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CHRONIC TOXICITY OF LEAD TO THREE FRESHWATER INVERTEBRATES—*BRACHIONUS CALYCIFLORUS*, *CHIRONOMUS TENTANS*, AND *LYMNAEA STAGNALIS*

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Abstract—Chronic lead (Pb) toxicity tests with *Brachionus calyciflorus*, *Chironomus tentans*, and *Lymnaea stagnalis* were performed in artificial freshwaters. The no-observable-effect concentration (NOEC), lowest-observable-effect concentration (LOEC), and calculated 20% effect concentration (EC20) for the rotifer *B. calyciflorus* were 194, 284, and 125 μg dissolved Pb/L, respectively. The midge *C. tentans* was less sensitive, with NOEC and LOEC of 109 and 497 μg dissolved Pb/L, respectively, and the snail *L. stagnalis* exhibited extreme sensitivity, evident by NOEC, LOEC, and EC20 of 12, 16, and <4 μg dissolved Pb/L, respectively. Our findings are presented in the context of other reports on chronic Pb toxicity in freshwater organisms. The *L. stagnalis* results are in agreement with a previous report on pulmonate snails and should be viewed in the context of current U.S. Environmental Protection Agency (U.S. EPA) hardness adjusted water quality criteria of 8 μg Pb/L. The present findings and earlier reports indicate that freshwater pulmonate snails may not be protected by current regulatory standards. Measurements of whole-snail Na^+ and Ca^{2+} concentrations following chronic Pb exposure revealed that Na^+ homeostasis is disturbed by Pb exposure in juvenile snails in a complicated pattern, suggesting two physiological modes of action depending on the Pb exposure concentration. Substantially reduced growth in the snails that exhibit very high Ca^{2+} requirements may be related to reduced Ca^{2+} uptake and thereby reduced shell formation.

Keywords—*Brachionus calyciflorus* *Chironomus tentans* *Lymnaea stagnalis* Lead Species sensitivity distribution

INTRODUCTION

The nonessential metal lead (Pb) occurs in the environment as a consequence of both natural and anthropogenic processes, with mining and smelting, coal burning, cement manufacturing, and use in gasoline contributing most to Pb contamination of aquatic environments ([1]; http://www.inchem.org/documents/ehc/ehc/ehc165.htm). A number of studies have investigated effects of prolonged Pb exposure on freshwater fish. These studies report a wide range of effects induced by chronic exposure to elevated Pb concentrations, including effects on pituitary function, gonadosomatic index, oocyte growth [2], anemia [3], reduced erythrocyte δ-amino levulinic acid dehydratase activity [4], neurological disorders (black tail), and scoliosis (spinal curvature) [5]. Fewer studies have examined the physiological and biochemical effects of prolonged Pb exposure on freshwater invertebrates, but reports of Pb toxicity evaluated by mortality are plentiful. Exceptions include reports of sublethal and reproductive effects in daphnia [6] and behavioral effects in pulmonate snails [7] possibly linked directly to Pb-induced modifications of neuron membrane properties [8,9].

As for other metals [10–12], large variation in sensitivity to Pb exists [13–15] that, together with the limited number of reports of chronic Pb-exposure studies, limits our ability to establish safe and realistic environmental regulations. The purpose of the present study was to expand the information about sensitivity to chronic Pb exposure in freshwater invertebrates. To pursue this objective, we exposed *Brachionus calyciflorus*, *Chironomus tentans*, and *Lymnaea stagnalis* to Pb. The pelagic rotifer *B. calyciflorus* and the benthic midge *C. tentans* were chosen for these experiments because no information is available on prolonged Pb exposure for either freshwater rotifers or aquatic insect larvae.

Rotifers offer the advantage of having one of the shortest life cycles of any animal, allowing for testing over several generations within a 48-h time period [16]; at the same time, they are a major component of zooplankton in freshwater ecosystems [17]. Various rotifer species are employed routinely in toxicity testing both in Europe and North America and standardized test conditions are established [17–19].

Chironomids have been used routinely in laboratory testing with early studies dating back more than a century [20]. They currently are recommended as standard toxicity test organisms in both Europe and North America [21,22]. *Chironomus tentans*, in particular, are used extensively in toxicity testing [20] and were exposed to Pb from shortly after hatching to emergence in the present study. This larval part of *C. tentans'* life cycle typically lasts on the order of 30 d and includes several stages of larval development (instars).

The final test organism, the common pond snail (*L. stagnalis*), was chosen because a large number of studies have reported on metal accumulation and effects in this and other pulmonate snails [7,9,23–27]. In particular, one early study by Borgmann et al. [13] revealed that the closely related *Lymnaea palustris* is highly sensitive to chronic Pb exposure. In the Borgmann study, early life stages of *L. palustris* were found to be extremely sensitive to low Pb levels, being impacted significantly at total Pb concentrations between 12 and 19 μg/L. With respect to chronic Pb exposure, these observations make *Lymnaea* the most-sensitive aquatic organisms tested to date. Because pulmonate snails exhibit rather high tolerance

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Table 1. Test medium pH and chemical composition (in mM) for studies on *Brachionus calyciforus* and *Lymnaea stagnalis*

	Concn.
Na^+	1.37
K^+	0.05
Ca^{2+}	0.73
Mg^{2+}	0.29
Cl^-	1.09
SO_4^{2-}	0.66
Total CO_2	1.39
pH	8.19

Table 2. Test medium pH and chemical composition (mM) for studies on *Chironomus tentans*

	Concn.
Na^+	0.31
K^+	0.02
Ca^{2+}	0.44
Mg^{2+}	0.04
Cl^-	0.33
SO_4^{2-}	0.28
Total CO_2	0.35
pH	7.9
TOC[a]	0.1

[a] TOC = total organic carbon.

to acute metal exposure, we exposed newly hatched snails for a period of 30 d in the present study.

