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Development and validation of an OECD reproductive toxicity test guideline with the mudsnail *Potamopyrgus antipodarum* (Mollusca, Gastropoda)

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ABSTRACT

Mollusks are known to be uniquely sensitive to a number of reproductive toxicants including some vertebrate endocrine disrupting chemicals. However, they have widely been ignored in environmental risk assessment procedures for chemicals. This study describes the validation of the *Potamopyrgus antipodarum* reproduction test within the OECD Conceptual Framework for Endocrine Disrupters Testing and Assessment. The number of embryos in the brood pouch and adult mortality serve as main endpoints. The experiments are conducted as static systems in beakers filled with artificial medium, which is aerated trough glass pipettes. The test chemical is dispersed into the medium, and adult snails are subsequently introduced into the beakers. After 28 days the reproductive success is determined by opening the brood pouch and embryo counting. This study presents the results of two validation studies of the reproduction test with eleven laboratories and the chemicals tributyltin (TBT) with nominal concentrations ranging from 10 - 1000 ng TBT-Sn/L and cadmium with concentrations from 1.56 - 25 µg/L.

The test design could be implemented by all laboratories resulting in comparable effect concentrations for the endpoint number of embryos in the brood pouch. After TBT exposure mean EC_{10}, EC_{50}, NOEC and LOEC were 35.6, 127, 39.2 and 75.7 ng Sn/L, respectively. Mean effect concentrations in cadmium exposed snails were, respectively, 6.53, 14.2, 6.45 and 12.6 µg/L.

The effect concentrations are in good accordance with already published data. Both validation studies show that the reproduction test with *P. antipodarum* is a well-suited tool to assess reproductive effects of chemicals.

Keywords: Standardisation, Mollusk, Reproduction, Endocrine Disruption, Tributyltin, Cadmium.

1. INTRODUCTION

The Organisation for Economic Co-operation and Development (OECD) is one of the focal institutions for the harmonization of test methods that are used for the risk assessment of chemicals (Gourmelon and Ahtiainen, 2007). In 2002 the Conceptual Framework for Endocrine Disrupters Testing and Assessment was agreed by the OECD providing a guide to the available *in silico*, *in vitro* and *in vivo* tests giving information for the assessment of endocrine disrupters with 5 levels of increasing complexity. Tests on invertebrates belong to level 4 and 5 of the Conceptual Framework and provide data on adverse effects on endocrine relevant endpoints (e.g. reproduction), although these tests are not of mechanistic nature like e.g. (*in vitro*) receptor binding assays: they may respond to various mechanisms caused by the general toxicity of a substance and may therefore also cover non-endocrine disrupting mechanisms (OECD, 2012a). They comprise species like *Daphnia magna*, *Chironomus riparius* and *Lumbriculus variegatus.* So far, a standard test for routine chemical testing with the species-rich phylum of mollusks has not yet been established within the Conceptual Framework, although mollusks are ecologically crucial organisms, which are essential to the biosphere and to the human economy (i.e. the shellfish industry) (Matthiessen, 2008; Shumway et al., 2003). Furthermore, they are highly sensitive to a number of endocrine disrupting chemicals (e.g. organotins) and other reproductive toxicants (Duft et al., 2007; Jorge et al., 2013; Matthiessen, 2008). A prominent example for the impact of a chemical on aquatic invertebrate populations in the field is the virilization effect on mollusks caused by tributyltin (TBT). It caused imposex and intersex development as two masculinization phenomena in more than 260 gastropod species, damaged the oyster-growing industry and caused severe losses of invertebrate biodiversity in coastal waters (Matthiessen and Gibbs, 1998; Titley-O'Neal et al., 2011). To close this gap OECD welcomed and supported the development of standard reproduction tests with mollusk species (Gourmelon and Ahtiainen, 2007; Matthiessen, 2008). In 2008, the German Environment Agency and the Department for

Environment, Food and Rural Affairs of the United Kingdom started the coordination for test method development. As a first step a detailed review paper on Mollusks Life-cycle Toxicity Testing was prepared summarising the state of knowledge on mollusk testing and proposing possible test designs and test species (OECD, 2010a). Beside the pond snail *Lymnaea stagnalis* (Gastropoda: Lymnaeidae) and the Pacific oyster *Crassostrea gigas*, (Bivalvia: Ostreidae) one of the most promising candidate species for a standardised test guideline was the New Zealand mudsnail *Potamopyrgus antipodarum* (Gastropoda: Tateidae). This species has already been subject of several ecotoxicological studies (summarized in Sieratowicz et al. 2011) and is known to be sensitive to a number of reproductive toxicants and endocrine disrupting chemicals (Duft et al., 2007; Gust et al., 2010; Jobling et al., 2004; Ruppert et al., 2016; Sieratowicz et al., 2011). Originating from New Zealand the mudsnail *P. antipodarum* was introduced to other parts of the world in the mid-19th century with the ballast water of ships (Ponder, 1988). It is common in aquatic ecosystems including lotic as well as lentic ecosystems (Alonso and Castro-Diez, 2012). But it also occurs in estuarine areas because it can tolerate salinities up to 15‰ (Jacobsen and Forbes, 1997) demonstrating its high ecological relevance. The shell length reaches up to 6 mm (Duft et al., 2007). During dry or cold periods, snails live completely buried in the sediment (Duft et al., 2003). *P. antipodarum* feeds on detritus, algae, and bacteria, being rasped from the surface of plants, stones, or the sediment (Macken et al., 2012). European populations consist almost exclusively of parthenogenetic females with embryos developing in the anterior part of the pallial oviduct section, which is transformed into a brood pouch from which juvenile snails are released through the vaginal opening (Fretter and Graham, 1994). Snails reach sexual maturity at an age of about 30 weeks at a size of about 3.5 mm (Jensen et al., 2001; Møller et al., 1994). Reproduction takes place throughout the year (Gust et al., 2011b).In Europe three clonal genotypes were identified: clone A can mainly be found at freshwater sites all over Europe whereas clone B and C characterize coastal and brackish-water habitats. Clone C is only

found in Wales and a few locations on continental Europe (Städler et al., 2005). Differences between clones have also been subject of several studies. A comparative sensitivity study with sediment-bound cadmium and four clones of *P. antipodarum* (clone A, B, C and one New Zealand clone) revealed that interclonal differences on life-history traits like specific growth-rate and reproductive output were within an order of magnitude (Jensen et al., 2001). Other studies show differences in life-history traits and feeding rates in response to a salinity gradient between clone A and B (Jacobsen and Forbes, 1997). In acute toxicity tests with *P. antipodarum* the widths of the tolerance also differed among clones. Due to its wide distribution in freshwaters, European clone A was used for further test development.

In 2011, the leading countries Germany, United Kingdom, France and Denmark promoted the inclusion of the development and validation of new test guidelines on mollusk reproductive toxicity testing within the OECD test guideline programme (project 2.36) (OECD, 2015). In a collaborative work between academia, industry and government the test protocols with the two gastropod species *P. antipodarum* and *L. stagnalis* were successfully optimised, pre-validated and validated (Ducrot et al., 2014; OECD, 2015, Charles et al., 2016), an intensive process including data mining, method standardization and optimization, and ring tests (OECD, 2005). In April 2016 the reproduction tests with *P. antipodarum* and *L. stagnalis* were officially approved by the national coordinators of the OECD member countries as test guidelines. They are the first aquatic non-arthropod-test, which were successfully validated within the Conceptual Framework for Endocrine Disrupters as a level 4 assay. The present study shows the results of the two first validation rounds with *P. antipodarum* using the substances cadmium and TBT with 11 European laboratories.

