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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**

Molluscs 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. The present study describes the development and validation of the New Zealand mudsnail *Potamopyrgus antipodarum* reproduction test within the OECD Conceptual Framework for Endocrine Disrupters Testing and Assessment. The number of embryos in the brood pouch, reflecting the individual reproduction effort in snails, and adult mortality serve as main endpoints. In this study the results of two validation studies of the reproduction test with eleven laboratories and the chemicals tributyltin (TBT) with nominal concentrations of 10 - 1000 ng TBT-Sn/L and cadmium with concentrations of 1.56 - 25 µg/L are presented.

The proposed test design could be implemented by all participating laboratories resulting in comparable effect concentrations. After TBT exposure mean EC10, EC50, NOEC and LOEC (with coefficient of variation) were 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. Mean effect concentrations in cadmium exposed snails were 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%).

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

*Keywords: Standardisation, Mollusc, Reproduction, Endocrine Disruption, Tributyltin, Cadmium.*

*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*

# 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: they may respond to various mechanisms 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 molluscs has not yet been established within the Conceptual Framework, although molluscs are ecologically crucial organisms, which are essential to the biosphere and to the human economy (i.e. the shellfish industry). 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). To close this gap OECD welcomed and supported the development of standard reproduction tests with mollusc 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 Molluscs Life-cycle Toxicity Testing was prepared summarising the state of knowledge on mollusc testing and proposing possible test designs and test species (OECD, 2010a). The most promising candidate species for a standardised test guideline were the New Zealand mudsnail *Potamopyrgus antipodarum* (Gastropoda: Hydrobiidae), the pond snail *Lymnaea stagnalis* (Gastropoda: Lymnaeidae) and the Pacific oyster *Crassostrea gigas* (Bivalvia: Ostreidae). In 2011, the leading countries Germany, United Kingdom, France and Denmark promoted the inclusion of the development and validation of new test guidelines on mollusc 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.

1. **MATERIALS AND METHODS**
   1. *Test organism*

Originating from New Zealand the mudsnail *Potamopyrgus antipodarum* (Mollusca, Gastropoda, Hydrobiidae) 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). 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) and reproduce throughout the year (Gust et al., 2011).

* 1. Implementation of the validation tests
     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). 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 NaHCO3) 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 16 ± 1°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.

* + 1. Principle of the reproduction test and experimental conditions

Adult *P. antipodarum* of a defined size class (Table 1) are exposed to a concentration range of the tested chemical and control groups for 28 d. Each exposure group, including a negative (water) and if required solvent controls, consists of four replicates containing ten individuals each. Test medium is changed three times a week to maintain exposure concentrations and adequate water quality parameters, e.g. O2, pH and conductivity, which are monitored before exposure medium renewals. Dead snails are counted and removed from the test vessels during medium renewal. Once a week, the test vessels are changed to prevent biofilm growth. After 28 d, snails are sacrificed at -20°C in a freezer or quick-frozen in liquid nitrogen, and stored at -80°C. The snails are dissected by removing the shell from the soft body and opening the brood pouch. The main endpoint, the number of embryos in the brood pouch of each individual, is assessed. Table 1 summarises the main experimental conditions.

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 NaHCO3 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 | 16 ± 1°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 |

* + 1. 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 a replication study at laboratory 2A. Because a statistically significant reduction of embryo numbers occurred only at the highest tested concentration in most of the reproduction tests, laboratories 2Ab and 2J performed the reproduction test with *P. antipodarum* in 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.

* + 1. *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
  1. *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 (EC10, EC50) 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 lowest r2.

## 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] | | | O2 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 |

* 1. *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)

*TBT*

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

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. |

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

* 1. *Effects of cadmium on P. antipodarum*

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

In all participating laboratories of validation I and II a concentration-dependent decrease of embryo numbers could be observed. Figure 1 shows the concentration-response curves fitted 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 EC10 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 EC10 values ranging from 3.46 µg/L to 10.3 µg/L. EC50 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 EC50 values was observed between the studies. Due to the bad health status 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.