MATERIALS AND METHODS

Experimental animals

Three species of freshwater invertebrates were used for the present investigation. Cysts of the rotifer *B. calyciforus* were obtained from Florida Aqua farms in Dade City (Florida, USA), and egg masses of the midge *C. tentans* were obtained from Aquatic Biosystems (Fort Collins, CO, USA). An in-house culture of the freshwater pond snail *L. stagnalis* was established from approximately 100 adult individuals generously provided by N. Syed (University of Alberta, Calgary, AB, Canada).

General experimental procedures

All toxicity tests were conducted in standard artificial freshwater using the formulation of the U.S. EPA [28] unless otherwise stated. Resulting water chemistry is presented in Table 1. All tests were performed in temperature-controlled incubators or environmentally controlled rooms at 25 ± 1°C for the rotifers and 23 ± 1°C for the midges and snails. Tests with rotifers were performed in darkness to avoid algae cell divisions, and tests on the midges and snails were performed on a 16:8-h light:dark cycle. In all cases, lead was added as lead nitrate, $Pb(NO_3)_2$, obtained from Fisher Scientific (Atlanta, GA, USA).

Chronic toxicity testing on B. calyciforus

Two days before test initiation, cysts were incubated in a Petri dish containing the test medium (Table 1) placed in an incubator at 25 ± 1°C. The incubation medium containing the cysts was agitated gently at 45 rpm until hatch. The tests were conducted in accordance with previously described methods [17]. In brief, 15-ml glass test tubes containing 12 ml of test solution each, served as test chambers. Tests were set up in quadruplicate at nominal concentrations of 0, 187.5, 375, 750, 1,500, 3,000 μg Pb/L. An additional three test chambers per treatment were set up and served as controls for measurements of total and dissolved (defined as the fraction of Pb recovered after passing through a 45-μm cellulose nitrate syringe filter; Acrodisc, Pall Life Sciences, MI, USA) Pb concentration. These three additional test chambers per treatment were not seeded with rotifers and were taken down after 24 h; water samples were acidified to 1% HNO_3 (trace metal–grade) and stored at −20°C until analyzed as outlined below (see *Analytical techniques* section). Prior to seeding, each test chamber received a rotifer food source, the green algae *Nannochloropsis* at 3×10^6 cells/ml, after which Pb was added from a concentrated $PbNO_3$ stock solution. The test medium containing Pb and algae was allowed to equilibrate for 30 min before seeding with six newly hatched individuals per chamber. After seeding, all chambers were placed in a light-shielded incubator and shaken at 100 rpm. The test was terminated after 48 h, at which time the total number of rotifers in each test chamber was recorded using a dissecting microscope.

Chronic toxicity testing on C. tentans

Testing with *C. tentans* was initiated with 8-d-old larvae. Larvae ($n = 5$) were placed in 150-ml polypropylene test chambers containing 130 ml of the test solution and 15 ml of thoroughly rinsed beach sand that served as a substrate for the larvae to build tubes. The experimental design included five Pb concentrations and a control, each performed in triplicate. Nominal concentrations of 0, 100, 200, 400, 600, and 800 μg/L were tested. Individual test chambers were supplied with a test water flow-through of 15 to 20 ml/min. This flow rate represents a compromise between having constant, high water quality and not disturbing the sand substrate. The test water for experiments with *C. tentans* was a mixture of Virginia Key (Florida, USA) tap water (one-third) and deionized water (two-thirds) supplemented with Ca^{2+} (as CaSO4). Resulting water chemistry is listed in Table 2. The water chemistry again represents a compromise between providing suitable chemical conditions for larval development (supplemented with Ca^{2+}) and being able to maintain high Pb concentrations in solution (by maintaining low HCO_3^- and CO_3^{2-} levels). The reported approach represents the best of five different test procedures, the remaining four not being reported.

Throughout the test, animals were fed Tetramin slurry (40 g/L) at a rate of 0.05 ml/organism/d, and mortality and midge emergence were recorded daily. The test was terminated after 5 d of no emergence from any of the treatments (27 d of exposure). Water samples were taken for measurements of total and dissolved Pb concentrations as outlined below.

Chronic toxicity testing on L. stagnalis

Testing was conducted in 150-ml polypropylene cups containing 100 ml of the appropriate test solution and consisted of six Pb concentrations (nominal 8, 18, 30, 80, 187.5, and 375 μg/L) and a control. Each treatment was performed on 10 animals placed in individual test chambers in general accordance with the methods described by Sloof and Canton [29] and Bhargava [30]. In brief, newly hatched snails (<24-h-old) were placed in the test chambers and fed an excess of carrots, sweet potatoes, and lettuce. Uneaten food was removed at each complete water change, which occurred three times weekly. Snail survival was monitored daily. Water samples were obtained three times weekly for later analysis of total and dis-

solved Pb concentrations as outlined below (see *Analytical techniques* section).

At the end of the 30-d exposure, surviving snails were removed from the test chambers and blotted dry; their mass was determined to the nearest 100 μg before storage at −20°C until analysis. Because an obvious effect of Pb exposure was observed on snail growth, individual snails were placed in 1.5-ml ultracentrifuge tubes after mass determination. A large number of additional control animals were chosen to encompass the size range observed for the Pb-exposed snails, their masses were determined the same as above, and they also were placed in centrifuge tubes. Trace metal–grade nitric acid (1N) was added to each of these centrifuge tubes (5 vol:mass) and snails were digested overnight at 80°C.

