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Growth inhibition in early life-stage tests predicts full life-cycle toxicity effects of lead in the freshwater pulmonate snail, *Lymnaea stagnalis*

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ABSTRACT

The freshwater pulmonate snail, *Lymnaea stagnalis*, is the most sensitive freshwater organism tested to date for several metals (Co, Cu, Pb, Ni) based on 28 d early life-stage (ELS) tests in which growth was the most sensitive endpoint. The United States Environmental Protection Agency (USEPA) has expressed concern that growth in 28 d ELS tests with mollusks may overpredict toxicity because of the potential for recovery in a full life-cycle (LC) test. Consequently, the USEPA only accepts the survival endpoint for these tests in establishing water quality criteria (WQC). To address this concern, the current study aimed to test the sensitivity of *L. stagnalis* to Pb in a 56 d full LC test evaluating survival, growth, reproductive and embryonic growth endpoints and compare the estimated effect levels to those established using the 28 d ELS test design. The most sensitive endpoints in this study were 28 d growth and 56 d egg mass production, both with a NOEC of <1.0 $\mu g L^{-1}$ and a LOEC of 1.0 $\mu g L^{-1}$, showing that the ELS growth endpoint is predictive of the 56 d reproduction endpoint. Snails exposed to 1.0 and 2.7 $\mu g L^{-1}$ Pb showed full and partial recovery from growth inhibition between 28 and 56 d. While this recovery supports the USEPA's concern about the 28 d growth endpoint; considering the reproductive lifespan of *L. stagnalis* and the recovery dose-response, we conclude that the 28 d growth endpoint will be within a factor of 3 of full LC endpoints. This is consistent with the level of precision previously determined for fish ELS tests, which the USEPA accepts for WQC derivation, and suggests that tests using 28 d ELS growth endpoint for *L. stagnalis* may be acceptable for inclusion in WQC derivation.

1. Introduction

One of the most significant uses of toxicity testing is to derive water quality criteria (WQC) for the protection of aquatic organisms. In the United States, the Environmental Protection Agency (USEPA) derives both acute and chronic WQC for pollutants. The USEPA defines chronic toxicity tests used to derive chronic WQC by their duration; either as life-cycle (LC) tests, partial life-cycle (PLC) tests, or early life-stage (ELS) tests. These tests are used to determine Species Mean Chronic Values (SMCVs), and the distribution of SMCVs is used to set the chronic WQC (typically at the 5th percentile, which ideally protects 95% of taxa) (Stephen, 1985).

In deriving chronic WQC, the USEPA prefers the use of full life-cycle studies that begin with <24 h old organisms and continue through sexual maturation, reproduction, and typically survival and growth of early life stage F_1 organisms. For fish, full life-cycle studies can require 6–24 months to complete depending on the species. Because of the significant resources required for such studies, the USEPA accepts partial life-cycle (beginning with adult exposures and following through early life stage survival and growth of F_1 fish) and early life-stage (beginning with embryos and following hatching success, survival, and growth through 30 d post-hatch) studies when full life-cycle studies are not available. These shorter test designs for fish have been shown to be reasonably predictive of full life-cycle studies for a wide range of toxicants (McKim, 1977; Macek and Sleight, 1977).

In contrast, for invertebrates, the USEPA has relied almost exclusively on full life-cycle studies for chronic WQC derivation. Full life-cycle studies with invertebrates can typically be performed in ≤60 d and often ≤28 d and are comparable to the level of effort required to perform ELS and PLC studies with fish; however, over the last 25 years, a number of ELS test methods have been developed for invertebrates (i.e., amphipods, aquatic insects, and freshwater mollusks). While there is now a relatively large body of data using the ELS method for invertebrates, there has not been a systematic analysis of how well ELS studies for invertebrates predict results

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Table 1
Chemistry for test water used in 56 d Pb exposure on *L. stagnalis*. Data presented as mean ± SEM.

Parameter	Value
pH	6.89 ± 0.06
Temperature (°C)	24.8 ± 0.2
DO (g L^{-1})	6.86 ± 0.04
[Na^+] (mM)	1.18 ± 0.03
[Ca^{2+}] (mM)	0.69 ± 0.06
[K^+] (mM)	0.03 ± 0.00
[Mg^{2+}] (mM)	0.18 ± 0.01
[Cl^-] (mM)	1.19 ± 0.12
[SO_4^{2-}] (mM)	0.08 ± 0.00
[HCO_3^-] (mM)	0.37 ± 0.06
DOC (μM C)	330 ± 7.02

observed in LC studies. As a result, the USEPA has generally not considered invertebrate ELS data in deriving chronic WQC. This issue was highlighted in the USEPA's recent revision of the ammonia WQC, where the most chronically sensitive freshwater taxa are mollusks based on data from ELS studies (USEPA, 2009). In this case, the USEPA judged that it is unclear whether effects on freshwater mollusk growth in relatively short-term studies (28 d) translate to long-term effects, as these organisms may recover from initial growth inhibition in longer-term (e.g., 90 d) studies. In contrast, survival effects are not reversible; therefore, any observed survival effects during a 28 d exposure are expected to directly predict the outcome of a full life-cycle test if one was performed (USEPA, 2009). Thus, the USEPA judged that in the absence of data from LC studies; survival, but not growth effects, from 28 d invertebrate studies can be used in the derivation of WQC. Other regulatory jurisdictions have less rigorous definitions of what constitutes a chronic test. For example, in the EU, ELS studies with non-standard test organisms, for which there has been no validation of their power to predict LC results, are routinely accepted for purposes of setting water quality guidelines and performing regional risk assessements (Bodar et al., 2005).