2. MATERIALS AND METHODS

2.1 Implementation of the validation tests

2.1.1 Snail production, biological quality checking and acclimation

All snails used for this study were obtained from the long-term breeding stock established in our laboratory (Goethe University, Frankfurt, Germany) and belong to the clone A genotype according to Städler et al. (2005). In 2009, this culture was reinvigorated with snails originating from populations collected in the Kalbach, a small creek in Frankfurt, Hesse, Germany and in 2011 with snails from the Lumda, a small creek near Rabenau in Hesse, Germany. In the culture, the snails were kept at 16 ± 1°C and a light:dark regime of 16:8 h in 15 L glass aquaria with aerated reconstituted water (deionised water; pH 8 (± 0.5) adjusted with NaOH and HCl; conductivity 770 µS/cm adjusted with TropicMarin® sea salt (Dr. Biener GmbH, Wartenberg, Germany) and $NaHCO_3$) as proposed in OECD (2010a). Snails were fed *ad libitum* twice a week with finely ground TetraPhyll® (Tetra GmbH, Melle, Germany). Once a week, at least one third of the culture medium was renewed.

Before shipping to the partner laboratories, the reproductive output was checked in snails from the stock culture. Overall, about 4700 snails were shipped in 1 L glass beakers, containing 500 snails in 950 mL culture medium and finely ground TetraPhyll® (ad libitum). Shipping duration was 1 day, except for laboratory 2F where shipping took 2 days. In the participating laboratories, snails were acclimated between 5 and 68 days at 15-17°C. The different acclimation periods were due to the scheduling of the test period in the laboratories. Surviving snails of the first batch sent to laboratory 1B (see 3.1) were acclimated for 5 months. The experiments of validation I with cadmium were conducted between May and July 2010. Validation II experiments with cadmium and TBT were performed between June 2013 and March 2014.

171 *2.1.2 Principle of the reproduction test and experimental conditions*

172 Adult *P. antipodarum* of a defined size class (Table 1) are exposed to a concentration range of
173 the tested chemical and control groups for 28 d. Each exposure group, including a negative
174 (water) and if required solvent controls, consists of four replicates containing ten individuals
175 each. Test medium is changed three times a week to maintain exposure concentrations and
176 adequate water quality parameters, e.g. O_2, pH and conductivity, which are monitored before
177 exposure medium renewals. Dead snails are counted and removed from the test vessels during
178 medium renewal. Once a week, the test vessels are changed to prevent biofilm growth. After
179 28 d, snails are sacrificed at -20°C in a freezer or quick-frozen in liquid nitrogen, and stored at
180 -80°C. Using a stereo microscope the snails are dissected by cracking the shell with pliers and
181 removing the shell parts from the soft body with tweezers. The brood pouch is opened
182 carefully and the embryos are extracted using needles and tweezers. The main endpoint, the
183 number of embryos in the brood pouch of each individual, is assessed. Table 1 summarises
184 the main experimental conditions.

185

186 Table 1: Summary of main experimental conditions in the ring tests

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 (validation I) or 500 mL glass beaker (validation II) with lids
Water renewal	3 times per week
Temperature	15-17°C
Light intensity	300 – 500 lux
Photoperiod	16:8 h L:D
Food source	Finely ground Tetraphyll®
Feeding	0.25 mg/animal and day
Snails origin	Laboratory culture, which was established with snails from Kalbach Frankfurt, Germany (August 2009)
Test snails size	3.5 – 4.5 mm
Snails density	10 snails per 800 mL medium (validation I) 10 snails per 400 mL medium (validation II) (4 replicates per tested concentration for both validation I and II)
Core test endpoints	Survival, reproduction

2.1.3 Tested chemicals and exposure water sampling

Cadmium

For the validation studies with cadmium, different cadmium salts were used. In validation I, cadmium sulphate hydrate (CAS no. 7790-84-3) purchased from Merck KGaA (Darmstadt, Germany) was used. In validation II, cadmium chloride (CAS-No.: 10108-64-2, Sigma-Aldrich®, Germany) was tested. Both, coming from a single batch, were provided to the participating laboratories by Goethe University. In validation I, four laboratories performed the test with cadmium sulphate hydrate (laboratory codes 1A-1D) and five laboratories conducted the reproduction test with cadmium chloride for validation II (laboratory codes 2A, 2E-2K). The nominal cadmium concentrations were chosen based on pre-tests at Goethe University (data not shown). Five concentrations of cadmium were used with a factor of 2 between concentrations: 1.56, 3.13, 6.25, 12.5 and 25 µg/L. No carrier solvent was used. Stock solutions with a cadmium concentration of 250 µg/L were prepared by adding ultra-pure water to the substances. For lower concentrations, a dilution series was prepared from the stock solutions. In the first and second validation experiments, 80 µL and 40 µL, respectively, of the stock solutions were added to the 1 L and 500 mL test vessels, respectively, to obtain the nominal test concentrations. To calculate the time-weighted mean (TWM) concentrations of cadmium according to annex 6 of the OECD test guideline 211 (OECD, 2012b), 25 mL of water from all test concentrations and water control was sampled over two (validation I) and four (validation II) renewal intervals, respectively. Samples from freshly prepared exposure media were taken at medium renewal, and pooled samples of all replicates were taken before medium renewal.

Water samples were stored in 50 mL polypropylene tubes (Sarstedt, Nümbrecht, Germany) and acidified with 65% nitric acid (Suprapur®, Merck KGaA, Darmstadt, Germany). Chemical analysis was performed via inductively coupled plasma mass spectrometry (ICP-

MS, ELAN DCR-e, Perkin Elmer, Überlingen, Germany) in validation I at the International Graduate School Zittau, Chair Environmental Technology in validation I and at chemlab GmbH Bensheim, Germany in validation II, according to DIN EN ISO 17294-2 (2005). For the first and second validation studies, the limits of determination (LOD) were 0.01 µg/L and 0.03 µg/L, respectively; the limits of quantification (LOQ) were 0.025 µg/L and 0.5 µg/L, respectively.

Tributyltin

TBT was tested as tributyltin chloride (96% purity, CAS No. 1461-22-9, Merck Schuchardt OHG, Hohenbrunn, Germany) from a single batch, which was provided to the participating laboratories by Goethe University. The nominal TBT concentrations were chosen based on pre-tests at Goethe University (data not shown). All laboratories tested concentrations ranging from 10 to 400 ng TBT-Sn/L, except for laboratory 2J and 2Ab which considered a concentration range from 25 up to 1000 ng TBT-Sn/L. Glacial acetic acid (100% purity, CAS No. 64-19-7, Merck KGaA Darmstadt, Germany) containing max. 0.002% hydrochloric acid (Suprapur®, CAS No. 7647-01-0, Merck KGaA Darmstadt, Germany) was used as solvent for TBT exposure groups. The resulting solvent concentration in the test vessels of all TBT exposed groups and of the solvent control was 10 µL/L.

Analytical measurements for TBT were performed by chemlab GmbH, Bensheim, Germany, according to DIN EN ISO 17353-F13 by gas chromatography (Agilent 7890A with Agilent 5975C). Pooled water from all test concentrations and solvent controls was sampled over two renewal intervals. Water samples were stored in high density polyethylene amber bottles at 4°C in darkness before analysis. Sample volume was 1000 mL for the lowest test concentration and the solvent controls, 500 mL for samples of nominal 25 ng/L and 250 mL for the three highest test concentrations. The LOD was 0.82 ng TBT Sn/L, the LOQ was 2.05 ng TBT-Sn/L.

2.1.4 Test validity criteria

The following conditions were set as validity criteria for validation I and II:

- The dissolved oxygen value should be at least 60% of the air saturation value in the controls throughout the test
- Overall mortality in the control groups should not exceed 20% at the end of the test.

For validation II, a third validity criterion was added to align the draft test guideline with available guidelines for freshwater invertebrates (OECD, 2004, 2012b):

- Water temperature should be 16 ± 1°C throughout the test in all exposure groups

2.2 Raw data recording and analysis

Embryo number and shell length for each female were recorded and entered into an Excel® (Microsoft Corporation, Redmond, USA) spreadsheet previously prepared by the ring-test coordinator. Statistical analysis was carried out using GraphPad Prism® (Version 5.03, GraphPad Software Inc., San Diego, USA). Fisher's exact test was used to test for differences in survival between treatments and controls. Embryo numbers were analysed as arithmetic means of each replicate using one-way analysis of variance (one-way ANOVA) followed by Dunnett´s multiple comparison test to evaluate statistical differences from the respective control group, if requirements for these parametric tests were fulfilled (normal distribution and homogeneity of variance). If normal distribution and homogeneity of variances could not be achieved even after a logarithmic or square root transformation of data, significant differences between exposure groups were assessed using the Kruskal-Wallis test followed by Dunn´s multiple comparison test. Water and solvent controls were combined for TBT because they were not statistically significantly different (Green and Wheeler, 2013). For all comparisons α was set 0.05. The 10% and 50% effect concentrations (EC_{10}, EC_{50}) for reproductive toxicity and survival were derived using a LogNorm or Weibull nonlinear regression model (Kusk, 2003). The best-fitting model was chosen, i.e. the highest r^2.