Analytical techniques

Water samples for determining of total and dissolved concentrations of Pb were acidified immediately upon sampling by addition of HNO_3 (Fisher Scientific, trace metal–grade) to a final concentration of 1%. Lead concentrations in tissue and water (total and dissolved) samples were analyzed by graphite furnace atomic absorption (Varian 220Z, Varian, Walnut Creek, CA, USA). Test water was analyzed for Na^+, K^+, Ca^{2+}, and Mg^{2+} by atomic absorption (Varian 220FS); for Cl^- and SO_4^{2-} by anion chromatography (DIONEX DX120, Sunnyvale, CA, USA); for pH (PHM220, Radiometer, Copenhagen, Denmark fitted with an Accumet combination glass electrode); and for total CO_2 using a Corning 962 carbon dioxide analyzer (Malvern, Worcestershire, UK). Dissolved oxygen was measured in Chironomid test chambers by a handheld dissolved oxygen meter (WTW Oxi 340i, Weilheim, Germany). In addition, the whole-snail Na^+, Ca^{2+}, and Pb concentrations were determined after appropriate dilution by atomic absorption (instrumentation as above).

Statistical analysis and presentation

Data are presented at means ± 1 standard error of the mean as a function of measured dissolved Pb unless otherwise stated. Tissue concentrations of Pb, Na^+, and Ca^{2+} are expressed in wet weight units. Reproduction, survival, and growth data (rotifers, chironomids, and snails, respectively) were evaluated using the statistical computer package ToxCalc™ Version 5.0 (Tidepool Scientific, McKinleyville, CA, USA). In all cases, the no-observable-effect concentration (NOEC) and lowest-observable-effect concentration (LOEC) were determined using analysis of variance and the 20% effect concentration (EC20) was determined by linear regression. Intrinsic growth rate for rotifers was calculated as described by Snell and Moffat [17]. For the chironomid test, five different nominal Pb concentrations resulted in only four significantly different dissolved Pb concentrations. Consequently, data are presented as a function of these four measured dissolved Pb concentrations only.

RESULTS

B. calyciflorus

Addition of Pb to the test medium resulted in measured mean dissolved concentrations of 0.1, 67, 194, 284, 390, and 700 μg Pb/L. Both the total number of rotifers at the end of the 48 h of incubation and the intrinsic rate of population increase exhibited classical concentration-dependent responses (Fig. 1). Considering the number of rotifers at the end of the test, the NOEC, LOEC, and EC20 were 194, 284, and 125 (95% confidence interval: 13–176) μg/L, respectively. For intrinsic rate of population increase, NOEC, LOEC, and EC20 were 67, 194, and 307 (95% confidence interval: 78–493) μg/L, respectively.

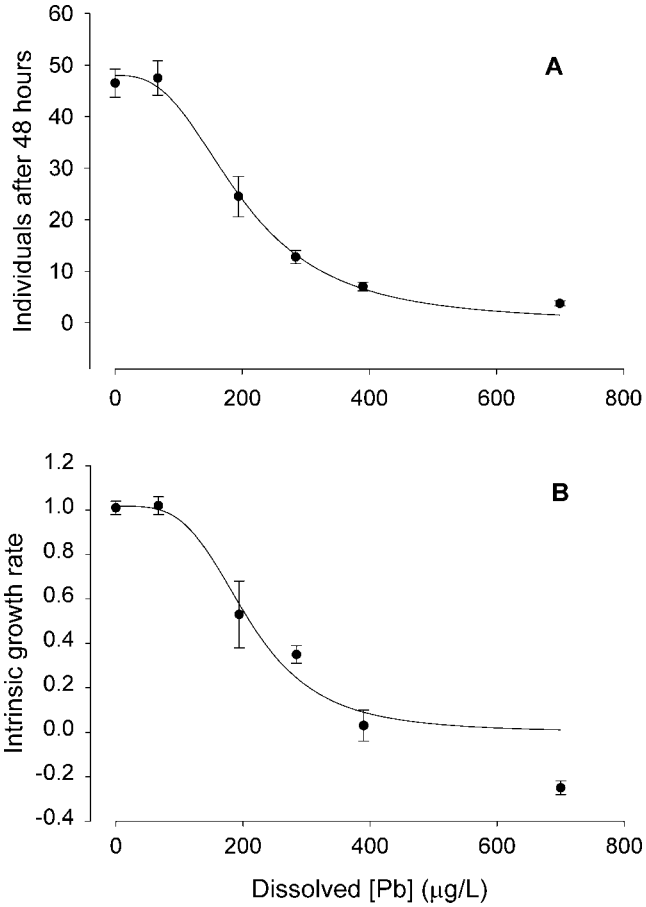

Fig. 1. (**A**) Total number of *Brachionus calyciflorus* at the end of 48 h of incubation at different [Pb] (Mean ± standard error of mean, 4 replicates). (**B**) Intrinsic rate of population increase calculated from data presented in **A** as defined by Snell and Moffat [17].

