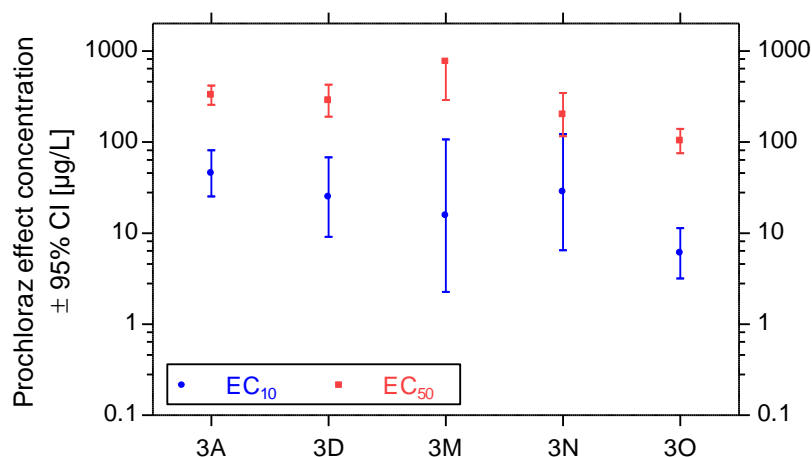


Figure 15: Calculated effect concentration (EC₁₀ in blue, EC₅₀ in red) values (µg/L) including 95% confidence intervals of the reproduction test with *Potamopyrgus antipodarum* with prochloraz from all valid tests (laboratory 3L excluded) conducted in the participating laboratories.

6.5 Comparison of present results to published and grey literature

105. Snails exposed to trenbolone in the tested concentration range did not show concentration dependent effects on reproduction. The assessed sensitivity differs from sensitivity data assessed with other species: Fecundity of the fathead minnow was significantly reduced by exposure to measured trenbolone test concentrations ≥ 27 ng/L (ANKLEY et al. 2003). In another study of HOLBECH et al. (2006) juveniles of *Danio rerio* were exposed to trenbolone-acetate. The sex ratio of exposed fish was significantly altered to an all-male population from exposure to 9.7 ng/L and above. In a study by OLMSTEAD et al. (2012) *Xenopus tropicalis* showed a significant shift in sex ratio toward males at 78 ng/L. In comparison to fish and amphibians *P. antipodarum* is less sensitive when exposed to trenbolone.

106. The effect concentrations for prochloraz obtained in this ring test are in good accordance to other species. Compared to studies with fish, *P. antipodarum* shows a comparable sensitivity towards prochloraz. THORPE et al (2011) investigated the sexual differentiation mode of the fathead minnow and zebrafish and found a LOEC of 100 µg/L for fathead minnow and 320 µg/L for zebrafish by a decreasing

proportion of females, respectively. In another study of ZHANG et al. (2008) *Oryzias latipes* was exposed towards prochloraz for ten days. The EC₅₀ value for the number of laid eggs per fish was 30 µg/L. Compared to other invertebrates the mudsnail also shows a comparable sensitivity to prochloraz. The assessed EC₁₀ and EC₅₀ for *Daphnia magna* in a 21-day reproduction test is 154 µg/L and 286 µg/L, respectively (HASSOLD & BACKHAUS, 2009).

6.6 Conclusions from validation III

107. In conclusion the findings from all partners showed a good agreement between the laboratories. Also the numbers of embryos in the control groups were in the same range among partners (with a coefficient of variation of 14.3%). Only two out of eight laboratories did not achieve the validity criteria.

108. None of the participating laboratories observed an effect of trenbolone on the reproduction of *P. antipodarum* which is also a consistent result.

109. Furthermore also the results of the reproduction tests with prochloraz showed a good match among partners. The mean values (with coefficient of variation) for EC₁₀, EC₅₀, NOEC and LOEC from laboratories 3A, 3D, 3M, 3N and 3O are 24.1 µg/L (61.3%), 336 µg/L (75.7%), 30.5 µg/L (26.7%) and 75.0 µg/L (89.7%).

110. The effect concentrations show a minimum of a 1.90-fold difference (NOECs) and a maximum of a 7.52-fold difference (EC₁₀ values). Overall it can be concluded that the robustness and the inter-laboratory reproducibility of the reproduction test with *P. antipodarum* with trenbolone and prochloraz could be demonstrated.

7. VALIDATION IV: TRICLOCARBAN AND TRICLOSAN

7.1 Organisation of the validation test

111. Four laboratories participated in the fourth ring test with *P. antipodarum* which started in September 2014. The partners were provided with snails, salts for preparing the medium and also the test substances. Triclocarban (TCC) and triclosan (TCS) were chosen as test chemicals, because in pre-tests with *P. antipodarum* a concentration-dependent effect of embryo numbers compared to controls was observed.

7.1.1 Snail production, biological quality checking and shipping

112. Snails used for the experiments came from the same laboratory culture which has been run under the same conditions as described in section 5.1.1 for validation II and validation III.

113. Table 44 summarizes the shipping and acclimation duration and gives an overview of the test schedules. Post shipping mortality did not occur in the partner laboratories.

Table 44: Shipping, acclimation and test schedules for the partner laboratories.

