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Optimizing the design of a reproduction toxicity test with the pond snail *Lymnaea stagnalis*

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ABSTRACT

This paper presents the results from two ring-tests addressing the feasibility, robustness and reproducibility of a reproduction toxicity test with the freshwater gastropod *Lymnaea stagnalis* (RENILYS strain). Sixteen laboratories (from inexperienced to expert laboratories in mollusc testing) from nine countries participated in these ring-tests. Survival and reproduction were evaluated in *L. stagnalis* exposed to cadmium, tributyltin, prochloraz and trenbolone according to an OECD draft Test Guideline. In total, 49 datasets were analysed to assess the practicability of the proposed experimental protocol, and to estimate the between-laboratory reproducibility of toxicity endpoint values. The statistical analysis of count data (number of clutches or eggs per individual-day) leading to ECx estimation was specifically developed and automated through a free web-interface. Based on a complementary statistical analysis, the optimal test duration was established and the most sensitive and cost-effective reproduction toxicity endpoint was identified, to be used as the core endpoint. This validation process and the resulting optimized protocol were used to consolidate the OECD Test Guideline for the evaluation of reproductive effects of chemicals in *L. stagnalis*.

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1. Introduction

In 2010, the Organization for Economic Cooperation and Development (OECD) recommended the development of a new test guideline for reprotoxicity testing in freshwater molluscs (OECD, 2010). Between 2011 and 2013, a 56-days reproductive semi-static-renewal test protocol was evaluated in a prevalidation ring-test using *Lymnaea stagnalis* (L.) and involved seven laboratories in Europe (Ducrot et al., 2014). Subsequent statistical analyses provided robust estimates of x% lethal and effective concentrations (LCx and ECx) for both clutch- and egg-based endpoints, and between-laboratory comparison demonstrated a low variability in LCx and ECx values. In addition, a consolidated draft of the standard operating protocol was provided with detailed rearing and toxicity test procedures as well as their application to evaluate reproductive toxicants (Ducrot et al., 2014). Consequently, both the OECD Validation Management Group for Ecotoxicity testing (VMG-Eco) and the OECD ad-hoc Expert Group on Invertebrate Testing further supported a validation ring-test.

The aim of the validation ring-test was threefold: (i) assessing the reproducibility of the test results among a larger number of laboratories with different levels of experience in mollusc testing (from inexperienced to experts); (ii) assessing consistency and reproducibility of toxicity thresholds (i.e., ECx values estimated for all laboratories) between the two ring-tests (i.e., prevalidation vs. validation steps); (iii) assessing responses of snails to a larger number of chemicals. In addition, key issues related to optimization of the test design also deserved elucidation: (i) costs, benefits and feasibility in reducing the exposure duration (i.e., could the test duration be reduced while safeguarding accuracy and precision of ECx estimates?); (ii) benefits of recording both the number of clutches and the number of eggs per clutch (i.e., does the choice of the recorded endpoint matter when estimating toxicity thresholds?).

The validation ring-test was conducted from October 2013 to October 2014 according to the draft standard operating procedure. In total, 13 laboratories from academia, government, industry and consultancy, in Europe and North-America, participated, collecting raw data and water samples for statistical and chemical analyses, respectively. Six laboratories (all new compared to the laboratories involved in the prevalidation ring-test) were in charge of testing cadmium (Cd), which had been used in the prevalidation ring-test (Ducrot et al., 2014). Five laboratories tested tributyltin (TBT), four laboratories tested prochloraz (PRO), and two laboratories tested trenbolone (TRB). The choice of these substances was based upon recommendations from the OECD VMG-Eco (Table 1). They were assumed to cause adverse effects on snail reproduction (as confirmed in pre-tests that were conducted for all chemicals except trenbolone). These substances reflect different levels of complexity in terms of toxicity testing; Cd is an “easy-to-test” substance, whereas TBT, PRO and TRB are more difficult substances to test (e.g., use of solvent required for TBT; limited stability of PRO in water, both difficulties being encountered for TRB (OECD, 2000)). Hence, performing the validation ring-test with difficult test substances could contribute to further demonstrate the robustness of the experimental protocol and to identify the most relevant reproduction endpoint in *L. stagnalis*.