C. tentans

A control and five additional concentrations of 100, 200, 400, 600, and 800 μg Pb/L (nominal) resulted in only four significantly different measured concentrations in addition to the control treatment. Nominal concentrations of 400 and 600 μg Pb/L yielded measured concentrations of 502 ± 42 and 493 ± 43 μg Pb/L, respectively. Data from these two treatments have been combined and results are represented for controls (0.5 μg Pb/L) and mean Pb concentrations of 42, 109, 497, and 724 μg Pb/L (Table 3). Three endpoints were evaluated in these experiments: Survival, emergence, and time-to-emergence. For all experimental groups, 100% emergence of surviving larvae was observed so responses of survival and emergence were the same (Table 3). Mean time-to-emergence did not differ significantly from the control value of 21.7 d, although time-to-emergence for animals exposed to 497 μg Pb/L exhibited a trend toward delay (25.0 d). Midge survival was reduced significantly in the two highest treatments relative to the control, resulting in a NOEC and LOEC of 109 of 497 μg dissolved Pb/L, respectively.

L. stagnalis

Mean measured dissolved Pb concentrations were <0.5 μg Pb/L in control waters and 4, 12, 16, 42, 113, and 245 μg

Table 3. Survival/emergence and time-to-emergence for *Chironomus tentans* as a function of dissolved Pb exposure concentration

Exposure concn. (μg/L)	Survival/emergence (%)	Time to emergence (d)
<0.5	53 ± 13	21.7 ± 1.0
42 ± 4	73 ± 18	22.0 ± 0.7
109 ± 9	53 ± 7	20.6 ± 0.4
497 ± 42	13 ± 7*	25[a]
724 ± 58	0	NA[b]

[a] Only two animals emerged, preventing calcualation of standard error. The asterisk (*) denotes statistically significant difference from control ($p < 0.05$).
[b] NA = not applicable.

dissolved Pb/L for the six Pb-exposed groups. Newly hatched *L. stagnalis* exhibited greatly reduced growth in response to Pb exposure. Snails did not exceed 1 mg average body mass at the highest Pb concentrations as opposed to control animals that reached a mean body mass of more than 60 mg at the end of the 30-d exposure period (Fig. 2A). Interestingly, no Pb-induced mortality was observed. Survival was 80% in the control animals and 100, 80, 100, 70, 70, and 70% in snails exposed to 4.3, 12, 16, 42, 113, and 245 μg Pb/L, respectively. For the growth endpoint, the NOEC was 12 μg Pb/L, the LOEC was 16 μg Pb/L, and the EC20 was found to be less than the lowest concentration tested, 4 μg Pb/L.

The apparent extreme sensitivity to chronic waterborne Pb exposure prompted measurements of snail Pb accumulation and whole-animal (including shell) concentrations of two of the major physiological cations, Na^+ and Ca^{2+}. Accumulated Pb concentrations in snails did not exhibit traditional saturation kinetics but rather a linear or even exponential increase with increasing environmental Pb concentrations reaching almost 5,000 μg Pb/g at the highest exposure concentration (245 μg Pb/L; Fig. 2B). Considering Pb accumulation in the whole snail rather than as concentration per gram tissue reveals a different pattern (Fig. 2C). The highest amount of Pb accumulated was observed in snails exposed to only 12 μg Pb/L, reaching values of approximately 11 ng/snail compared to 4 to 5 ng/snail after exposure to the highest Pb concentrations (Fig. 2C).

Two major cations often influenced by metal exposure also were measured in the snails after the end of the 30-d Pb exposure. An apparent effect of Pb exposure was seen on whole-animal Ca^{2+} concentrations (Fig. 3A), with significantly elevated Ca^{2+} concentrations in snails exposed to the two lowest Pb concentrations. However, sampling of control snails at a representative size range revealed a biphasic pattern to whole-snail Ca^{2+} concentration (Fig. 3B). Very small snails contained Ca^{2+} concentrations as low as 500 μmol/g, whereas snails in the 10- to 20-mg range contained Ca^{2+} concentrations of 1,500 to 2,000 μmol/g. Larger snails (>40 mg) contained between 1,000 and 1,500 μmol Ca^{2+}/g. This biphasic pattern of whole-snail Ca^{2+} concentrations was observed in both Pb-exposed and nonexposed snails (Fig. 3B); therefore, there were no differences between Pb-exposed snails and size-matched controls.

Whole snail Na^+ concentrations also appeared to be elevated as a result of exposure to low Pb concentrations and clearly were reduced in snails exposed to higher concentrations (Fig. 4A). Size-dependent Na^+ concentrations in control snails were less apparent than the size-dependent Ca^{2+} concentrations in control snails (Fig. 4B) and small Pb-exposed snails showed