Partner	Laboratory 4A	Laboratory 4C	Laboratory 4G	Laboratory 4L
Snail shipping date	-	09/10/2014	16/10/2014	02/09/2014
Snails received	-	09/10/2014	16/10/2014	03/09/2014
Number of snails sent	-	500	500	500
Acclimation duration	-	32 d	25 d	15 d
Test starting date	06/10/2014	10/11/2014	10/11/2014	17/09/2014
Test ending date	03/11/2014	08/12/2014	08/12/2014	15/10/2014

7.2 Implementation of the 28-day reproduction test

7.2.1 Principle of the test

114. The principle of the reproduction test with *P. antipodarum* and the test procedure are the same as in validation III (see section 6.2.1).

7.2.2 Chemicals

115. As test chemicals TCC (CAS-No.: 101-20-2, Sigma-Aldrich®, Germany) and TCS (CAS-No.: 3380-34-5, Sigma-Aldrich®, Germany) were chosen. For both substances DMSO was used as a solvent at a concentration of 10 µL/L. Therefore, an additional solvent control group was considered. TCC and TCS are used as antimicrobial agents and both were tested at the following nominal concentrations:

0.1 µg/L, 0.3 µg/L, 1 µg/L, 3 µg/L, 10 µg/L.

7.2.3 Experimental conditions

116. The experimental conditions were the same as in validation III. Only the amount of food was adjusted to 62 µg/snail x day to reduce the amount of unconsumed food and the resulting risk of fungus growth which may caused an increased mortality of the test organisms. Experimental conditions are summarized in table 45.

Table 45: Summary of main experimental conditions.

Test duration	28 days
Test water	Reconstituted water (with 0.3 g Tropic Marin® salt and 0.18 g NaHCO ₃ per 1 litre de-ionised water) water quality requirements: pH 7.5 – 8.5, conductivity 770 ± 100 µS/cm, oxygen concentration > 80% ASV (air saturation value)
Test vessels	500 mL glass beakers with lids
Water renewal	3 times per week
Temperature	16 ± 1°C
Light intensity	500 ± 100 lux
Water sampling	From all test concentrations and solvent control water was sampled over four renewal intervals for trenbolone and prochloraz
Photoperiod	16:8 h L:D
Food source	Fine grounded Tetraphyll®
Feeding	62.5 µg/animal and day
Snails origin	Laboratory culture, which was built up with snails from Lumda Hesse, Germany
Test snails size	3.5 – 4.5 mm
Snails density	6 snails per 400 mL medium (6 replicates per tested concentration)
Core test endpoints	Mortality, reproduction

7.3 Chemical analysis and biological data analysis

117. In the first, the middle and in the last week of the test, samples from TCC and TCS exposure groups and solvent control were taken for chemical analysis. Therefore, on day 0, day 14 and day 25 water samples were taken from freshly prepared exposure media and on day 2, day 16 and day 28 from old water which was pooled from every replicate of one treatment group. Samples were stored in HDPE-bottles at 4°C until analysis.

118. Chemical analysis of TCC and TCS was performed by liquid chromatography-mass spectrometry (LC/MS, Agilent HPLC 1200 series with triple quadrupole mass spectrometer 6410, Santa Clara, USA) after solid-phase extraction (SPE), based on HALDEN AND PAULL (2005) at chemlab GmbH in Bensheim, Germany.

119. SPE cartridges (Oasis® HLB 6 cc vac, 200 mg, Waters GmbH, Eschborn, Germany) were conditioned with 2 mL n-heptane, 2 mL acetone, 2 x 3 mL methanol and 2 x 4 mL ultrapure water. Up to a nominal concentration of 1 µg/L, including solvent control, 200 mL of water samples were extracted. At nominal concentrations of 3 and 10 µg/L 100 mL of water samples were extracted. After sample loading, cartridges were dried under a gentle stream of nitrogen. For elution 1:1 methanol:acetone mixture with 10 mM acetic acid were used. Sample volumes were reduced under a constant nitrogen flow to 500 µL.

120. TWM concentrations of the chemicals were calculated according to Annex 6 of OECD guideline 211 (OECD 2012).

121. Biological raw data were reported by the participating laboratories using an Excel® spread sheet which had been provided by Goethe University. From the raw data means were calculated for biological parameters (shell height, embryo numbers). Effect concentrations were calculated by Dunnett's test (NOEC, LOEC).

7.4 Results

7.4.1 Compliance with validity criteria

122. For a test to be valid the conditions were chosen based on available guidelines for freshwater invertebrates and as proposed in OECD (2010). For the purpose of validation IV, the following validity criteria should be fulfilled:

- mortality in the controls should not exceed 20%,
- mean embryo number per snail in the controls should be ≥ 5 ,
- dissolved oxygen saturation must have been at least 60% of the air saturation value (ASV) and
- water temperature should be $16 \pm 1^\circ\text{C}$ throughout the test.

123. All laboratories met the given validity criteria of the water temperature and the dissolved oxygen saturation. The water temperature ranged between 15.3°C (laboratory 4A) and 16.5°C (laboratory 4C). The measured oxygen saturation was between 95.8% (laboratory 4A) and 100% (laboratory 4L). Laboratory 4G exceeded the validity criterion for the maximum control mortality in the solvent control (30.6%) for unknown reasons (Figure 16). In the dilution-water control the mortality was 13.9%. Also in the TCC exposed groups an increased mortality was observed with a maximum of 27.8% at the highest test concentration. In the TCS groups a maximum of 11.1% of snails died during the test. Hence, the tests from laboratory 4G were not valid and test results from this laboratory are not considered in the following evaluation of biological responses (chapter 7.4.3.2).