This paper presents the results of the validation ring-tests for Cd, TBT and PRO, in comparison with those of the prevalidation ring test where applicable. ECx were estimated for each laboratory and then compared in order to assess their reproducibility between laboratories. We also investigated the consequence of reducing the exposure duration on both ECx median value and uncertainty. Finally, after having confirmed the low between-laboratory variability when reducing the exposure duration, we considered the possibility of recording only one core endpoint to be used in the OECD test guideline for the reproduction toxicity tests with *L. stagnalis*.

2. Materials and methods

2.1. Implementation of the validation ring-test

The experimental design used to collect raw data during the validation ring-test followed the one used for the prevalidation ring-test. All details about test organisms, snail acclimation, tested chemicals, experimental conditions, sampling and analysis of test media, and collection of raw data are available in Ducrot et al (Ducrot et al., 2014) and summarized in Supplementary Information (Table S0). The principle of the reproduction toxicity test and the specificities of the validation ring-test are here recalled.

2.1.1. Principle of the reproduction toxicity test

The primary objective of the test was to assess the effect of chemicals on the reproductive output of *L. stagnalis*. To this end, reproducing adults of *L. stagnalis* were exposed to a range of 5 concentrations of the test chemical and a control (water/medium only or, when required, a solvent control) and monitored for 56 days for survival and reproduction. No less than 6 replicates of 5 snails were exposed to each concentration (i.e., 30 snails per treatment and per control). Prior to the test, snails were sampled from a laboratory parasite-free culture, checked for identical size (27 ± 2 mm), and introduced into test vessels for a few days (acclimation period), until reproduction restarted. As soon as the exposure to the test chemical started (i.e., day 0 of the test), survival and fecundity were recorded at least twice a week, before feeding the snails *ad libitum* with (organic) round-headed lettuce and renewing the water/medium. Dead snails were counted and withdrawn from the test vessels. Both the number of clutches and the number of eggs per clutch were counted. Raw data were collected in a spreadsheet automatically providing a text file under the appropriate format for the statistical analyses.

2.1.2. Tested chemicals and exposure water sampling and analysis

Specifications of the test chemicals are provided in Table 1. Nominal concentrations for Cd were chosen based on the prevalidation ring-test, namely 25, 50, 100, 200, 400 µg L^{-1}. Nominal concentrations were 87.5, 175, 350, 700, 1400 ng L^{-1} and 10, 32, 100, 320, 1000 µg L^{-1} for TBT and PRO, respectively. Water samples were collected before and after water/medium renewal, at the beginning, mid-term and end of each experiment for the determination of actual exposure concentrations (42 samples per experiment). Actual Cd concentrations in water/medium were measured in 50 mL acidified samples (triplicates) by atomic adsorption spectrometry (limit of detection: 0.8 µg L^{-1}). Actual TBT concentrations in water/medium were measured in triplicate by coupled capillary gas chromatography to mass spectrometry (GC-MS-MS; ITQ100, Thermo Scientific, USA) according to Giusti et al (Giusti et al., 2013) with slight modifications. The limit of detection (LOD) was 6 ng TBT L^{-1} and the limit of quantification (LOQ) was 18 ng TBT L^{-1} (concentrations are expressed in ng TBT L^{-1}: equivalent in ng Sn L^{-1} can be calculated by dividing these values by a factor 2.44). The mean recovery efficiency was 99% ± 18.6% and was in good agreement with requirements of the SANCO guidance document (SANCO/12571/2013, 2014). PRO samples were analysed directly from filtered samples by LC-MS-MS (LOD: 3.9 µg L^{-1}, LOQ: 1.56 µg L^{-1} and mean recovery efficiency: 70% ± 6.3%).