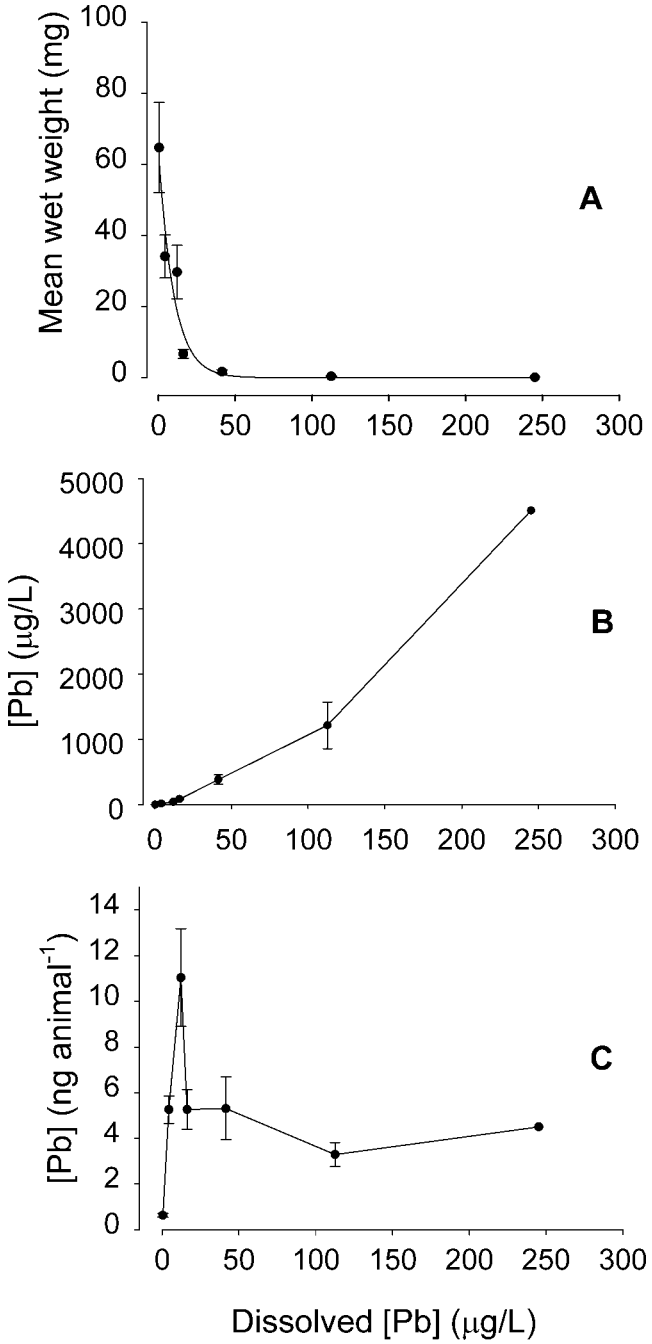

Fig. 2. (**A**) Mean juvenile *Lymnaea stagnalis* mass (mg) after 30 d of exposure to a range of dissolved [Pb]. (**B**) Accumulated whole-animal [Pb] (μg/g) at day 30. (**C**) Accumulated whole-animal Pb content (μg individual^{-1}). The $n = 10$ in all cases (mean ± standard error of mean).

markedly reduced whole-body Na^+ concentrations compared to controls. In contrast, Pb-exposed snails of intermediate size (20–40–mg range; low Pb–exposure concentrations) contained higher Na^+ concentrations than control snails of similar mass (Fig. 4B).

DISCUSSION

The three organisms tested in the present study all displayed sensitivity to aqueous Pb exposure, with *C. tentans* being the most tolerant and *L. stagnalis* being the most sensitive (Table 4). Our findings agree with earlier reports on extremely high

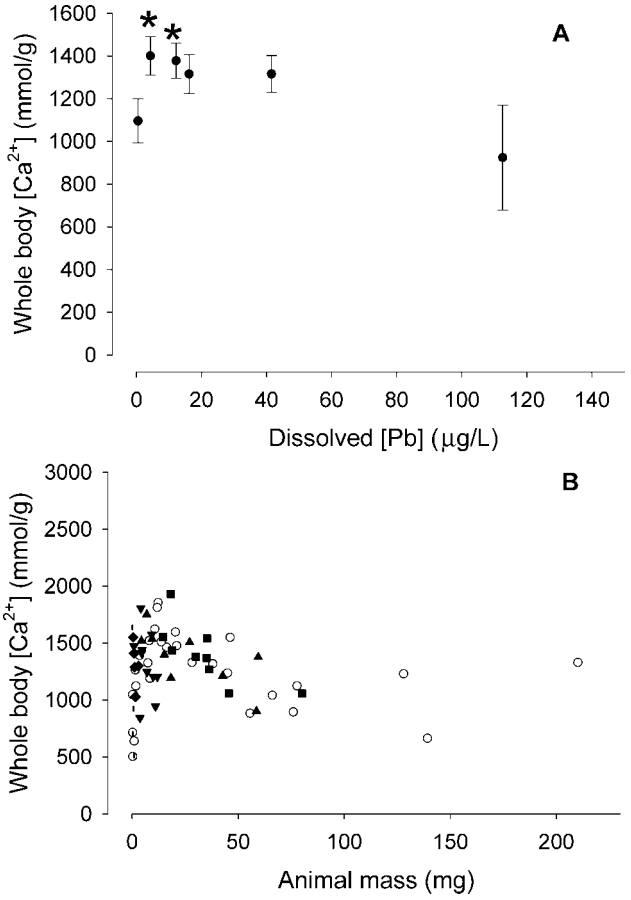

Fig. 3. (**A**) Mean whole-body [Ca^{2+}] (μmol/g) in juvenile *Lymnaea stagnalis* at the end of a 30-d exposure to a range of [Pb] (n = 10 in all cases; mean ± standard error of mean). (**B**) Individual measurements of whole-animal [Ca^{2+}] in juvenile snails at the end of 30-d Pb exposure; ○ control, ■ 4.3 μg/L, ▲ 12.2 μg/L, ▼ 16.2 μg/L, ◆ 41.5 μg/L, ▮ 6 μg/L. Control animals include juveniles sampled from the main culture to ensure comparable size ranges for Pb-exposed and nonexposed controls. Snails exposed to 245.2 μg/L were not analyzed for [Ca^{2+}] due to difficulties determining accurate mass of individuals. The asterisk (*) indicates statistically significant difference from control value.