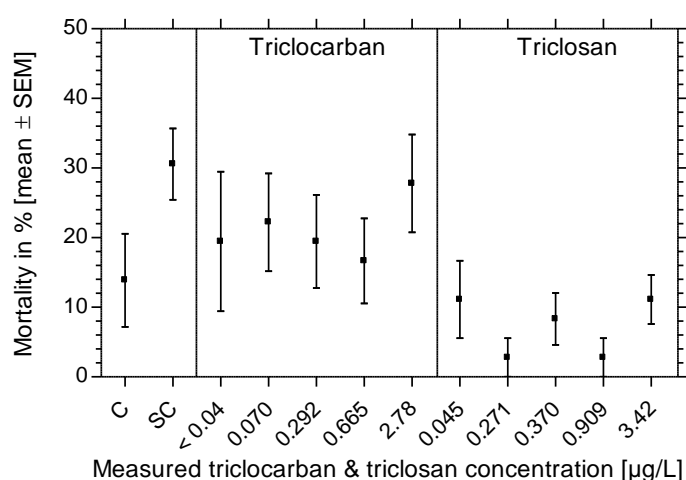


Figure 16: Mortality [in %] of *Potamopyrgus antipodarum* during four weeks of exposure in the dilution-water control (C), solvent control (SC) and in triclocarban (TCC) and triclosan (TCS) exposure groups at laboratory 4G (6 replicates with 6 snails each).

7.4.2 Physico-chemical parameters

124. Table 46 summarizes the means of the physico-chemical parameters for all laboratories. The pH values ranged between 8.20 (laboratory 4C) to 8.43 (laboratory 4L) which is in the required range of 7.5 - 8.5. The same applies to the values for conductivity which were similar among all laboratories.

Table 46: Mean physico-chemical parameters of all laboratories.

Laboratory	pH			Conductivity [$\mu\text{S}/\text{cm}$]			Temperature [$^{\circ}\text{C}$]			O ₂ saturation [%]		
	mean	SD	n	mean	SD	N	mean	SD	n	mean	SD	n
4A	8.36	0.693	145	825	45.3	145	15.3	0.767	145	95.8	1.61	144
4C	8.20	0.684	145	721	15.9	146	16.5	0.478	146	98.0	2.47	146
4G	8.35	0.691	145	738	32.7	145	16.0	0.327	145	99.2	0.822	145
4L	8.43	0.039	144	702	30.7	144	16.3	1.37	144	100	2.00	144

7.4.3 Results of validation IV with triclocarban

7.4.3.1 Actual exposure concentrations

125. The results of the analytical measurement of TCC for all laboratories are shown in tables 47 - 50. In total, 26.6% of the nominal concentrations were measured analytically. Therefore, TWM concentrations were used for the calculations of effect concentrations. The initial concentrations varied between 7.94% and 90.0% and after two or three days values varied between 4.25% and 82.1% of nominal concentrations. In all laboratories the measured concentrations of TCC in solvent control groups were below the LOD of 0.04 $\mu\text{g}/\text{L}$.

Table 47: Results of triclocarban analyses in exposure media from laboratory 4A [$\mu\text{g}/\text{L}$].

Nominal concentration	Measured concentration						Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 14: fresh medium	Day 16: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
0.1	0.0362	0.0821	0.0900	0.0373	0.0544	0.0620	0.058	58.0
0.3	0.173	0.117	0.112	0.0379	0.214	0.0887	0.121	40.3
1	0.561	0.256	0.178	0.312	0.450	0.311	0.340	34.0
3	0.350	0.398	0.541	0.463	0.603	0.766	0.542	18.1
10	3.03	1.46	3.29	1.54	3.06	2.90	2.55	25.5

LOD = Limit of determination (0.04 $\mu\text{g}/\text{L}$) n.c. = not calculable

Table 48: Results of triclocarban analyses in exposure media from laboratory 4C [$\mu\text{g/L}$].

Nominal concentration	Measured concentration						Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 14: fresh medium	Day 16: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
0.1	< LOD	< LOD	< LOD	0.0562	0.0581	< LOD	n.c.	n.c.
0.3	0.0369	< LOD	< LOD	< LOD	0.103	0.0584	n.c.	n.c.
1	0.141	0.110	0.0794	0.0425	0.200	0.217	0.14 2	14.2
3	0.371	0.367	0.253	0.234	0.397	0.509	0.36 8	12.3
10	1.27	1.16	0.968	1.33	2.81	1.77	1.64	16.4

LOD = Limit of determination (0.04 $\mu\text{g/L}$) n.c. = not calculable

Table 49: Results of triclocarban analyses in exposure media from laboratory 4G [$\mu\text{g/L}$].

Nominal concentration	Measured concentration						Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 14: fresh medium	Day 16: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
0.1	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
0.3	< LOD	0.0650	0.100	0.0651	0.0690	0.0823	0.07 0	23.3
1	0.118	0.237	0.369	0.264	0.317	0.404	0.29 2	29.2
3	0.471	0.399	0.850	0.631	0.634	0.928	0.66 5	22.2
10	1.41	2.21	2.96	2.84	2.74	4.11	2.78	27.8

LOD = Limit of determination (0.04 $\mu\text{g/L}$) n.c. = not calculable

Table 50: Results of triclocarban analyses in exposure media from laboratory 4L [$\mu\text{g/L}$].

