Effects of Lead on Na^+, K^+-ATPase and Hemolymph Ion Concentrations in the Freshwater Mussel *Elliptio Complanata*

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ABSTRACT: Freshwater mussels are an imperiled fauna exposed to a variety of environmental toxicants such as lead (Pb) and studies are urgently needed to assess their health and condition to guide conservation efforts. A 28-day laboratory toxicity test with Pb and adult Eastern elliptio mussels (*Elliptio complanata*) was conducted to determine uptake kinetics and to assess the toxicological effects of Pb exposure. Test mussels were collected from a relatively uncontaminated reference site and exposed to a water-only control and five concentrations of Pb (as lead nitrate) ranging from 1 to 245 μg/L in a static renewal test with a water hardness of 42 mg/L. Endpoints included tissue Pb concentrations, hemolymph Pb and ion (Na^+, K^+, Cl^-, Ca^{2+}) concentrations, and Na^+, K^+-ATPase enzyme activity in gill tissue. Mussels accumulated Pb rapidly, with tissue concentrations increasing at an exposure-dependent rate for the first 2 weeks, but with no significant increase from 2 to 4 weeks. Mussel tissue Pb concentrations ranged from 0.34 to 898 μg/g dry weight, were strongly related to Pb in test water at every time interval (7, 14, 21, and 28 days), and did not significantly increase after day 14. Hemolymph Pb concentration was variable, dependent on exposure concentration, and showed no appreciable change with time beyond day 7, except for mussels in the greatest exposure concentration (245 μg/L), which showed a significant reduction in Pb by 28 days, suggesting a threshold for Pb binding or elimination in hemolymph at concentrations near 1000 μg/g. The Na^+, K^+-ATPase activity in the gill tissue of mussels was significantly reduced by Pb on day 28 and was highly correlated with tissue Pb concentration ($R^2 = 0.92$; $P = 0.013$). The Na^+, K^+-ATPase activity was correlated with reduced hemolymph Na^+ concentration at the greatest Pb exposure when enzyme activity was at 30% of controls. Hemolymph Ca^{2+} concentration increased significantly in mussels from the greatest Pb exposure and may be due to remobilization from the shell in an attempt to buffer the hemolymph against Pb uptake and toxicity. We conclude that Na^+, K^+-ATPase activity in mussels was adversely affected by Pb exposure, however, because the effects on activity were variable at the lower test concentrations, additional research is warranted over this range of exposures. Environ Toxicol 27: 268–276, 2012.

Keywords: freshwater mussels; *Elliptio complanata*; lead; bioavailability; Na^+, K^+-ATPase

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INTRODUCTION

The activity of Na^+, K^+-ATPase functions to maintain ionic regulation in eukaryotes by transporting sodium ions out of, and potassium ions into, cell membranes (Kaplan,

2002). Na^+, K^+-ATPase activity has been shown to be significantly reduced with lead (Pb) exposure (Rajanna et al., 1991; Krstić et al., 2005; Krinulović and Vasić, 2006), including a freshwater fish (Rogers et al., 2005), through noncompetitive inhibition and thus, may similarly be affected in other freshwater taxa of interest. For example, waterborne Pb exposure in fish has been shown to cause disruption of Ca^{2+} homeostasis and imbalances of Na^+ and Cl^- regulation, in part through inhibition of Na^+, K^+-ATPase activity and Ca^{2+} uptake at the gill epithelium (Rogers et al., 2003, 2005; Rogers and Wood, 2004; Birceanu et al., 2008). Waterborne Pb has also been shown to cause glucose and ion disruption in rainbow trout (*Oncorhynchus mykiss*) and whitefish (*Coregonus spp*.) (Haux and Larsson, 1982; Haux et al., 1986), ion disruption in the freshwater red crab (*Dilocarcinus pagei*) (Amado et al., 2006) and Na^+ imbalances in snails (*Lymnaea stagnalis*) (Grosell et al., 2006a).