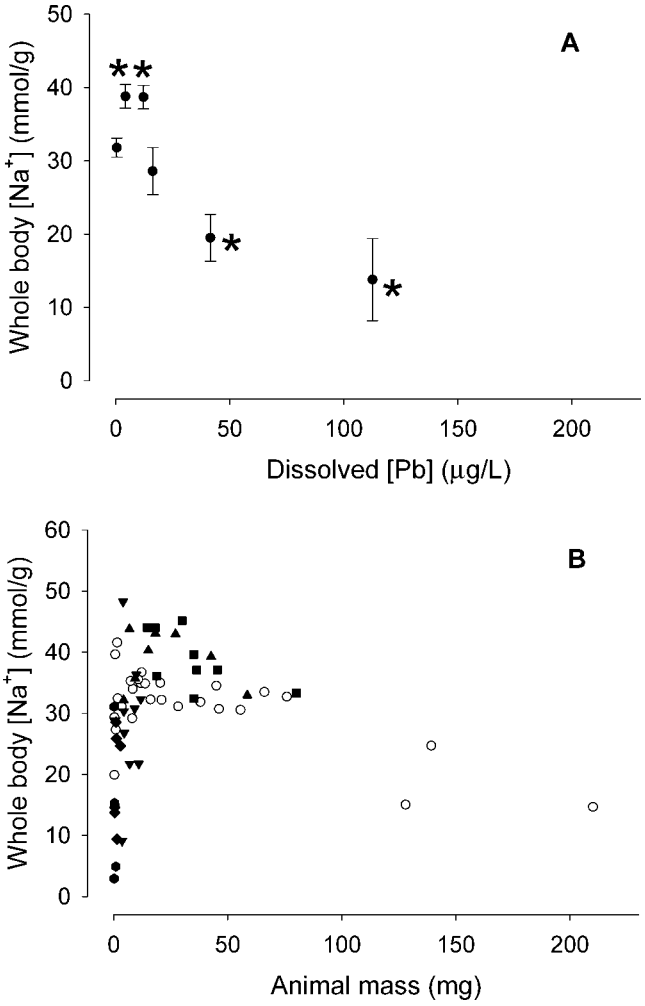

Fig. 4. (**A**) Mean whole-body [Na^{+}] (μmol/g) in juvenile *Lymnaea stagnalis* at the end of a 30-d exposure to a range of [Pb], n = 10 in all cases (Mean ± standard error of mean). (**B**) Individual measurements of whole-animal [Na^{+}] in juvenile snails at the end of 30-d Pb exposure; ○ control, ■ 4.3 μg/L, ▲ 12.2 μg/L, ▼ 16.2 μg/L, ◆ 41.5 μg/L, ▮ 112.6 μg/L. Control animals include juveniles sampled from the main culture to ensure comparable size ranges for Pb-exposed and nonexposed controls. Snails exposed to 245.2 μg/L were not analyzed for [Na^{+}] due to difficulties determining accurate mass of individuals. The asterisk (*) indicates statistically significant difference from control value.

Pb sensitivity in freshwater pulmonate snails [13] and are placed in context in Figure 5, which shows the species-sensitivity distribution (using chronic NOECs) for a range of freshwater organisms. The displayed sensitivity distribution should be seen in the context of normally occurring concentrations of 0.6 to 120 μg Pb/L [15].

Control reproductive output in *B. calyciflorus* as total number of individuals and as intrinsic growth rate was similar to previous reports [17] and displayed little variation among the four replicates in each treatment (Fig. 1A and B). The classical concentration-dependent response in the rotifer experiment demonstrates the utility of these organisms and associated experimental procedures for toxicity testing. Based on dissolved Pb concentration measurements, the rotifers, with an EC20 of 67 μg Pb/L, fall within the middle of the range of chronic toxicity values reported in Figure 5. It should be noted, however, that the test was performed in the presence of food (the green algae *Nannochloropsis*) and that the presence of organic ligands in the test solutions arising from *Nannochloripsis* cells cannot be ruled out. Any such ligands would have rendered Pb less bioavailable, decreasing uptake and toxicity, and so might have led to an underestimation of waterborne Pb sen-

Table 4. No-observable-effect concentration (NOEC), lowest-observable-effect concentration (LOEC), and 20% effect concentrations (EC20) for three invertebrate species exposed to Pb. The EC20 could not be calculated for this dataset

Species	NOEC (μg/L)	LOEC (μg/L)	EC20 (μg/L)
Brachionus calyciflorus[a]	194	284	125 (13–176)
B. calyciflorus[b]	67	194	307 (78–493)
Chironomus tentans	109	497	NA[c]
Lymnaea stagnalis	12	16	<4

[a] Based on total number of rotifers at end of test.
[b] Based on intrinsic rate of population increase.
[c] NA = applicable.