Nominal concentration	Measured concentration						Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 14: fresh medium	Day 16: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
0.1	0.0400	0.0400	0.0400	< LOD	0.18 0	< LOD	0.04 7	47.0
0.3	0.0900	0.150	0.110	0.0700	0.220	0.0900	0.11 7	39.0
1	0.350	0.270	0.340	0.360	0.260	0.350	0.32 0	32.0
3	0.850	0.350	0.590	0.640	1.08	0.680	0.68 1	22.7
10	2.19	1.56	1.56	1.35	1.31	1.19	1.52	15.2

LOD = Limit of determination (0.04 $\mu\text{g/L}$) n.c. = not calculable

7.4.3.2 Biological responses

126. Laboratory 4G exceeded the validity criterion for the maximum control mortality in the solvent control (30.6%). Because the test from laboratory 4G was not valid the test results from this laboratory are not considered in the following evaluation of biological responses.

Mortality

127. At laboratory 4L no mortality was observed in any of the exposure groups and controls. The maximum mortality was observed at laboratory 4A at the highest test concentration of TCC (2.55 $\mu\text{g/L}$), where 2.78% snails died.

Reproduction

128. Figure 17 shows the results of the valid reproduction tests with TCC at laboratories 4A, 4C and 4L. Laboratory 4A observed a significant decrease of the embryo numbers with increasing TCC concentrations (NOEC = 0.121 $\mu\text{g/L}$; LOEC = 0.340). Laboratory 4L found a significant effect of the solvent DMSO on reproduction with a decreased number of embryos. Compared to the solvent control, this laboratory found a significant decrease of the embryo numbers at the highest test concentration of 1.52 $\mu\text{g/L}$ (= LOEC; NOEC = 0.681 $\mu\text{g/L}$). At laboratory 4C the embryo numbers of all exposure groups were on the level of the control.

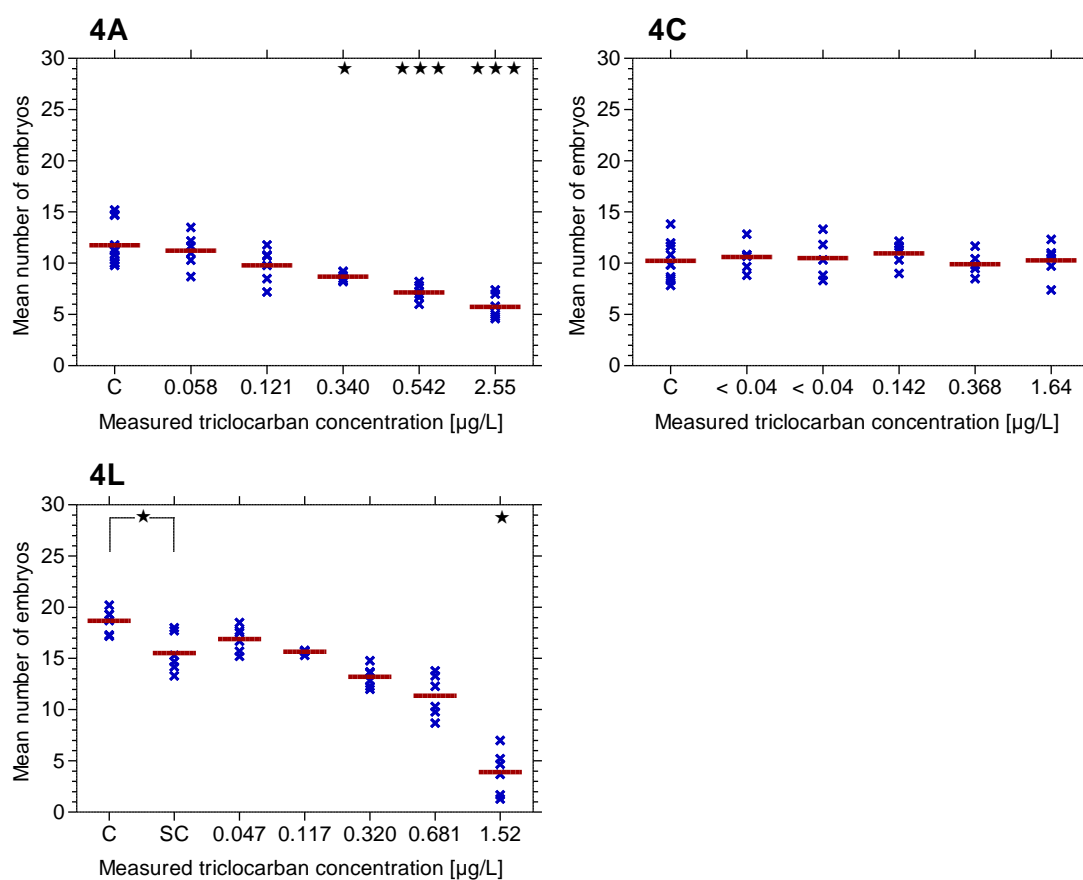


Figure 17: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks of exposure to triclocarban at laboratories 4A, 4C and 4L (x: replicate mean, —: mean of the treatment group, 6 replicates with 6 snails each). Asterisks indicate significant differences compared to control (Dunnett's test), $\star = p < 0.05$, $\star\star\star = 0.001$.

7.4.4 Results of validation IV with triclosan

7.4.4.1 Actual exposure concentrations

129. The results of the analytical measurement of TCS for all laboratories are shown in tables 51 - 54. In total, only 31.1% of the nominal concentrations were measured analytically. Therefore, TWM concentrations were used for the calculation of effect concentrations. The initial concentrations varied between 2.34% and 88.2% and after two or three days values varied between 2.27% and 88.3% of nominal concentrations. In all laboratories the measured concentrations of TCS in solvent control groups were below the LOD of 0.04 $\mu\text{g/L}$.