Cadmium dose-response curves Validation I and II

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, EC10 and EC50 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 |
| EC10 | 4.52  (2.52-6.51) | *0.689*  *(0.25-1.91)* | 3.46  (2.28-5.25) | 4.49  (2.81-7.17) | 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) | 35.5 |
| EC50 | 11.3  (9.48-13.2) | *4.59*  *(3.00-7.02)* | 11.4  (9.30-13.9) | 13.2  (10.8-16.0) | 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) | 21.8 |

* 1. *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 EC10, EC50, NOEC and LOEC are summarised in Table 6. NOECs showed a 5.98-fold difference between laboratories. Calculated ECx values are similar and most of the 95%-confidence intervals are overlapping. EC50 value fold difference was 1.79, with an inter-laboratory coefficient of variation of 39.3%.

TBT embryo numbers validation II

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, EC10 and EC50 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 |
| EC10 | 45.0  (28.4–71.2) | 12.7  (5.73–28.3) | 89.1  (53.2-149) | 22.4  (15.6–32.3) | 36.8  (26.1–51.7) | 36.5  (20.4–65.0) | 6.62  (0.95–45.9) | 76.9 |
| EC50 | 153  (109-213) | 124  (77 - 200) | 188  (157-226) | 37.9  (28.5-50.4) | 88.8  (73.5-107) | 137  (93.2-200) | 159  (48.8-519) | 39.3 |

###### DISCUSSION

* 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 EC50 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 EC10 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 mollusc *L. stagnalis* Ducrot et al. (2014) found coefficients of variations of 29.5% and 71.5% for EC10 and EC50 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 coefficients of variation of values obtained in the studies with TBT are most probably caused by the fact that TBT has a much lower water solubility and higher degradation in the test system. 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.

*Cadmium*

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 EC10 values of 1.30 µg/L and 9.73 µg/L and EC50 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 mollusc species, *L. stagnalis* (Ducrot et al., 2014), *P. antipodarum* displayed a higher sensitivity to cadmium. These authors found that the mean EC50 was 94.5 µg/L, which is 6.7-fold higher compared to the mean EC50 of 14.2 µg/L in the present study.

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.

*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 EC10 and EC50 values of 37.8 ng TBT-Sn/L and 115 ng TBT-Sn/L, respectively reported by Duft et al. (2003) 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. 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 mollusc 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).

Overall, the assessed results with *P. antipodarum* exposed to TBT (lowest NOEC: 16.1 ng TBT-Sn/L) would have led to a PNEC of 1.6 ng TBT-Sn to be used in the chronic risk assessment of TBT. Although the resulting PNEC would still not be protective for marine molluscs, 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.

* 1. *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 m2. 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.

* 1. *Outlook*

After the successful completion of these first validation studies, two further ring tests with the substances prochloraz, trenbolone, triclosan and triclocarban were performed. Here, the test design was slightly amended (six replicates with six snails per replicate) to improve the statistical power of the reproduction test. The used test design with 4 replicates and 10 snails per replicate was originally designed for an evaluation with the weighted mean embryo number per treatment group. This evaluation is based on common error propagation rules and considers the tank as the experimental unit, but takes into account the within-tank (*i.e.* individual animal) variability (OECD, 2010a). To adapt the draft technical guideline to the standards of OECD guidelines, the evaluation method was changed and a further validity criterion was added: the mean embryo number per snail in the controls should be ≥ 5. This criterion was also fulfilled in all reproduction tests presented in this study.

The draft test guideline of the reproduction test with *P. antipodarum* has been acknowledged by the experts of the “validation management group on ecotoxicity testing” (VMG-Eco) of the OECD and the OECD *ad hoc* Expert Group on Invertebrate testing who supported the submission of the draft test guideline. After a successful second international commenting round by OECD member states the guideline was approved by the Working Group of the National Coordinators for the Test guidelines Programme (WNT) in April 2016.

###### CONCLUSIONS

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, EC10 and EC50 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.

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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