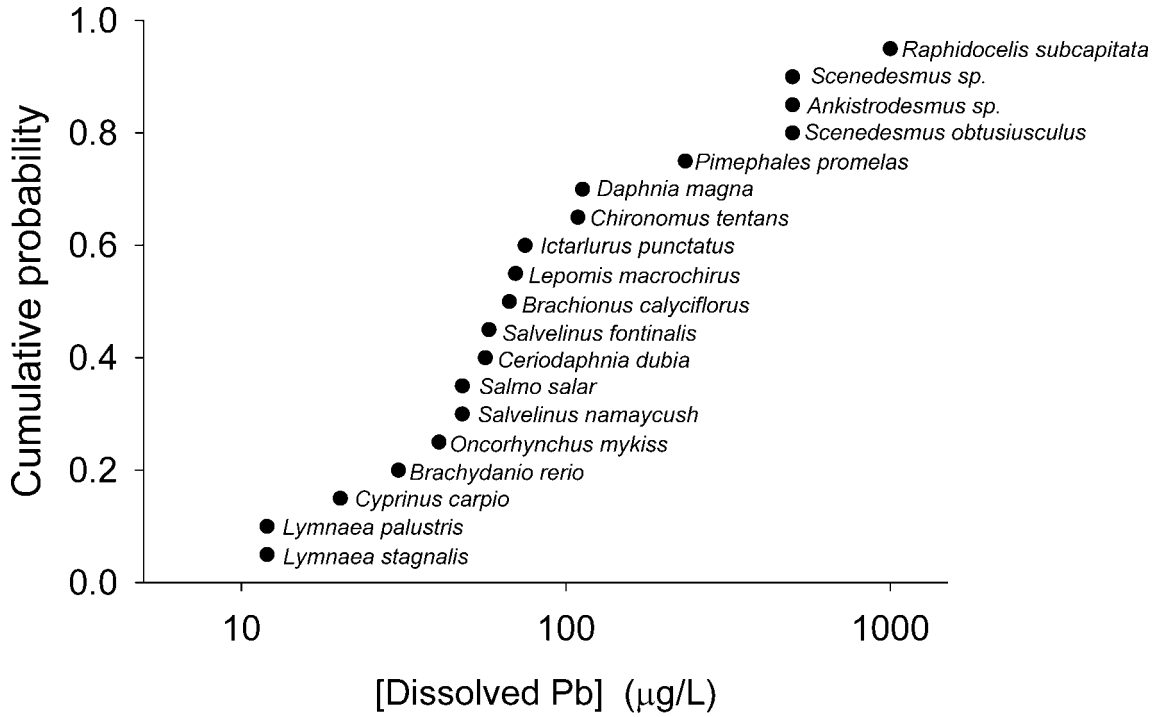

Fig. 5. Species-sensitivity distribution for chronic Pb exposure in freshwaters expressed as species mean chronic values for no-observable-effect concentrations of dissolved Pb. Values, in order of sensitivity from highest to lowest, were obtained from the following references: *Lymnaea stagnalis*, present study; *Lymnaea palustris* [13]; *Cyprinus carpio* [44]; *Brachydanio rerio* [45]; *Onchorhynchus mykiss* [46–48]; *Salvelinus namaycush* [47]; *Salmo salar* [49]; *Ceriodaphnia dubia* [14–50]; *Salvelinus fontinalis* [47]; *Brachionus calyciflorus*, present study; *Lepomis macrochirus* [47]; *Ictarlurus punctatus* [47]; *Chironomus tentans*, present study; *Daphnia magna* [6,51,52]; *Pimephales promelas* [14–53]; *Scenedesmus obtusiusculus* [54]; *Ankistrodesmus* sp. [54]; *Scenedesmus* sp. [54]; and *Raphidocelis subcapitata* [54].

sitivity relative to ligand-free water. On the other hand, the high density of food might have resulted in dietary Pb exposure due to Pb accumulation by *Nannochloropsis* during the experiment, which might have contributed to the observed toxicity. Regardless, in these chronic-exposure experiments, the presence of food, of course, is necessary for normal reproduction and survival and also represents environmentally relevant conditions.

The midge larvae *C. tentans* was the most tolerant of the three species tested with a NOEC of 109 μg dissolved Pb/L. Initial experiments were performed under static renewal conditions, but problems were encountered with maintaining Pb in solution at concentrations high enough to induce effects. Similar problems were encountered even with subsequent flow-through experiments. The experiments reported here for *C. tentans* were performed using an artificial freshwater designed to reduce $PbCO_3$ precipitation under flow-through conditions (see Table 2 for chemical composition). At the high Pb concentrations necessary to induce effects in *C. tentans*, waters rich in HCO_3^- result in $PbCO_3$ precipitation. To avoid this problem, experiments were conducted in water of general low-ionic strength with low total CO_2 levels (almost exclusively present as HCO_3^-). This water was supplemented with $CaSO_4$ to ensure sufficient Ca^{2+} in the water for larval survival and development.

Despite these artificial conditions, more than 50% survival and emergence was observed in the control groups, which is comparable to previous reports on *C. tentans* [20] with time-to-emergence being approximately 22 d after initiation of exposure. When considering this value for time-to-emergence, it should be noted that exposure was initiated with 8-d-old larvae and that emergence thus occurred approximately 30-d post-hatch, which again is similar to previously reported values [20].

In view of these observations, Pb toxicity in *C. tentans* as a result of waterborne exposure only perhaps is unlikely to occur under natural conditions. At HCO_3^- concentrations typical of most freshwaters, $PbCO_3$ precipitation would prevent dissolved Pb concentrations from reaching levels required to induce toxicity in *C. tentans*. Possible exceptions to this interpretation include environments with low Ca^{2+} concentrations. The Ca^{2+} addition to the test water in the present study might have reduced Pb uptake and thereby toxicity because of Pb/Ca^{2+} antagonism [31,32].

Our experiments with *L. stagnalis* yielded the highest sensitivity to chronic Pb exposure of any organism tested to date with a NOEC of 12 and an EC20 of <4 μg dissolved Pb/L. Our results agree fully with the early findings of Pb hypersensitivity in *L. palustris* [13], which suggests that pulmonate snails in general form a group of organisms with extreme sensitivity to Pb. Our observations should be viewed in the context of the U.S. EPA water-quality criteria of 8 μg Pb/L, for waters at the hardness employed in the present study [33], which suggests that this group of freshwater organisms might not be protected under current regulatory guidelines. The observed hypersensitivity correlates with high Pb accumulation in the juvenile snails at the end of the 30-d exposure period. Bioconcentration factors for metals generally are higher at low-exposure concentrations [34] but, even for the low concentrations used in this snail experiment, the observed bioconcentration factor is extremely high (Fig. 2). An early study by Spehar et al. [35] employing similar exposure concentrations on several freshwater invertebrates confirm high bioconcentration factors for lead in some freshwater invertebrates, in-

cluding a snail. However, the bioconcentration factor observed in the present study exceeds those reported by Spehar et al. [35] by four- to fivefold, and the tissue Pb concentrations found in *L. stagnalis* in the present study are the highest ever reported [34].