Table 51: Results of triclosan analyses in exposure media from laboratory 4A [$\mu\text{g/L}$].

Nominal concentration	Measured concentration						Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 14: fresh medium	Day 16: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
0.1	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
0.3	< LOD	< LOD	< LOD	0.0400	< LOD	0.1000	n.c.	n.c.
1	< LOD	0.0400	< LOD	0.100	< LOD	0.340	0.0708	7.08
3	< LOD	0.160	< LOD	0.240	0.380	0.260	0.180	6.00
10	< LOD	< LOD	0.100	2.57	1.68	1.84	1.36	13.6

LOD = Limit of determination (0.04 $\mu\text{g/L}$) n.c. = not calculable

Table 52: Results of triclosan analyses in exposure media from laboratory 4C [$\mu\text{g/L}$].

Nominal concentration	Measured concentration						Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 14: fresh medium	Day 16: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
0.1	< LOD	0.0475	0.0488	0.0560	0.0882	0.0688	0.057	57.0
0.3	0.129	0.137	0.163	0.254	0.198	0.171	0.175	58.3
1	0.422	0.364	0.558	0.599	0.656	0.482	0.519	51.9
3	0.946	0.686	1.24	0.837	1.21	1.14	1.03	34.3
10	2.42	2.14	3.87	2.07	5.12	3.01	3.17	31.7

LOD = Limit of determination (0.04 $\mu\text{g/L}$) n.c. = not calculable

Table 53: Results of triclosan analyses in exposure media from laboratory 4G [$\mu\text{g/L}$].

Nominal concentration	Measured concentration						Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 14: fresh medium	Day 16: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
0.1	0.0424	0.0371	0.0538	< LOD	0.0354	0.0815	0.045	45.0
0.3	0.121	0.0812	1.24	0.265	0.167	0.124	0.271	90.3
1	0.442	0.293	0.465	0.0227	0.585	0.468	0.370	37.0
3	0.943	0.911	0.0702	0.116	1.93	1.05	0.909	30.3
10	1.23	2.69	0.808	0.928	7.10	5.33	3.42	34.2

LOD = Limit of determination (0.04 $\mu\text{g/L}$) n.c. = not calculable

Table 54: Results of triclosan analyses in exposure media from laboratory 4L [$\mu\text{g/L}$].

Nominal concentration	Measured concentration						Time weighted mean	% of nominal-concentration
	Day 0: fresh medium	Day 2: old Medium	Day 14: fresh medium	Day 16: old Medium	Day 25: fresh medium	Day 28: Old medium		
Solvent control	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	n.c.	n.c.
0.1	0.0414	< LOD	0.0411	0.0400	0.0385	0.0400	0.0364	36.4
0.3	0.0780	0.0554	0.0694	0.0600	0.0683	0.0769	0.0677	22.6
1	0.502	0.112	0.464	< LOD	0.238	0.280	0.220	22.0
3	1.03	0.185	0.632	0.430	0.454	0.398	0.480	16.0
10	1.46	0.737	1.09	0.320	1.28	1.14	0.964	9.6

LOD = Limit of determination (0.04 $\mu\text{g/L}$) n.c. = not calculable

7.4.4.2 Biological responses

130. Laboratory 4G exceeded the validity criterion for the maximum control mortality in the solvent control (30.6%). Because the test from laboratory 4G was not valid the test results from this laboratory are not considered in the following evaluation of biological responses.

Mortality

131. At laboratory 4L no mortality was observed in any of the TCS exposure groups and controls. The maximal observed mortality was 5.56% at laboratory 4A at the lowest test concentration.

Reproduction

132. Figure 18 shows the results of the valid reproduction tests with TCS at laboratories 4A, 4C and 4L. Laboratory 4A found a significant decrease of embryo numbers at the lowest test concentration, which was not detected at the higher test concentrations. For laboratory 4L a significantly lower embryo number compared to the solvent control was observed at a TCS concentration of 0.964 µg/L (= LOEC; NOEC = 0.480 µg/L). Laboratory 4C did not detect any effect on the reproductive output of *P. antipodarum*.