3 RESULTS

3.1 Post shipping mortality

No striking post-shipping mortality occurred after sending the snails to the laboratories and during the acclimation phase, except in laboratory 1B. Here, many snails died for unknown reasons. Hence, a second batch of snails was sent to this laboratory. Surviving snails from the two batches were combined and used for the test.

3.2 Water quality parameters and compliance with validity criteria

Table 2 summarises the mean physico-chemical parameters for all participating laboratories of validation I and II. The pH values ranged between 7.95 (2E) and 8.39 (2F) which is in the determined range of 7.5-8.5. Also the measured conductivity and mean oxygen concentrations were similar among laboratories. All laboratories which conducted the reproduction tests with cadmium (1A-2H) fulfilled the given validity criteria for temperature and oxygen concentrations and recommended pH and conductivity.

In all laboratories control mortality was between 0% and 2.5%, except for laboratory 2J. Here, a mean mortality of 30% was observed in the solvent control. All laboratories performing the reproduction tests with TBT in validation II achieved the defined temperature scale, except for laboratory 2K. Here, a mean temperature of 19°C was measured instead of 16°C. Therefore, the non-valid test results of laboratories 2J and 2K are not considered in the evaluation of reproduction data but can be found in the Supporting Information.

Table 2: Mean of the physico-chemical parameters during the validation studies I and II

Laboratory	pH			Conductivity [µS/cm]			Temperature [°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
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
2J	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	1.050	91	86.9	10.4	91

3.3 Actual exposure concentrations

Cadmium

Table 3 summarises the calculated TWMs of measured cadmium concentrations from both validation studies. More detailed information can be found in the Supporting Information. Overall, the measured cadmium concentrations were similar among the laboratories. Most of the TWMs were below nominal concentrations and varied between 60.9% and 91.2% of nominal cadmium concentrations. Only in laboratory 2G one measured value was 135% higher than the nominal concentration of 6.25 µg/L. In a few control samples very low background concentrations were measured in the range of ng/L. Only in laboratory 1B 9.89 µg cadmium/L occurred in one sample of old control water, which was most probably the result of a sample tube mix up, as no cadmium could be measured in the freshly prepared sample 2 days before. Therefore, this sample was not included in the TWM calculation. All effect concentrations reported in this study are based on TWM concentrations in order to facilitate the comparison of results between labs.

Table 3: Time weighted mean concentrations of cadmium in validation I and II

Nominal concentration [µg Cd/L]	Laboratories Measured concentration [µg Cd/L]								
	1A	1B	1C	1D	2A	2E	2F	2G	2H
Control	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1.56	1.13	1.18	1.12	1.21	1.07	1.06	1.06	1.41	0.95
3.13	2.46	2.54	1.95	2.44	2.09	2.14	2.37	2.48	2.21
6.25	5.61	4.83	5.62	4.69	4.62	4.24	4.74	8.42	4.78
12.5	10.9	9.71	7.70	9.45	11.1	9.03	10.6	10.1	9.19
25	20.0	19.1	22.8	19.0	20.8	17.8	21.0	15.9	18.6

n.d.: not detected (below LOD)

306 *TBT*

307 In Table 4, calculated TWMs of measured TBT concentrations are shown. In the solvent
308 controls no TBT was detected. In most laboratories TWMs were below nominal
309 concentrations. The average TWM concentration was 44.2% of nominals and values varied
310 between 10.1% and 121%. Initial concentration varied between 6.31% and 285% whereas
311 measured concentrations of old samples varied between 1.79% and 299%. Therefore, TWMs
312 were used to calculate effect concentrations. More detailed information on the actual exposure
313 concentrations of each participating laboratory can be found in the Supporting Information.

314

315 Table 4: Time weighted mean concentrations of TBT in validation II

Nominal concentration [ng TBT-Sn/L]	Laboratories Measured concentration [ng TBT-Sn/L]						
	2Aa	2Ab	2E	2F	2G	2H	2I
Solvent control	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	4.11	n.t.	5.40	2.61	4.30	3.04	6.39
25	30.5	18.6	28.8	4.66	12.5	7.75	9.43
65	30.7	27.8	36.8	9.30	39.2	17.6	16.2
160	56.1	50.8	96.3	16.1	41.4	35.7	38.0
400	120	229	198	41.8	132	94.9	69.7
1000	n.t.	838	n.t.	n.t.	n.t.	n.t.	n.t.

316 n.d.: not detected (below LOD), n.t.: not tested

317

318 *3.4 Effects of cadmium on P. antipodarum*

319 In both validation studies, the mortality of snails across treatments was similar between the
320 laboratories and did not exceed 5.1% after 28 days, except in laboratories 1A and 2F. There,
321 mortalities of 32.5% and 37.5%, respectively, were observed at the highest test concentrations
322 of 20 µg/L and 21 µg/L, which were statistically significant compared to the water controls
323 (Fisher's exact test, $p < 0.001$).

324 In all participating laboratories of validation I and II a concentration-dependent decrease of
325 embryo numbers could be observed. Figure 1 shows the concentration-response curves fitted
326 to the experimental data. Obtained effect concentrations are summarised in Table 5. In

comparison to the other laboratories, laboratory 1B detected considerably lower effect concentrations, resulting in an EC_{10} of 0.69 µg/L and a NOEC of 1.18 µg/L. Effect concentrations´ 95%-confidence intervals of laboratory 1B did not overlap with confidence intervals of effect concentrations from other laboratories of the validation studies. These showed similar results with EC_{10} values ranging from 3.46 µg/L to 10.3 µg/L. EC_{50} values show a 4.2-fold difference between the laboratories. NOEC values were between 1.95 µg/L and 11.1 µg/L. When excluding laboratory 1B, a 1.72-fold difference in EC_{50} values was observed between the studies. Due to the bad health status which was most likely due to shipping stress this most probably resulted in a higher sensitivity of the snails from the mixed cohorts (see 3.3) in laboratory 1B, therefore, the obtained effect concentrations were excluded from the calculation of the coefficients of variation.