Freshwater mussels of the bivalve order Unionoida are long-lived (40–100 years), benthic aquatic organisms that are among the most sensitive and rapidly declining faunal groups in the world, and especially in North America. Of the estimated 840 species of freshwater mussels globally, ~300 are native to North America, but nearly 70% of these are extinct or vulnerable to extinction (Bogan, 1993; Williams et al., 1993; Graf and Cummings, 2007). Because unionoids are such an imperiled fauna, and as near-continuous filter- and deposit-feeders are exposed to a variety of toxicants that are dissolved in surface waters and associated with particles throughout their life (Cope et al., 2008), it is critical to develop research and conservation management strategies that involve nonlethal sampling techniques to assess health, condition, and status. Fortunately, the sampling and analysis of freshwater mussel hemolymph has recently been shown to be a viable nonlethal tool for assessing unionid mussel health, condition, and potential exposure to toxicants (Gustafson et al., 2005a).

As the majority of toxicity tests conducted with unionids have been acute tests with survival as the endpoints, very few sublethal endpoints are known for use in unionids (relative to other faunal groups). Thus studies are needed to examine nonlethal endpoints of exposure for a variety of contaminants. Because Pb has been shown to cause disruption to proteins in unionids (Moura et al., 2000), cause osmotic imbalances in unionids (Newton and Cope, 2006), and ATPase enzyme inhibition reduces Na^+ uptake in *Daphnia magna* (Bianchini and Wood, 2008), we hypothesized that exposure to waterborne Pb would cause the disruption of Na^+, K^+-ATPase activity and hemolymph ion (Na^+, K^+, Cl^-, Ca^{2+}) concentrations in the Eastern elliptio (*Elliptio complanata*) mussel. The past detection and measurement of Na^+, K^+-ATPase activity in unionid mussel tissue has been equivocal, largely depending on the species evaluated. For example, Na^+, K^+-ATPase activity was not detected in the mantle tissue of *Anodonta cataracta* (Wheeler and Harrison, 1982), whereas activity was detected in *Anodonta cygnea* (Pivovarova et al., 1992) and found in mantle and gills of other freshwater bivalves of the genera *Carunculina* (Dietz and Findley, 1980; Dietz, 1985), *Lampsilis* (a unionid), *Corbicula*, *Rangia* (Dietz, 1985), and the marine clam *Tapes* (Pagliarani et al., 2008), albeit, at relatively low levels of activity in each. In this study, we assessed a suite of nonlethal (concentrations of osmoregulatory ions (Na^+, K^+, Cl^-, Ca^{2+}) and concentrations of Pb in hemolymph) and lethal (Na^+, K^+-ATPase activity in gill tissue) endpoints to examine the effects of waterborne Pb exposure on the freshwater mussel *Elliptio complanata*.

MATERIALS AND METHODS

Collection, Transport, and Holding of Mussels

A total of 64 *Elliptio complanata* were collected from a rural, forested, and relatively uncontaminated section of the Eno River near Hillsborough, NC, USA (NCDENR, 2009) for the study. Test mussels averaged 78 mm in length, ranging from 65 to 98 mm, and had a mean wet weight of 55 g, ranging from 31 to 92 g. Upon collection, mussels were promptly placed into ice chests to maintain their temperature near the 21°C river water, covered with damp mesh bags to prevent desiccation and temperature change, and transported directly to the laboratory (30-min transportation time). Upon arrival at the laboratory, each mussel was scrubbed with a soft-bristle brush and rinsed with deionized (DI) water. Six mussels were randomly chosen for baseline measurements of the test endpoints (Na^+, K^+-ATPase activity in gill tissue, Pb concentration in body tissue, and Pb and ion concentrations in hemolymph) for comparative purposes, and the remaining mussels were placed into 3-L glass aquaria for the duration of the experiment. The aquaria contained 2 L of ASTM soft water (ASTM, 1993) maintained at 20°C (by central room thermostat) that was gently aerated (5–15 bubbles/s) by a central aeration unit (Sweet Water Air Pump SL24 Aquatic Eco-Systems, Apopka, FL, USA). For the test, there were six target Pb treatment concentrations (0, 1.95, 7.8, 31.25, 125, and 500 μg/L), and nine replicates per treatment with one mussel per replicate. The 57 test mussels (including three for the control day 0 sampling) were then acclimated to test conditions for 5 days, with a complete renewal of fresh ASTM soft water on the third day of acclimation. The baseline and day 0 mussels were weighed and measured, and hemolymph and tissue samples were collected as described in Experimental Procedures. Immediately prior to the start of the test on day 0, the mussel in each aquarium was fed 20 mL of a suspension containing 2 mL of Instant Algae® Shellfish Diet and 1 mL *Nannochloropsis* concentrate (Reed Mariculture, Campbell, CA, USA) in 1 L of deionized water. The mussel in each jar was allowed to siphon and feed for 2 h, after which a complete water renewal and toxicant spiking commenced. Mussel feeding and water