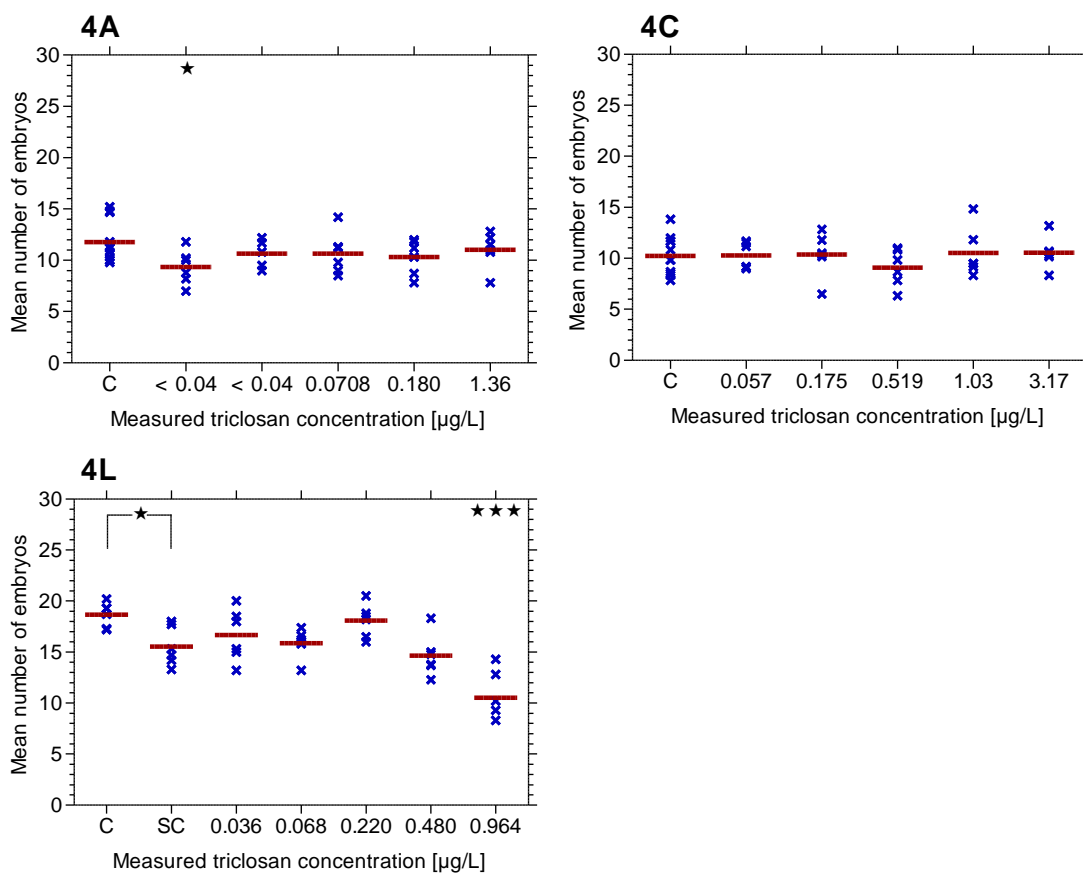


Figure 18: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks of exposure to triclosan at laboratories 4A, 4C and 4L (x: replicate mean, —: mean of the treatment group, 6 replicates with 6 snails each). Asterisks indicate significant differences compared to control (Dunnnett’s test), ★ = p<0.05, ★★ = 0.001.

7.5 Comparison of the embryo numbers in controls from all validation studies

133. In figure 19 the mean embryo numbers of the negative controls from all partners of all validation studies are shown. In total, the arithmetic mean values ranged between 10.1 and 24.3 embryos per female. In validation I, II and III snails were fed with 250 µg/snail x day and mean embryo numbers between 12.0 and 24.3 were found in the brood pouch of the snails. In validation IV the food dose was reduced to

62.5 µg/snail x day and mean embryo numbers ranged between 10.1 and 18.7. No significant effect in the embryo numbers could be observed between all validation studies (one-way ANOVA with Bonferroni's test, $p = 0.273$). Also by comparison of the control groups of validation IV with the results from the other three validation studies, no significant effect could be detected (unpaired t -test, $p = 0.237$).

134. Furthermore, the snails in validation IV were not starving as there still remained left-overs of TetraPhyll® in the test vessels. The reduced feeding rate reduces the potential development of fungi which may cause an increased mortality of the snails.

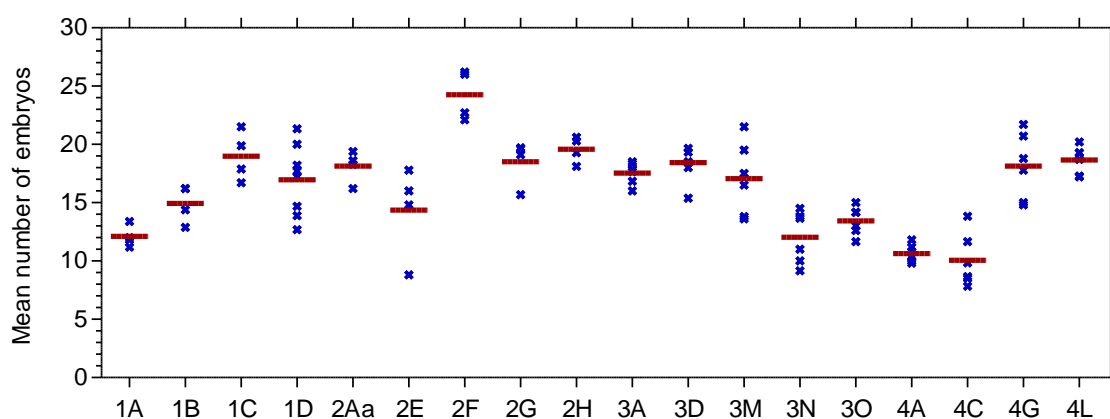


Figure 19: Total embryo numbers of *Potamopyrgus antipodarum* after four weeks of in the dilution-water control groups of all laboratories in four validation studies (x: replicate mean, —: mean of the treatment group, 6 replicates with 6 snails each). The initial number in the laboratory codes refers to the respective validation exercise.

7.6 Comparison of present results to published and grey literature

135. Within validation IV, TCC and TCS exposed snails showed a concentration-dependent decline of embryo numbers in two and one laboratories, respectively. In none of the exposure groups a significant increase of embryo numbers was found. This contrasts to already published and grey literature. GIUDICE AND YOUNG (2010) reported a concentration-dependent increase in embryo numbers of *P. antipodarum* with NOEC and LOEC values of 0.05 µg/L and 0.2 µg/L, respectively. HEIDELBACH (2014) tested TCC and TCS in a reproduction test with *P. antipodarum* and found an inverted u-shaped concentration-response relationship, with a stimulation of reproduction at low concentrations followed by an inhibition at higher test concentrations.