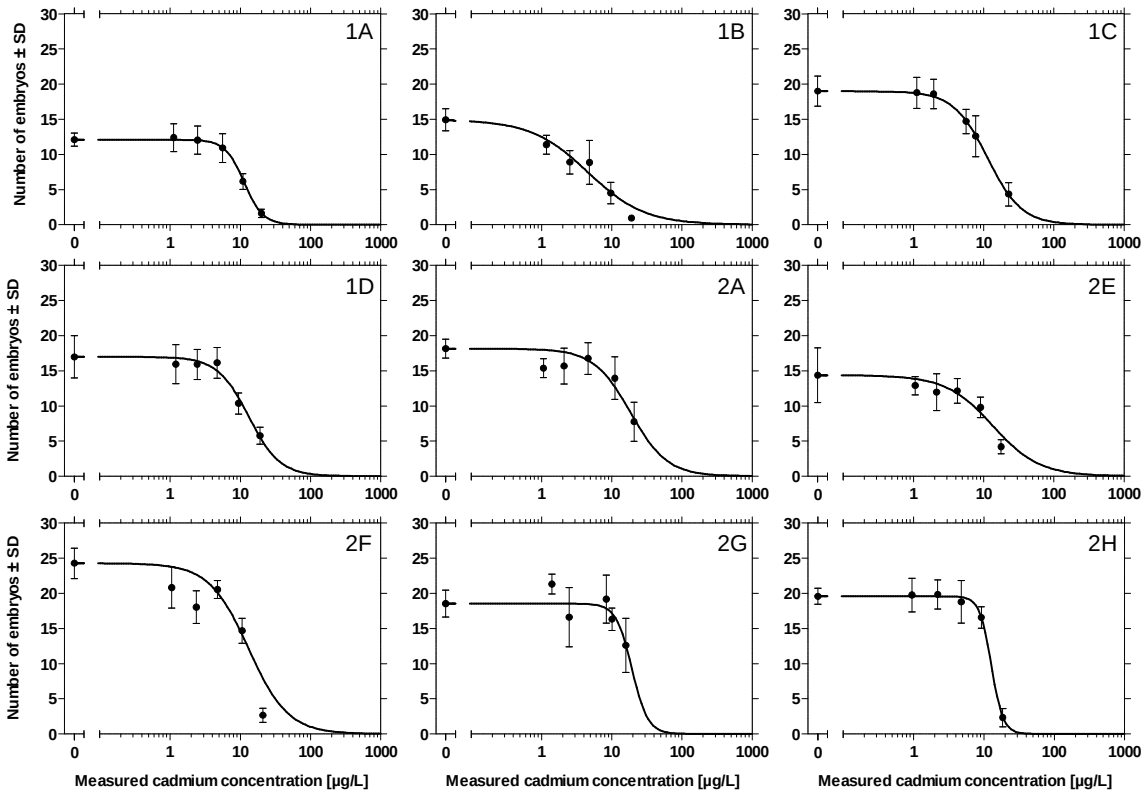

Figure 1: Total embryo numbers (mean ± standard deviation (SD)) after 28 days exposure to measured cadmium concentrations in laboratories reporting valid test results of validation I and II (n = 4 replicates per group).

Table 5**:** Effect concentrations (NOEC, LOEC, EC_{10} and EC_{50} with 95%-confidence intervals in brackets) for total embryo number based on time weighted means of measured concentrations in µg Cd/L and corresponding coefficients of variation (CV%), excluding laboratory 1B.

Nominal concentration	Laboratories									CV%
	1A	*1B*	1C	1D	2A	2E	2F	2G	2H	
NOEC	5.61	*1.18*	1.95	4.69	11.1	4.24	4.74	10.1	9.19	50.5
LOEC	10.9	*2.54*	5.62	9.45	20.8	9.03	10.6	15.9	18.6	42.2
EC_{10}	4.52	*0.689*	3.46	4.49	7.19	6.10	7.74	10.3	8.47	35.5
	(2.52-6.51)	*(0.25-1.91)*	(2.28-5.25)	(2.81-7.17)	(4.37-11.8)	(3.88-9.59)	(6.3-9.78)	(6.61-16.2)	(7.15-10.0)	
EC_{50}	11.3	*4.59*	11.4	13.2	18.5	13.5	13.1	19.4	12.8	21.8
	(9.48-13.2)	*(3.00-7.02)*	(9.30-13.9)	(10.8-16.0)	(15.1-22.5)	(11.3-16.0)	(11.6-14.8)	(13.5-27.9)	(11.4-14.3)	

3.5 Effects of tributyltin on P. antipodarum

In the exposure groups of laboratories reporting valid test results with a maximum nominal concentration of 400 ng TBT-Sn/L, snail mortality was ≤5%. Laboratory 2Ab tested a maximum nominal concentration of 1000 ng TBT-Sn/L. At this concentration, mortality was significantly increased (87.5%, $p<0.001$).

Figure 2 shows the results of the validation studies from the 7 laboratories reporting valid test results. In every experiment a significant decrease in the embryo numbers occurred in a concentration-response manner. Obtained effect concentrations EC_{10}, EC_{50}, NOEC and LOEC are summarised in Table 6. NOECs showed a 5.98-fold difference between laboratories. Calculated EC_x values are similar and most of the 95%-confidence intervals are overlapping. EC_{50} value fold difference was 1.79, with an inter-laboratory coefficient of variation of 39.3%.

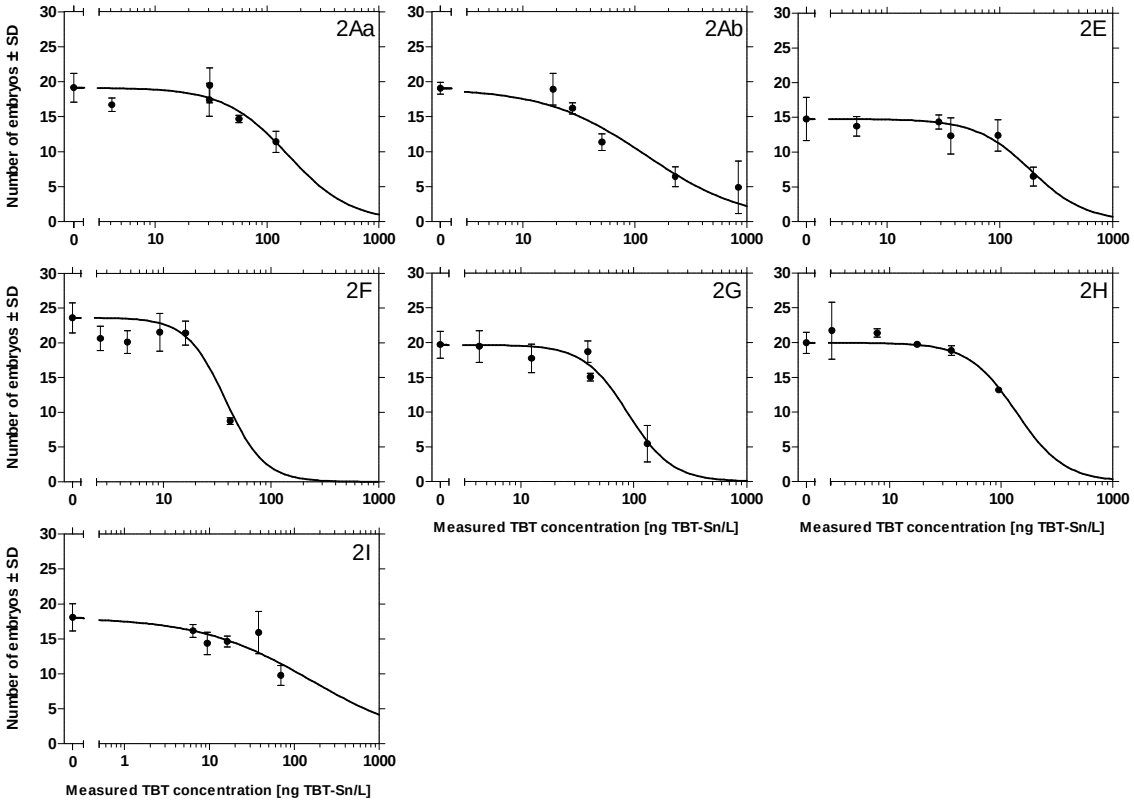

Figure 2: Total embryo numbers (mean ± standard deviation (SD)) after 28 days exposure to measured TBT concentrations in laboratories reporting valid test results of validation II (n = 4 replicates per group, 8 for merged controls)

Table 6**:** Effect concentrations (NOEC, LOEC, EC_{10} and EC_{50} with 95%-confidence intervals in brackets) for total embryo number based on time weighted means of measured concentrations in ng TBT-Sn/L and corresponding coefficients of variation (CV%).