and toxicant renewals were conducted three times per week during the 28-day test in this same manner.

EXPERIMENTAL PROCEDURES

All laboratory methods followed the ASTM guide for conducting laboratory toxicity tests with freshwater mussels (ASTM, 2006), with modifications for testing adult mussels. Water samples (5 mL) were taken from three of the nine replicate test aquaria per treatment at 0, 48, and 72 h postrenewal for verification of Pb exposure concentration. These samples were stored preserved (75 μL of concentrated trace metal grade nitric acid) until analysis. Alkalinity, hardness, pH, temperature, and dissolved oxygen were measured in each aquarium before test initiation and then three times per week thereafter. Water temperature and dissolved oxygen were measured with a calibrated multi-probe (YSI Model 556 MPS, Yellow Springs Instruments, Yellow Springs, OH, USA). Water pH was measured with a calibrated Beckman Model Φ 240 (Beckman Instruments, Fullerton, CA, USA) meter. Alkalinity and hardness were measured by standard titrametric methods (APHA et al., 1995). Physiochemical characteristics of test water averaged 20.2°C (range 18.9–21.0), dissolved oxygen 8.7 mg/L (range 8.4–9.2), pH 7.8 (range 7.5–8.0), alkalinity 30 mg/L as $CaCO_3$ (range 28–32), and hardness 42 mg/L (range 40–44).

On day 0 of the test, three mussels from the control treatment were selected as day 0 controls to assess potential differences in test endpoints that may have occurred from the time of collection (baseline samples) to the end of the 5-day acclimation period. As with the baseline mussels they were removed from their aquaria, weighed and measured, gently pried open, and a 25-gauge syringe was used to withdraw 1 mL of hemolymph from the anterior adductor muscle of each. This was then divided into two, 1.2-mL cryotubes for ion (Na^+, K^+, Cl^-, and Ca^{2+}) and Pb analysis. These mussels were then dissected and three samples of gill tissue (~15 mg each) were removed per mussel for Na^+, K^+-ATPase activity assessment, and each placed in 1-mL centrifuge tubes, on ice, with 100 μL of SEI (sucrose 250 mM, Na2EDTA*2H_2O 10 mM, imidazole 50 mM, pH 7.3) buffer solution. Hemolymph samples for ion analyses, along with gill samples, were then stored at −80°C. The mussels were then bagged and stored, along with hemolymph samples for Pb analysis, at −20°C. The remaining aquaria were renewed with fresh ASTM soft water and dosed with the appropriate Pb treatment for the replicate. On days 7, 14, and 28, three mussels from each Pb treatment concentration were sampled as previously described.