136. The sensitivity to TCC and TCS differs with other species. A chronic toxicity test with *Daphnia magna* to TCS resulted in a NOEC after 21 days of 40 µg/L (ORVOS et al. 2002). For TCS exposed eggs of *Oryzias latipes* a reduced hatching rate was found at a concentration of 313 µg/L (IISHIBASHI et al. 2004). The exposure of *Ceriodaphnia dubia* to TCC resulted in a NOEC of 1.90 µg/L after 8 days (TAMURA et al. 2012).

7.7 Conclusions from validation IV

137. For the exposure to TCC, two out of three laboratories reporting valid test results found a concentration-dependent decrease in the embryo numbers. For the reproduction tests with TCS, only one of the three laboratories observed a significant decrease in the reproduction of snails at the highest test concentration. The non-apparent effect on the reproduction of *P. antipodarum* is probably due to the very low measured concentrations of TCC and TCS.

138. The results also demonstrate that the reduced food level does not affect the reproductive output of the snails compared to the other three validation studies but is advantageous because the amount of unconsumed food and therefore the risk of fungus growth and increased snail mortality are reduced.

8. OVERALL CONCLUSION

139. The robustness and the reproducibility of the reproduction test with *Potamopyrgus antipodarum* has been demonstrated in three validation exercises with four test compounds. In total 17 partners from 10 countries participated in these round robins coming from industries, government and academia. Within the four validation studies, 43 reproduction tests have been performed, thereof one laboratory had to repeat the reproduction test with TBT due to very low concentrations of the test substance and five laboratories did not achieve the given validity criteria. One laboratory had technical issues to satisfy the temperature between 15°C and 17°C and four other laboratories did not meet the biological criteria (maximum control mortality; or minimum embryo number in control groups in snails coming from a different culture). This suggests that the given criteria are appropriate and achievable.

140. For all tested chemicals the inter-laboratory reproducibility of the test has been shown as most of the laboratories detected comparable NOEC, LOEC, EC₁₀ and EC₅₀ values with overlapping 95%-confidence intervals for the latter, even if difficult to handle substances were chosen as test compounds (e.g. TBT or trenbolone). Furthermore in validation I and II the repeatability/intra-laboratory reproducibility could be demonstrated as laboratory A repeated the reproduction test with TBT and cadmium.

141. For these reasons the drawing up of a guideline-proposal should be supported.

9. LITERATURE REFERENCES

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ANNEX 1. RESULTS FOR THE NUMBER OF EMBRYOS WITH AND WITHOUT SHELL

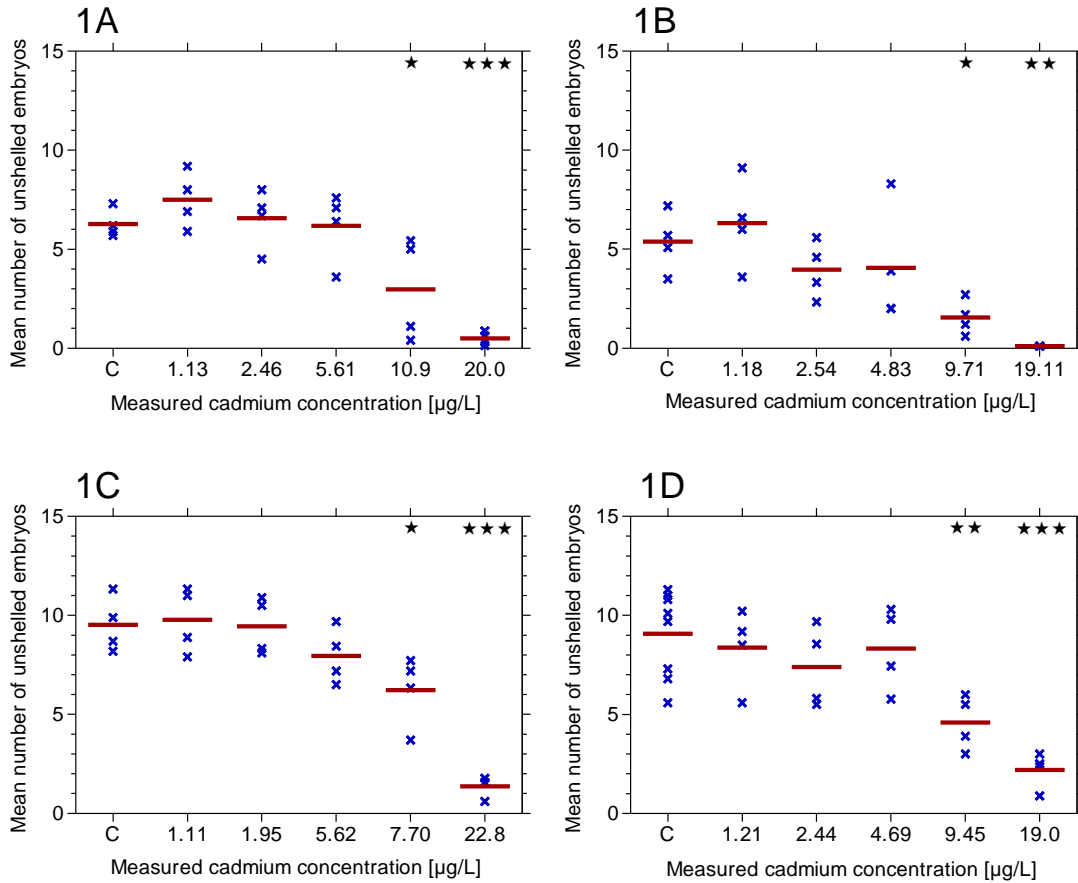


Figure 1: Unshelled embryo numbers of *Potamopyrgus antipodarum* after four weeks of exposure to cadmium at laboratories 1A, 1B, 1C and 1D (x: replicate mean, —: mean of the treatment group, 4 replicates with 3 - 10 snails each). Asterisks indicate significant differences compared to control (Dunnett's test), ★ = p<0.05, ★★ = p<0.01, ★★★ = p < 0.001).