The apparent hyperaccumulation of Pb in *L. stagnalis* correlates well with the high sensitivity to chronic Pb exposure and raises the question of potential dietary transfer of Pb from snails to higher trophic levels.

Although our study on *L. stagnalis* agrees with the earlier study on *L. palustris* [13] with respect to hypersensitivity to Pb, it differs with respect to the most-sensitive endpoint. Although our studies on *L. stagnalis* revealed no Pb-induced mortality but greatly reduced growth rate, Borgmann et al. [13] found mortality to be a more sensitive endpoint than growth (based on shell length). Although similarly high sensitivity to Pb in these two species suggests commonality in the toxic action of Pb, the difference in affected endpoints between the two species might suggest slightly different physiology in *L. stagnalis* and *L. palustris*.

The explanation for the observed hypersensitivity to chronic Pb exposure likely can be associated with the physiology of these animals and observations of physiological responses during Pb exposure might shed light on the physiological and biochemical mechanism(s) of Pb toxicity. Sampling of the juvenile snails at the end of the 30-d exposure allowed for measurements of whole-snail Na^+ and Ca^{2+} concentrations.

Sodium homeostasis appears to be disrupted during chronic Pb exposure even at the lowest Pb concentration tested, which is in agreement with a recent report on acute Pb exposure of rainbow trout [31]. However, the effects of Pb on rainbow trout Na^+ homeostasis were observed at 1.2 mg Pb/L, several orders of magnitude higher than the concentrations applied in the present study. The Na^+ homeostasis response to chronic Pb exposure in *L. stagnalis* displayed a biphasic pattern with elevated whole-body Na^+ concentrations at the lowest Pb concentrations and reduced whole-body Na^+ concentrations at the higher Pb concentrations. Size-matched controls clearly show that the observed response directly is induced by Pb exposure. Possible explanations for these two responses include Pb-induced acidosis at low Pb concentrations and direct effects on Na^+ transport proteins at higher Pb concentrations.

It is well established that Na^+ uptake in freshwater fish and other freshwater organisms including *Lymnaea* sp. occurs by direct or indirect exchange for H^+ [36–41]. The elevated Na^+ concentration in response to chronic exposure to low Pb concentrations thus might reflect a chronic respiratory or metabolic acidosis that is (partly) compensated by increased exchange of H^+ and Na^+. In support of this suggestion are observations of elevated haemolymph Na^+ concentrations and reduced pH (acidosis) in *L. stagnalis* held in acidic water [41]. The reduced whole-body Na^+ concentration seen at higher Pb concentrations is in agreement with recent observations on rainbow trout showing reduced Na^+ uptake and plasma Na^+ concentrations and may reflect inhibition of Na^+/K^+ adenosine triphosphatase activity in *L. stagnalis* at higher Pb concentrations as seen in rainbow trout [31]. These likely explanations for the observed disturbance of Na^+ homeostasis remain to be validated but offer clear, testable hypotheses.

Lead exposure appeared to increase whole-body Ca^{2+} concentrations, but size-matched controls demonstrate that the differences in Ca^{2+} concentrations were a function of body mass rather than Pb concentration. It appears that newly hatched snails have the lowest Ca^{2+} concentrations and that these concentrations increase as snails increase in size, reaching a plateau at 1,200 to 1,500 mmol/kg. The apparent lack of effect of Pb exposure in whole-snail Ca^{2+} concentration does not necessarily mean that Ca^{2+} homeostasis is not disrupted. The shell of mollusks almost entirely consists of $CaCO_3$ [42], which means that snails exhibiting reduced growth have accumulated less Ca^{2+} during the 30-d Pb exposure. The majority of Ca^{2+} for *L. stagnalis* shell formation is derived directly from the water via uptake across the integument [43]. It cannot be ruled out that the reduced growth seen in response to Pb exposure is the result of impaired Ca^{2+} uptake from the water caused by waterborne Pb exposure and that growth is reduced or even arrested when Ca^{2+} is limiting. Considering the very high whole-animal Ca^{2+} concentrations in snails, which are two to three orders of magnitude higher than corresponding values for fish, it is obvious that Ca^{2+} requirements for snails (and shell-forming animals in general) are very high. Calcium^{2+} homeostasis clearly is disrupted by reduced Ca^{2+} uptake in rainbow trout exposed to high Pb concentrations and a similar response in pulmonate snails seems likely. However, it remains to be tested whether the high sensitivity to exposure of the Ca^{2+} antagonist Pb in these animals is related to their high Ca^{2+} requirements.

CONCLUSION

It is clear from the present study that toxicity arising from chronic Pb exposure is unlikely to occur in rotifers and midge larvae under environmentally realistic conditions, but also that great variability in sensitivity to Pb exists among species. Pulmonate snails clearly are the most Pb-sensitive organisms tested to date and may not be protected fully under the current environmental guidelines. Furthermore, nothing is known about how water chemistry might affect pulmonate snail sensitivity to Pb exposure; snails exposed to Pb at lower or higher Ca^{2+} concentrations might be more or less sensitive, respectively. Our observations also raise the question about the sensitivity of pulmonate snails to chronic exposure to other metals and clearly identify an area in need of further studies.

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