Nominal concentration	Laboratories							CV%
	2Aa	2Ab	2E	2F	2G	2H	2I	
NOEC	30.7	18.6	96.3	16.1	39.2	35.7	38.0	68.3
LOEC	56.1	27.8	198	41.8	41.4	94.9	69.7	77.0
EC_{10}	45.0	12.7	89.1	22.4	36.8	36.5	6.62	76.9
	(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	39.3
	(109-213)	(77 - 200)	(157-226)	(28.5-50.4)	(73.5-107)	(93.2-200)	(48.8-519)	

4 DISCUSSION

4.1 Reproducibility of test results among laboratories and comparison with literature

The proposed test protocols were successfully applied by all participating laboratories. The assessed effect concentrations are comparable and in an adequate range of acceptance when comparing with other validation studies for chronic tests with invertebrates and dealing with reproductive endpoints. For example, Taenzler et al. (2007) and Tassou and Schulz (2009) performed a small scale ring test with a total of 4 laboratories for OECD guideline No. 233, life-cycle toxicity test with *Chironomus riparius*. For pyriproxifen the NOECs varied between 4 µg/L and 20 µg/L (fold difference of 5), and the coefficient of variation was 58.5%, which is documented in the validation report for this test guideline (OECD, 2010b). In the ring test for OECD guideline No. 225 (OECD, 2007), Sediment-Water *Lumbriculus* Toxicity Test Using Spiked Sediment, with 15 laboratories and the substance pentachlorophenol coefficients of variation varied between 37.9% for the EC_{50} and 68.6% for the LOEC. The maximum inter-laboratory factor was 23.5 for the LOEC, which is even higher compared to the maximum inter-laboratory factor of 13.5 for the EC_{10} in the *P. antipodarum* studies with TBT reported in this study, which is notoriously tricky to work with.

In another ring test study with the mollusk *L. stagnalis* Ducrot et al. (2014) found coefficients of variations of 29.5% and 71.5% for EC_{10} and EC_{50} values, respectively, for 5 valid laboratories looking at an endpoint of eggs per individual-day and the heavy metal cadmium. This is comparable with the variability of effect data in our study.

The higher variability of data in the TBT study may be due to the lower water solubility, the higher adsorption potential and degradation of the test compound. As a consequence the actual exposure concentrations will also vary among laboratories. Additionally, approximately 1000-fold lower test concentrations were tested, which also caused a higher experimental error in the laboratories and measurements of concentrations were only conducted at two renewal intervals. This would have caused higher experimental error in the calculation of the

TWM for each laboratory. However, another point demonstrating the usability of the reproduction test with *P. antipodarum* is that only 2 out of 18 tests failed to meet the validity criteria. In laboratory 2K this was due to technical issues causing the mean temperature to fluctuate outside the proposed validity criteria limit of 16 ± 1ºC. In laboratory 2J the mortality in the solvent control was 30% for unknown reasons. The snails in the water control group in this test were not negatively affected. The mean embryo numbers in water and solvent controls only varied slightly between laboratories and were in a range of natural variability in laboratory cultures (Geiß et al., 2016; Sieratowicz et al., 2011).

4.2 Cadmium

The non-essential trace metal cadmium is listed by the US Environmental Protection Agency as one of 126 priority pollutants and has multiple effects on the cellular level. It affects proliferation, differentiation and causes apoptosis. Indirectly it provokes the generation of reactive oxygen species and DNA damage. (Bertin and Averbeck, 2006; Waisberg et al., 2003). Due to its persistent nature and ability to accumulate in organisms, cadmium is an important stressor in the environment. The estimated effect concentrations in the studies with cadmium are in the range of effect data of already published data with *P. antipodarum*. In similar reproduction tests, Sieratowicz et al. (2011) and Ruppert et al. (2016) found EC_{10} values of 1.30 µg/L and 9.73 µg/L and EC_{50} values of 11.5 µg/L and 11.3 µg/L, respectively. Data obtained by Sieratowicz et al. (2011) were based on nominal concentrations. In comparison to the standard test organism *D. magna*, *P. antipodarum* shows a similar sensitivity. Borgmann et al. (1989) found a reproductive inhibition of about 82.1% at 7.78 µg/L for *D. magna*. In comparison to a study with the gastropod mollusk species, *L. stagnalis* (Ducrot et al., 2014), *P. antipodarum* displayed a higher sensitivity to cadmium. These authors found that the mean EC_{50} was 94.5 µg/L, which is 6.7-fold higher compared to the mean EC_{50} of 14.2 µg/L in the present study. In the eastern oyster *Crassostrea virginica*

cadmium exposure resulted in impaired oxygen uptake in the gills and led to a reduction in the aerobic scope. This may negatively affect the organism´s fitness and lower the energy reserves which were potentially destined for e.g. reproduction (Ivanina et al., 2008).The reason for the comparably high sensitivity of snails in laboratory 1B could be that the tested snails were partially from the cohort sent with the first batch, which showed a high post-shipping mortality. Therefore, part of the tested snails might have been in a poor condition for unknown reasons resulting in lower effect concentrations. Because we cannot exclude such confounding factors, like for example illness, that could have influenced the outcome of this study, we excluded laboratory 1B from the overall evaluation.

4.3 TBT

The effects of TBT on *P. antipodarum* reproduction occurred at very low concentrations within the order of ng/L and are in accordance with EC_{10} and EC_{50} values of 37.8 ng TBT-Sn/L and 115 ng TBT-Sn/L, respectively reported by Duft et al. (2007) for an 8-weeks experiment. Considering that only two measuring intervals were used to calculate the time weighted mean measured concentration and subsequent effect concentrations with a difficult-to-handle substance like TBT, the results of the validation studies showed a very good accordance among participating laboratories. In a whole-sediment biotest with TBT conducted by Duft et al. (2003) *P. antipodarum* exhibited a decline in embryo numbers resulting in an EC_{10} of 0.98 µg TBT-Sn/kg representing its high sensitivity at environmentally relevant concentrations. The higher or comparable sensitivity of *P. antipodarum* towards this endocrine disrupting substance in comparison to other standard test organisms could also be demonstrated. In a study with *D. magna* and TBT-oxide, the detected LOEC was 1.8 µg TBTO/L (≡ 716 ng TBT-Sn/L) and the NOEC was 1.0 µg TBTO/L (≡ 398 ng TBT-Sn/L) (Mathijssen-Spiekmann, 1989). In experiments conducted by McAllister and Kime (2003) with zebrafish (*Danio rerio*) 0.1 ng TBT/L and higher concentrations induced a male

biased population producing a high incidence of sperm lacking flagella after a 70-days post-hatch exposure. In other caenogastropod species TBT is known to induce imposex, an imposition of male characteristics in females (Giraud-Billoud et al., 2013). One of the most sensitive mollusk species to TBT is the dogwhelk, *Nucella lapillus*. Female specimens show an increase of imposex after 4 weeks at concentrations below 5 ng TBT-Sn/L (Stroben et al., 1992). The detailed biochemical mechanism of TBT causing imposex in snails is still a matter of debate (Oehlmann et al., 2007). One of them is that TBT inhibits the cytochrome P450-dependent aromatase, which is responsible for the conversion of androgens to estrogens (Bettin et al., 1996). A further possible mechanism is that TBT acts as an agonist of the retinoid X receptor (Nishikawa et al., 2004). Overall, in the chronic risk assessment of TBT an assessment factor of 10 could be used due to a broad dataset on effects of TBT on aquatic organisms with effect data from at least 3 trophic levels. The assessed results with *P. antipodarum* exposed to TBT (lowest NOEC: 16.1 ng TBT-Sn/L) would have led to a Predicted No Effect Concentration (PNEC) of 1.6 ng TBT-Sn. Although the resulting PNEC would still not be protective for marine mollusks, it underlines the specific sensitivity of this phylum of invertebrates to this chemical. This demonstrates the advantages of *P. antipodarum* as a potential test species for freshwater invertebrate risk assessment.

4.4 Resource and expertise requirements

The similar results between the laboratories in this ring test study demonstrate the ability of all participating persons to perform the reproduction tests with *P. antipodarum* regardless of their previous expertise with this species. The draft test guideline circulated to the participating laboratories, offered precise guidance for performing the test including the evaluation of the reproductive performance of the snails, with detailed instructions on shell removal, brood pouch opening and counting of embryos. For culturing and testing the snails, no unusual or hard to obtain equipment is needed and minimal space is required. A 15 L

aquarium can be used to culture up to 1500 snails (Sieratowicz et al., 2013). For a reproduction test with five test concentrations of a chemical, including water control and solvent control, 28 beakers are used, which only requires an area of around 1 m^2. The performance of the static test is comparatively simple and inexpensive to perform. A total of 280 animals are needed by using the test design proposed in this study. The water consumption amounts to only 170 L, if three water renewals per week are performed in 500 mL beakers. Test preparation and implementation require 4-6 hours of manpower and for three water renewals per week during the test performance (including the measurement of physico-chemical parameters), the work input amounts to 8-10 hours per week. For the final evaluation of the reproductive performance using a binocular microscope the required manpower is, depending on the number of embryos, 7-9 hours.