Na^+, K^+-ATPase Activity Assay

The Na^+, K^+-ATPase activity was determined using the method of McCormick (1993) as used for detection in rainbow trout gills (Rogers et al., 2003), modified for use with mussel tissue. This method of detection requires the inhibition of Na^+, K^+-ATPase in order to compare total ATPase activity and then calculate the amount of activity caused solely by Na^+, K^+-ATPase. Ouabain is sometimes used to inhibit Na^+, K^+-ATPase activity by binding to the α subunit of the enzyme after it has released $3Na^+$ and before it binds $2K^+$, thus stopping the cycle (Kaplan, 2002). However, ouabain proved insufficient in inhibiting Na^+, K^+-ATPase activity in *Elliptio complanata* gill tissue. Overall, there were no consistent differences between the nonouabain and ouabain inhibited activities of total ATPase. It is known that there are various isoforms of the α subunit of Na^+, K^+-ATPase that are species specific, and have varying degrees of sensitivity to ouabain inhibition (Anner et al., 1992; Pierre et al., 2007). It has also been shown that certain regions of the α subunit of Na^+, K^+-ATPase, when mutated, will reduce ouabain sensitivity (Croyle et al., 1997). Thus, we used a K^+-free salt solution for inhibition instead of ouabain, and much stronger and more consistent results were achieved. This method of detection has been used in conjunction with ouabain inhibition to verify that Na^+, K^+-ATPase activity was not present in measurable amounts in the mussel *Anodonta cataracta*, with both salt depletion and ouabain showing negative results (Wheeler and Harrison, 1982). This method has also been used to detect Na^+, K^+-ATPase activity in the gills of goldfish, which are insensitive to ouabain, requiring an extremely high concentration (10 mM) for inhibition (Chasiotis and Kelly, 2008). Chasiotis and Kelly (2008) found that inhibition with a K^+-free salt solution was just as effective as using the high concentration of ouabain, and less costly. Because the other ATPase that would be affected by K^+ removal, H^+, K^+-ATPase, also known as gastric H^+, K^+-ATPase, is found in gastric tissues responsible for maintaining high acid content, and the dominant ATPase for epithelial acid-base state in unionids is H^+, Na^+-ATPase (Byrne and Dietz, 1997), the majority of inhibition observed in gill tissue should be Na^+, K^+-ATPase activity. Because salt depletion is considered to show less activity than would be generated by total inhibition of the enzyme and, therefore, be a conservative estimate of the activity, and the fact that we found high activity (about 50% of total ATPase in *Elliptio complanata* gill tissue), we postulate that *Elliptio complanata* contain Na^+, K^+-ATPase with an α-isoform highly resistant to inhibition by ouabain. For these reasons the K^+-free salt solution was selected in place of ouabain to determine the mussels' Na^+, K^+-ATPase activity.

For the assay, a refrigerated centrifuge (AllegraTM 25R Centrifuge, Beckman Coulter, Fullerton, CA, USA) was set to 4°C, and aliquots of phosphoenolpyruvate (PEP, 21 mM) and adenosine diphosphate (ADP, 4 mM) were removed from a −80°C freezer and thawed for about 30 min. The assay mixture (AM) was prepared fresh each day. Nicotinamide adenine dinucleotide (NADH) (5.45 mg) and adenosine triphosphate (ATP) (13.5 mg) were weighed and rinsed