Previous studies have established members of the freshwater pulmonate snail genus *Lymnaea* as some of the most sensitive freshwater organisms to dissolved lead (Pb) exposure (Borgmann et al., 1978; Brix et al., 2012; Grosell and Brix, 2009; Grosell et al., 2006b). In particular, the pond snail, *Lymnaea stagnalis*, is the most sensitive freshwater organism tested to date, with an EC20 of <3 μg L^{-1} for growth (Brix et al., 2012). Unlike ammonia, where survival effects occur at concentrations just slightly higher than observed growth effects, no significant effects on snail survival have been observed in these ELS studies at concentrations up to 120 μg L^{-1} Pb.

Consequently, the objective of the current study was to test the sensitivity of *L. stagnalis* to chronic Pb in a full LC exposure, specifically evaluating the USEPA's concern that growth effects observed in previous 28 d ELS studies may not predict effect in full LC studies. Endpoints for this study included survival, growth, and reproduction through 56 d, as well as embryonic growth of F_1 organisms through 10 d of exposure.

2. Materials and methods

2.1. Experimental animals

Adult snails were obtained from an in-house culture maintained in flow-through dechlorinated City of Miami tap water (Table 1). The culture was fed a mix of lettuce and sweet potatoes, and egg masses were transferred from the main culture tanks to static-renewal nursery tanks for hatching before being used in toxicity studies.

2.2. Effect of prolonged Pb exposure on snail weight, specific growth rate, survival and reproduction

A full life-cycle chronic toxicity test (56 d) was performed using newly hatched snails (≤24 h old) in a flow-through system. Dechlorinated Virginia Key tap water was supplied via gravity to a single source container with a volume of 44 L, then to a series of 6 mixing chambers. Total water flow from the source container into the mixing chambers, with volumes of 7 L each, was ~180 mL min^{-1}, where mixing was achieved by vigorous aeration. A constant water level was achieved in the mixing chambers via an overflow drain. Stock solutions of Pb (as $PbNO_3$ in Milli-Q water) were added to the mixing chambers via Mariotte bottles. Each mixing chamber supplied test solution (Pb added) to four replicate 1.5 L test chambers, each containing five newly-hatched snails, at a flow rate of ~8–10 mL min^{-1}.

Prior to test initiation, the flow-through system was operated for 5 d without snails and a small piece of sweet potato was introduced to each exposure chamber. This allowed a biofilm to be established in the exposure beakers upon which the juvenile snails could feed. Snails were exposed to control (<0.18), 1.0 ± 0.16, 2.7 ± 0.38, and 8.4 ± 1.05 μg L^{-1} Pb (measured concentrations). Snail survival was monitored daily. Weekly, snails were blotted dry on paper towels after which total body mass (wet weight) was determined to the nearest 0.1 μg on an analytical balance (Mettler, Toledo). Water samples were collected at the beginning of the experiment and weekly thereafter for measurement of dissolved Pb concentrations (defined as passing through a 0.45 μM filter). In addition, water samples were collected three times weekly for temperature, dissolved oxygen and pH measurements and twice monthly to measure dissolved Na^+, Ca^{2+}, K^+, Mg^{2+}, Cl^-, SO_4^{2-}, and HCO_3^- concentrations. Three samples were collected throughout the experiment for DOC analysis.

Following 32 d of exposure, when the first egg masses appeared in the control treatment, egg masses, if present, were collected daily from each treatment tank and preserved in 10% formalin solution. The number of egg masses per day was counted for each treatment tank. The number of embryos per egg mass was determined on preserved egg masses using a dissecting microscope.

2.3. Embryonic Pb toxicity

Following 56 d of exposure, treatment tanks were cleared of adult snails and egg masses. One egg mass per container was kept and maintained under experimental conditions. Photographs of each egg mass were taken at 4 d and 7 d using a Nikon SMZ800 precision microscope (Nikon Instruments, Melville, NY, USA) equipped with a Firei400 digital camera (Unibrain, San Ramon, CA, USA) under 10×, 15×, and 30× magnification. Egg capsule diameter and snail embryo diameter were determined under 15× magnification using ImageJ software (National Institute of Health, 2011).

2.4. Analytical chemistry

Water samples for determination of Pb exposure concentrations were passed through a 0.45 μm cellulose nitrate syringe filter (Acro-disc, Pall Life Sciences, MI, USA) and acidified by addition of HNO_3 (Fisher Scientific, Trace metal grade) to a final concentration of 1%. Lead concentrations were analyzed by graphite furnace atomic absorption (Varian 220Z, Varian, Walnut Creek, CA, USA) via multiple injection, in which samples were analyzed in duplicate.

Concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} in water samples were determined using atomic absorption spectrophotometry (VarianAA 220FS, Mulgrave, Victory, Australia). Concentrations of Cl^- and SO_4^{2-} were determined using anion chromatography (DIONEX DX 120, Sunnyvale, CA, USA). Concentrations of HCO_3^- were