4.5 Ecological relevance and further scope of application

Because mollusks are second to the arthropods as the invertebrate group with the most species (>130,000), chronic toxicity tests with mollusks may be more amenable to ecological extrapolation than tests with representatives of smaller taxonomic groups (Matthiessen, 2008). Of this species-rich phylum, the known gastropod species contribute ca. 85% of all mollusks (Gruner, 1993) and play important ecological roles in various ecosystems contributing significantly to the biomass (Oehlmann et al., 2007). The sensitivity of many mollusk species towards pollutants is also due to their limited ability to eliminate toxicants via excretory organs in comparison to other invertebrate groups (Lee, 1985; Legierse et al., 1998). The assessed results in this study demonstrate the susceptibility of the test organism *P. antipodarum* to the tested reference substances. The decrease of embryo numbers in the snail caused by chemicals can affect population and community levels in its natural environment. Apart from the fact that *P. antipodarum* is an invasive species in other parts of the world, in New Zealand *P. antipodarum* plays an important role as a grazing invertebrate in

the maintenance of littoral freshwater communities by grooming macrophyte hosts of periphytic algae and removing settled sediment. It increases the resistance of macrophytes to sedimentation and affects the algal composition, biomass, and production (Bennett et al., 2015; Gust et al., 2011a; James et al., 2000). It is a common species in many different environments. As an inhabitant of freshwater environments it lives in the upper layers of aquatic sediments of streams, lakes and estuaries (Winterbourn, 1970). In the past years its sensitivity towards pollutants and endocrine disrupting chemicals has been demonstrated both in the laboratory and in the field (Geiß et al., 2016; Giudice and Young, 2010; Gust et al., 2011a; Ruppert et al., 2016; Schmitt et al., 2008; Sieratowicz et al., 2011). In an overview portrait, Duft et al. (2007) compared three prosobranch snail species, *Marisa cornuarietis*, *Nassarius reticulatus* and *P. antipodarum*, with regard to their (1) sensitivity towards tested model compounds at environmentally relevant concentrations in laboratory tests and also towards field sediment samples, (2) their "user-friendliness", (3) the possibility of culturing the snails in the laboratory and (4) their fields of application (e.g. estuarine and freshwater, sediment). *P. antipodarum* has been proposed as a suitable model organism and representative of the molluskan species for chemical risk assessment (Duft et al., 2007; Matthiessen, 2008).

The application possibilities of *P. antipodarum* in toxicity tests are diverse. In addition to exposure via water the testing of substances via sediments is also possible. It has also been used in several effect monitoring studies (Duft et al., 2007; Giebner et al., 2016; Gust et al., 2011a) demonstrating its assets as a test organism and broad scope of application for toxicity testing. Furthermore, it is possible to study additional endpoints with *P. antipodarum* like velocity, space utilization or time to reach food or to start normal movement after manipulation when exposed to a toxicant (Alonso and Camargo, 2004; Alonso and Camargo, 2012; Alonso et al., 2016). Alonso et al. (2016) developed a video-based behavioral bioassay with pulse exposures of acetone. These endpoints were also very sensitive and ecologically relevant as an indicator of the state of animal fitness and a good link between physiological

528 and ecological effects. For the proposed test design of the reproduction test it is indeed
529 necessary to observe especially abnormal behavior like avoidance of water, avoidance of food
530 or lethargy. But the guideline recommends only selective observations (at least three times per
531 week) because it is quite difficult to systematically observe the animals in the reproduction
532 test without removing and hence, additionally disturbing them. This could have additional
533 effects on the reproduction of the snails (Sieratowicz et al., 2013).
534
535 *4.6 Outlook*
536 After the successful completion of these first validation studies reported here, two further ring
537 tests with the substances prochloraz, trenbolone, triclosan and triclocarban were performed
538 (Geiß et al., 2017). Here, the test design was slightly amended (six replicates with six snails
539 per replicate) to improve the statistical power of the reproduction test. The used test design
540 with 4 replicates and 10 snails per replicate was originally designed for an evaluation with the
541 weighted mean embryo number per treatment group. This evaluation is based on common
542 error propagation rules and considers the tank as the experimental unit, but takes into account
543 the within-tank (*i.e.* individual animal) variability (OECD, 2010a). To adapt the draft
544 technical guideline to the standards of OECD guidelines, the evaluation method was changed
545 and a further validity criterion was added: the mean embryo number per snail in the controls
546 should be ≥ 5. This criterion was also fulfilled in all reproduction tests presented in this study.
547 The draft test guideline of the reproduction test with *P. antipodarum* has been acknowledged
548 by the experts of the "validation management group on ecotoxicity testing" (VMG-Eco) of the
549 OECD and the OECD *ad hoc* Expert Group on Invertebrate testing who supported the
550 submission of the draft test guideline. After a successful second international commenting
551 round by OECD member states the guideline was approved by the Working Group of the
552 National Coordinators for the Test guidelines Programme (WNT) and became effective as
553 OECD Guideline for the Testing of Chemicals, Section 2, No. 242.

4 CONCLUSIONS

As a common species in many different environments *Potamopyrgus antipodarum* has been subject in several studies and demonstrated its sensitivity towards pollutants, including endocrine disrupting chemicals. As a representative of the very diverse group of mollusks it has been proposed as a suitable model organism for the chemical risk assessment. The presented test design with *P. antipodarum* was implemented by all participating laboratories resulting in comparable concentration-response characteristics and effect concentrations. The robustness and the intra- as well as inter-laboratory reproducibility of the reproduction test has been proven, with most of the laboratories finding comparable NOEC, LOEC, EC_{10} and EC_{50} values with overlapping 95%-confidence intervals for the latter. The outcome of this ring test is in an adequate range of acceptance when comparing with other validation studies for chronic tests. The results obtained in the tests with the difficult to handle chemical TBT demonstrate the sensitivity of *P. antipodarum* in comparison to other standard test organisms and shows that the presented reproduction test with *P. antipodarum* is a well suited tool for assessing reproductive effects of chemicals. Additionally, its broad scope of application for toxicity testing and monitoring studies offers multiple possibilities for the risk assessment of chemicals. Several additional endpoints allow to link physiological and ecological processes. Therefore, the obtained results will contribute to the further development of test methods and evaluation concepts for the regulation of reprotoxic chemicals in REACh, as well as pesticides, biocides and pharmaceuticals.