into a graduated cylinder with SEI buffer. Lactate dehydrogenase (LDH) (4.6 U/mL) and pyruvate kinase (PK) (5.1 U/mL) were added in the same tube and centrifuged at 12,000 $\times$ *g* for 8 min at 4°C. The supernatant was removed, resuspended with SEI buffer, and added to the cylinder. Once the PEP was thawed, 4.7 mL was added to the cylinder. The volume was brought to 37.5 mL with SEI buffer, and the completed AM was mixed well. Salt dilutions were prepared with a 3:1, AM:salt solution ratio. For the A solution, which measures total ATPase activity, 15 mL of AM was mixed with 5 mL of salt solution prepared previously (imidazole 50 mM, NaCl 189 mM, $MgCl_2$ * $6H_2O$ 10.5 mM, KCl 42 mM, pH 7.5). For the B solution, which measures ATPase activity minus Na^+, K^+-ATPase activity, 15 mL of AM was mixed with 5 mL of the K^+-free salt solution prepared previously (same as the salt solution without KCl added). Both salt dilutions A and B were kept on ice throughout the experiment. The ADP standard curve was prepared from thawed 4-mM ADP stock. To the ADP standard tubes, 0, 25, 50, and 100 μL of 4 mM ADP and 200, 175, 150, and 100 μL SEI buffer, respectively, were added for 0, 5, 10, and 20 nmoles ADP/10 μL concentrations. A Fusion™ Universal Microplate Analyzer (A153600 Meriden, CT, USA) was allowed to warm for 30 min prior to analysis. Mussel gill samples were thawed immediately prior to running the assay. Sample tubes were homogenized with 25 μL 0.3% SEID (3X concentrate; 0.0751 g Na deoxycholic acid in 25 mL SEI buffer) for 20–30 s using 1.5-mL centrifuge tube pellet pestles. Activity decreases after homogenization, so all samples were read within 30 min of processing. The homogenates were centrifuged at 5000 $\times$ *g* for 1 min at 4°C, and 10 μL was pipetted into each of four wells per sample. The ADP standard curve was run once at the beginning of each batch of samples, 10 μL per three wells per concentration was pipetted into the plate, and 200 μL of ice cold A w/salt was added to each well. The ADP standard curve rapidly decreases in the first 2–3 min, but then stabilizes, and is read in mOD/nmole ADP. Then, 200 μL of ice cold B w/salt (K^+) was added to two of the four wells for each sample, and 200 μL of ice cold A w/salt was added to the other two wells. The plate was then promptly read on the plate reader at 340 nm for 10 min at 1-min intervals. ATPase activity was measured as mOD/10 μL/min. The Bradford Protein Assay (Bradford, 1976) kit (IBI/Shelton Scientific; VWR No. 14221-496) was used to determine protein concentration, and the final Na^+, K^+-ATPase activity as expressed as μmoles ADP/mg protein/h.

Ion and Lead Analytical Procedures

All samples of mussel tissue and hemolymph were analyzed for Pb with standard methods at Research Triangle Institute (Research Triangle Park, NC, USA) according to good laboratory practices and strict quality assurance procedures. Briefly, mussel tissue was lyophilized and homogenized, with a nominal weight of 250 mg aliquoted and heated with a mixture of concentrated nitric and hydrochloric acids. Hydrogen peroxide was added to aid in the decomposition of organic material. Samples were then analyzed by magnetic sector inductively coupled plasma mass spectrometry (Thermo Element 2 Magnetic Sector ICP-MS). The average recovery of Pb from samples of mussel tissue was 109.3%, and ranged 99.6–119%. Recovery of Pb in samples (n = 17) of test water averaged 100.7% and ranged 95–103%. Hemolymph Pb recovery averaged 96% and ranged 84–101%.

All hemolymph samples were analyzed for ion concentrations at the Analytical Service Laboratory in the Department of Soil Sciences at North Carolina State University (Raleigh, NC, USA) according to standard methods, good laboratory practices and strict quality assurance procedures with two Dionex Ion Chromatographs (DX-500 and 4000, Dionex Corporation, Sunnyvale, CA, USA). Using an autosampler (Dionex AS-50) fitted with two injection valves, the samples were simultaneously analyzed for anions and cations. Chloride analysis was performed using a separation column AS 22 and conductivity detection for the cations analysis was done on a Dionex CS 12A column. The concentration of each analyte was determined by comparing the peak area in the chromatograms (using Dionex Software Peak Net 5.21) to those generated with standard solutions.

Statistical Analysis

Data for Na^+, K^+-ATPase activity and Pb and ion concentrations were analyzed following a generalized linear model with concentration and time considered as fixed-effect factors, and mussels considered as random factors. Analyses were performed using the Proc MIXED procedure in SAS v9.1.3 (SAS Institute, Cary, NC, USA). Data were evaluated by residual plot and $\log_e$ transformed prior to analysis when necessary to achieve homogeneity of variances. Estimated least squares and their 95% confidence intervals were back-transformed for presentation purposes and statistical significance was determined at $\alpha = 0.05$ for all tests, unless otherwise stated.