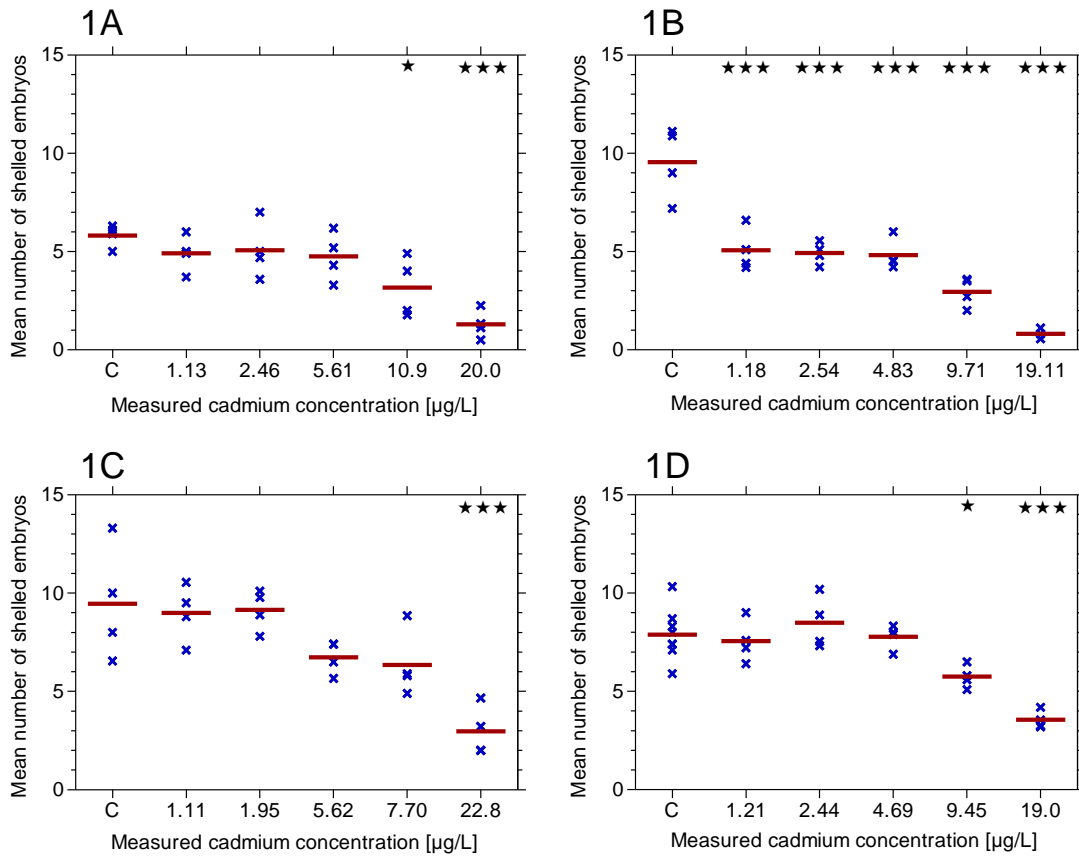


Figure 2: Unshelled embryo numbers of *Potamopyrgus antipodarum* after four weeks of exposure to cadmium at laboratories 1A, 1B, 1C and 1D (x: replicate mean, —: mean of the treatment group, 4 replicates with 3 - 10 snails each). Asterisks indicate significant differences compared to control (Dunnett's test), $\star = p < 0.05$, $\star\star = p < 0.01$, $\star\star\star = p < 0.001$.

ANNEX 2. RESULTS OF LABORATORY 2JA

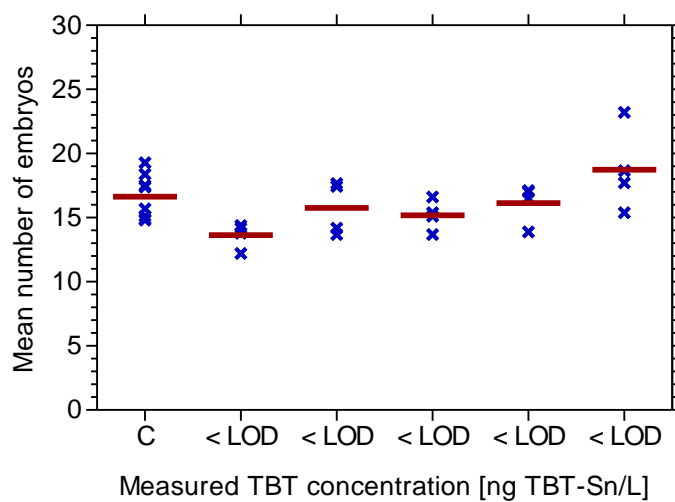


Figure 1: Total embryo numbers of *Potamopyrgus antipodarum* after 4 weeks of exposure to TBT at laboratory 2Ja (x: replicate mean, —: mean of the treatment group, 4 replicates with 8 - 10 snails each, in 2Ja at 838 ng/L: 3 replicates with 1-3 snails each) .