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582 **REFERENCES**

583

584 Alonso, A., Camargo, J. A., 2004. Sub-lethal responses of the aquatic snail *Potamopyrgus*
585 *antipodarum* (Hydrobiidae, Mollusca) to unionized ammonia: A tolerant invading
586 species. Fresenius Environ. Bull. 13**,** 607-615.
587 Alonso, A., Camargo, J. A., 2012. A video-based tracking analysis to assess the chronic toxic
588 effects of fluoride ion on the aquatic snail *Potamopyrgus antipodarum* (Hydrobiidae,
589 Mollusca). Ecotoxicol. Environ. Saf. 81**,** 70-75.
590 Alonso, A., Castro-Diez, P., 2012. The exotic aquatic mud snail *Potamopyrgus antipodarum*
591 (Hydrobiidae, Mollusca): state of the art of a worldwide invasion. Aquat. Sci. 74**,** 375-
592 383.
593 Alonso, A., Garcia-Perinan, E., Camargo, J. A., 2016. Development of a Low-Cost
594 Ecotoxicological Bioassay Based on the Feeding Behaviour of the Aquatic Snail
595 *Potamopyrgus antipodarum* (Hydrobiidae, Mollusca). Arch. Environ. Contam.
596 Toxicol. 71**,** 553-560.
597 Bennett, D. M., Dudley, T. L., Cooper, S. D., Sweet, S. S., 2015. Ecology of the invasive New
598 Zealand mud snail, *Potamopyrgus antipodarum* (Hydrobiidae), in a mediterranean-
599 climate stream system. Hydrobiologia. 746**,** 375-399.
600 Borgmann, U., Ralph, K. M., Norwood, W. P., 1989. Toxicity test procedures for *Hyalella*
601 *azteca*, and chronic toxicity of cadmium and pentachlorophenol to *Hyalella azteca*,
602 *Gammarus fasciatus*, and *Daphnia magna*. Arch. Environ. Contam. Toxicol. 18**,** 756-
603 764.
604 Bertin, G., Averbeck, D., 2006. Cadmium: cellular effects, modifications of biomolecules,
605 modulation of DNA repair and genotoxic consequences (a review). Biochimie. 88**,**
606 1549-1559.
607 Bettin, C., Oehlmann, J., Stroben, E., 1996. TBT-induced imposex in marine neogastropods is
608 mediated by an increasing androgen level. Helgolander Meeresuntersuchungen. 50**,**
609 299-317.
610 Charles, S., Ducrot, V., Azam, D., Benstead, R., Brettschneider, D., De Schamphelaere, K.,
611 Filipe Goncalves, S., Green, J. W., Holbech, H., Hutchinson, T. H., Faber, D.,
612 Laranjeiro, F., Matthiessen, P., Norrgren, L., Oehlmann, J., Reategui-Zirena, E.,
613 Seeland-Fremer, A., Teigeler, M., Thome, J.-P., Tobor Kaplon, M., Weltje, L.,
614 Lagadic, L., 2016. Optimizing the design of a reproduction toxicity test with the pond
615 snail *Lymnaea stagnalis*. Regul Toxicol Pharm. 81, 47-56.
616 DIN EN ISO 17294-2, 2005. Water quality - Application of inductively coupled plasma mass
617 spectrometry (ICP-MS) - Part 2: Determination of 62 elements. Beuth-Verlag, Berlin,
618 Germany.
619 Ducrot, V., Askem, C., Azam, D., Brettschneider, D., Brown, R., Charles, S., Coke, M.,
620 Collinet, M., Delignette-Muller, M. L., Forfait-Dubuc, C., Holbech, H., Hutchinson,
621 T., Jach, A., Kinnberg, K. L., Lacoste, C., Le Page, G., Matthiessen, P., Oehlmann, J.,
622 Rice, L., Roberts, E., Ruppert, K., Davis, J. E., Veauvy, C., Weltje, L., Wortham, R.,
623 Lagadic, L., 2014. Development and validation of an OECD reproductive toxicity test
624 guideline with the pond snail *Lymnaea stagnalis* (Mollusca, Gastropoda). Reg.
625 Toxicol. Pharmacol. 70, 605-614.
626 Duft, M., Schulte-Oehlmann, U., Tillmann, M., Markert, B., Oehlmann, J., 2003. Toxicity of
627 triphenyltin and tributyltin to the freshwater mudsnail *Potamopyrgus antipodarum* in a
628 new sediment biotest. Environ. Toxicol. Chem. 22**,** 145-152.
629 Duft, M., Schmitt, C., Bachmann, J., Brandelik, C., Schulte-Oehlmann, U., Oehlmann, J.,
630 2007. Prosobranch snails as test organisms for the assessment of endocrine active

chemicals - an overview and a guideline proposal for a reproduction test with the freshwater mudsnail *Potamopyrgus antipodarum*. Ecotoxicology. 16**,** 169-182.

Fretter, V., Graham, A. British prosobranch molluscs. Their functional anatomy and ecology; The Ray Society; London, England, 1994.

Geiß, C., Ruppert, K., Heidelbach, T. and Oehlmann, J., 2016. The antimicrobial agents triclocarban and triclosan as potent modulators of reproduction in *Potamopyrgus antipodarum* (Mollusca: Hydrobiidae). J. Environ. Sci. Health A. 51, 1173-1179.

Geiß, C., Ruppert, K., Askem, C., Barroso, C., Faber, D., Ducrot, V., Holbech, H., Hutchinson, T.H., Kajankari, P., Lund Kinnberg, K., Lagadic, L., Matthiessen, P., Morris, S., Neiman, M., Penttinen, O.-P., Sanchez-Marin, P., Teigeler, M., Weltje, L., Oehlmann, J., 2017. Validation of the OECD Reproduction Test Guideline with the New Zealand mudsnail *Potamopyrgus antipodarum* using trenbolone and prochloraz. Ecotoxicology.

Giebner, S., Ostermann, S., Straskraba, S., Oetken, M., Oehlmann, J., Wagner, M., 2016. Effectivity of advanced wastewater treatment: reduction of in vitro endocrine activity and mutagenicity but not of in vivo reproductive toxicity. Environmental Science and Pollution Research. 1-12.

Giraud-Billoud, M., Vega, I. A., Wuilloud, R. G., Clement, M. E., Castro-Vazquez, A., 2013. Imposex and novel mechanisms of reproductive failure induced by tributyltin (TBT) in the freshwater snail *Pomacea canaliculata*. Environ. Toxicol. Chem. 32, 2365-2371.

Giudice, B. D., Young, T. M., 2010. The antimicrobial triclocarban stimulates embryo production in the freshwater mudsnail *Potamopyrgus antipodarum*. Environ. Toxicol. Chem. 29**,** 966-970.

Gourmelon, A., Ahtiainen, J., 2007. Developing test guidelines on invertebrate development and reproduction for the assessment of chemicals, including potential endocrine active substances - The OECD perspective. Ecotoxicology. 16**,** 161-167.

Green, J., Wheeler, J. R., 2013. The use of carrier solvents in regulatory aquatic toxicology testing: Practical, statistical and regulatory considerations. Aquat. Toxicol. 144**,** 242-249.

Gruner, H.-E., 1993. Lehrbuch der speziellen Zoologie, Band 1: Wirbellose Tiere, 3. Teil: Mollusca, Echiurida, Annelida, Onychophora, Tardigrada, Pentastomida; Gustav Fischer Verlag: Jena, Stuttgart, New York.

Gust, M., Buronfosse, T., Geffard, O., Coquery, M., Mons, R., Abbaci, K., Giamberini, L., Garric, J., 2011a. Comprehensive biological effects of a complex field poly-metallic pollution gradient on the New Zealand mudsnail *Potamopyrgus antipodarum* (Gray). Aquat. Toxicol. 101**,** 100-108.

Gust, M., Garric, J., Giamberini, L., Mons, R., Abbaci, K., Garnier, F., Buronfosse, T., 2010. Sensitivity of New Zealand mudsnail *Potamopyrgus antipodarum* (Gray) to a specific aromatase inhibitor. Chemosphere. 79**,** 47-53.

Gust, M., Mouthon, J., Queau, H., Dussart, C., Buronfosse, T., Garric, J., 2011b. Natural variability and response interpretation of fecundity, vertebrate-like sex-steroid levels and energy status in the New Zealand mudsnail *Potamopyrgus antipodarum* (Gray). Gen. Comp. Endocrinol. 172**,** 243-250.

Ivanina, A. V., Cherkasov, A. S., Sokolova, I. M., 2008. Effects of cadmium on cellular protein and glutathione synthesis and expression of stress proteins in eastern oysters, *Crassostrea virginica* Gmelin. J. Exp. Biol. 211**,** 577-586.

Jacobsen, R., Forbes, V. E., 1997. Clonal variation in life-history traits and feeding rates in the gastropod, *Potamopyrgus antipodarum*: performance across a salinity gradient. Funct. Ecol. 11**,** 260-267.

James, M. R., Hawes, I., Weatherhead, M., 2000. Removal of settled sediments and periphyton from macrophytes by grazing invertebrates in the littoral zone of a large oligotrophic lake. Freshwat. Biol. 44**,** 311-326.

Jensen, A., Forbes, V. E., Parker, E. D., 2001. Variation in cadmium uptake, feeding rate, and life-history effects in the gastropod *Potamopyrgus antipodarum*: Linking toxicant effects on individuals to the population level. Environ. Toxicol. Chem. 20**,** 2503-2513.

Jobling, S., Casey, D., Rodgers-Gray, T., Oehlmann, J., Schulte-Oehlmann, U., Pawlowski, S., Baunbeck, T., Turner, A. P., Tyler, C. R., 2004. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. Aquat. Toxicol. 66, 207-222.

Lee, R. F., 1985. Metabolism of tributyltin oxide by crabs, oysters and fish. Mar. Environ. Res. 17**,** 145-148.

Legierse, K., Sijm, D., van Leeuwen, C. J., Seinen, W., Hermens, J. L. M., 1998. Bioconcentration kinetics of chlorobenzenes and the organophosphorus pesticide chlorthion in the pond snail Lymnaea stagnalis - a comparison with the guppy *Poecilia reticulata*. Aquat. Toxicol. 41**,** 301-323.

Jorge, M. B., Loro, V. L., Bianchini, A., Wood, C. M., Gillis, P. L., 2013. Mortality, bioaccumulation and physiological responses in juvenile freshwater mussels (*Lampsilis siliquoidea*) chronically exposed to copper. Aquat. Toxicol. 126**,** 137-147.

Kusk, K. O., 2003. Program manual for statistical data treatment of toxicity test results. KOK-456T2 program dealing with data from tests with continuous response parameters using curve fitting. Technical University of Denmark, Odense, Denmark.

Macken, A., Le Page, G., Hayfield, A., Williams, T. D., Brown, R. J., 2012. Effects of test design and temperature in a partial life-cycle study with the freshwater gastropod *Potamopyrgus antipodarum*. Environ. Toxicol. Chem. 31**,** 1989-94.

Mathijssen-Spiekmann, E. A. M. C., J. H.; Roghair, C. J., Onderzoek naar de toxiciteit van TBTO voor een aantal zoetwaterorganismen (Research into the toxicity of TBTO for a number of freshwater organisms). Report No.: 668118 001. Dutch National Institute for Public Health and the Environment, Bilthoven, The Netherlands: RIVM, 1989.

Matthiessen, P., 2008. An assessment of endocrine disruption in mollusks and the potential for developing internationally standardized mollusk life cycle test guidelines. Integr. Environ. Assess. Manage. 4**,** 274-284.

Matthiessen, P., Gibbs, P. E., 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. Environ. Toxicol. Chem. 17**,** 37-43.

McAllister, B. G., Kime, D. E., 2003. Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (*Danio rerio*). Aquat. Toxicol. 65**,** 309-316.

Møller, V., Forbes, V., Depledge, M., 1994. Influence of acclimation and exposure temperature on the acute toxicity of cadmium to the freshwater snail *Potamopyrgus antipodarum* (Hydrobiidae). Environ. Toxicol. Chem. 13**,** 1519-1524.

Nishikawa, J., Mamiya, S., Kanayama, T., Nishikawa, T., Shiraishi, F., Horiguchi, T., 2004. Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. Environ. Sci. Technol. 38**,** 6271-6276.

Oehlmann, J., Di Benedetto, P., Tillmann, M., Duft, M., Oetken, M., Schulte-Oehlmann, U., 2007. Endocrine disruption in prosobranch molluscs: evidence and ecological relevance. Ecotoxicology. 16**,** 29-43.

OECD, 2004. OECD guideline for the testing of chemicals. Sediment-water chironomid toxicity test using spiked water No. 219. Organisation for Economic Co-operation and Development, Paris, France.

OECD, 2005. Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. ENV/JM/MONO(2005)14. . No. 34, Organisation for Economic Co-operation and Development, Paris.

OECD, 2007. Sediment-water *Lumbriculus* toxicity test using spiked sediment. No. 225, Organisation for Economic Co-operation and Development, Paris, France.

OECD, 2010a. Detailed review paper on molluscs life-cycle toxicity testing. Environmental Health and Safety Publications. Series on Testing and Assessment Number 121. ENV/JM/MONO(2010)9,Organisation for Economic Co-operation and Development, Paris, France.

OECD, 2010b. Validation report of the chironomid full life-cycle toxicity test No. 136, Organisation for Economic Co-operation and Development, Paris, France.

OECD, 2012a. Guidance document on standardised test guidelines for evaluating chemicals for endocrine disruption No. 150, Organisation for Economic Co-operation and Development, Paris, France.

OECD, 2012b. OECD guideline for the testing of chemicals. *Daphnia magna* reproduction test. No. 211, Organisation for Economic Co-operation and Development, Paris, France.

OECD, 2015. Work plan for the test guidelines programme. Organisation for Economic Co-operation and Development, Paris.

Ponder, W. F., 1988. *Potamopyrgus antipodarum* - a molluscan colonizer of Europe and Australia. J. Moll. Stud. 54**,** 271-285.

Ruppert, K., Geiß, C., Ostermann, S., Theis, C., Oehlmann, J., 2016. Comparative sensitivity of juvenile and adult *Potamopyrgus antipodarum* (Mollusca: Hydrobiidae) under chronic exposure to cadmium and tributyltin. J. Environ. Sci. Health A. doi:10.1080/10934529.2016.1170443.

Schmitt, C., Oetken, M., Dittberner, O., Wagner, M., Oehlmann, J., 2008. Endocrine modulation and toxic effects of two commonly used UV screens on the aquatic invertebrates *Potamopyrgus antipodarum* and *Lumbriculus variegatus*. Environ. Pollut. 152**,** 322-329.

Shumway, S. E., Davis, C., Downey, R., Karney, R., Kraeuter, J., Parsons, J., Rheault, R., Wikfors, G., 2003. Shellfish aquaculture–in praise of sustainable economies and environments. World Aquacult. 34**,** 8-10.

Sieratowicz, A., Stange, D., Schulte-Oehlmann, U., Oehlmann, J., 2011. Reproductive toxicity of bisphenol A and cadmium in *Potamopyrgus antipodarum* and modulation of bisphenol A effects by different test temperature. Environ. Pollut. 159**,** 2766-74.

Sieratowicz, A., Schulte-Oehlmann, U., Wigh, A., Oehlmann, J., 2013. Effects of test media on reproduction in *Potamopyrgus antipodarum* and of pre-exposure population densities on sensitivity to cadmium in a reproduction test. J. Environ. Sci. Health A 48**,** 481-8.

Städler, T., Frye, M., Neiman, M., Lively, C. M., 2005. Mitochondrial haplotypes and the New Zealand origin of clonal European *Potamopyrgus*, an invasive aquatic snail. Mol. Ecol. 14**,** 2465-73.

Stroben, E., Oehlmann, J., Fioroni, P., 1992. *Hinia reticulata* and *Nucella lapillus*. Comparison of two gastropod tributyltin bioindicators. Mar. Biol. 114, 289-296.

Titley-O'Neal, C. P., Munkittrick, K. R., MacDonald, B. A., 2011. The effects of organotin on female gastropods. J. Environ. Monit. 13**,** 2360-2388.

Tassou, K. T., Schulz, R., 2009. Effects of the insect growth regulator pyriproxyfen in a two-generation test with *Chironomus riparius*. Ecotox. Environ. Safe. 72, 1058-1062.

Taenzler, V., Bruns, E., Dorgerloh, M., Pfeifle, V., Weltje, L., 2007. Chironomids: suitable test organisms for risk assessment investigations on the potential endocrine disrupting properties of pesticides. Ecotoxicology. 16**,** 221-230.

780 Waisberg, M., Joseph, P., Hale, B., Beyersmann, D., 2003. Molecular and cellular
781 mechanisms of cadmium carcinogenesis. Toxicology. 192**,** 95-117.
782 Winterbourn, M., 1970. Population studies on the new zealand freshwater gastropod,
783 *Potamoptrgus antipodarum* (gray). J. Molluscan Stud. 39**,** 139-149.
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Highlights:

- *A new OECD reproductive toxicity test guideline with the New Zealand mudsnail* Potamopyrgus antipodarum *has been developed*
- *A draft test protocol was used to assess effects of cadmium and TBT in 11 laboratories in a validation study*
- *Effect values were reproducible and consistent with literature data*
- *Successful validation of the protocol was acknowledged by OECD by final acceptance of the test guideline in 2016*