RESULTS

Concentrations of Pb in test water averaged 101% of target concentrations after renewals over the 28-day study, ranging from 92% for the greatest concentration to 108% for the least. Thus, the treatments averaged concentrations of 2, 8, 31, 121, and 458 μg/L immediately following renewals. By 48 or 72 h postrenewal, the concentration of Pb in test water was significantly reduced to 7 and 6% of the target concentration, respectively, over the study. During the first

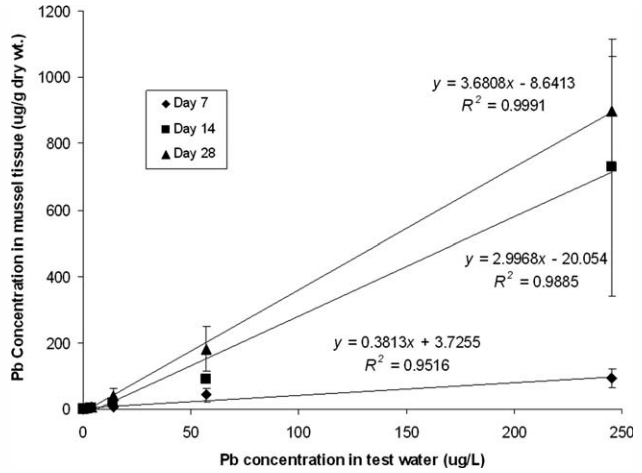

Fig. 1. Relations between mussel tissue Pb concentration and waterborne Pb exposure concentration on days 7, 14, and 28. Error bars represent 95% confidence intervals.

14 days of the study, Pb concentrations averaged 5.1% of target at 48 h and 4.1% of target at 72 h, which increased slightly for the second half of the study to 8.7% of target at 48 h and 7.3% of target at 72 h. The average daily exposure concentrations over the 28-day study were calculated as the weekly average, with three times 0 h (T0) for renewal days, three times 48 h (T48) for the day after each renewal and one time 72 h (T72) for the last day of each week 2 days after a renewal. This resulted in an average daily exposure of 1, 4, 14, 57, and 245 μg Pb/L, and those values were used hereafter to denote the actual treatment groups.

Average Pb concentrations in mussel tissue for each treatment group over the 28-day study ranged from 0.33 to 898 μg/g, and were strongly correlated on d 7, 14, and 28 ($R^2 = 0.952$–0.999; $P < 0.001$) with exposure concentration (Fig. 1). Tissue bioaccumulation factors (BAF) on d 28 ranged from 1.65 to 3.66, with an average of 2.66. The average BAF of the two lowest treatment groups of 1 and 4 μg Pb/L was 1.78. An increase in mussel tissue Pb concentration was observed with treatment group and time on days 7 and 14, with all treatment groups significantly different from each other by day 14. Mussels continued to accumulate Pb in their tissue throughout day 28, but not significantly from day 14.

Average Pb concentrations in mussel hemolymph for each treatment group over the 28-day study ranged from below the detection limit (0.25 μg/L) in the controls to 822 μg/L in the greatest treatment concentration. Pb in mussel hemolymph was more variable than tissue Pb concentrations. The hemolymph BAF on d 28 ranged from 1.07 to 3.93 in the 1 to 57 μg/L treatments, and then decreased to 0.23 for the 245 μg/L treatment (a decrease from 3.35 on day 7), with an average BAF of 1.58. The averages for concentrations of Pb in hemolymph from the 1 to 57 μg/L treatments all increased over the 28-day study (Fig. 2). The Pb concentrations of hemolymph in mussels from the 245 μg/L treatment increased in concentration by day 7, but decreased thereafter with a final day 28 concentration significantly less than that on day 7. This hemolymph Pb concentration on day 28 was also not statistically different from levels for the 57 μg/L Pb treatment group.

Calcium (Ca^{2+}) concentrations in hemolymph were significantly elevated from controls when mussels were exposed to the greatest Pb (245 μg/L) concentration [Fig. 3(A)]. There was no significant change in Cl^- and K^+ concentrations in mussel hemolymph among the Pb treatments on day 28 [Fig. 3(B,C)]. In contrast, Na^+ concentrations in hemolymph were significantly reduced by day 28 in the 245 μg/L Pb treatment relative to controls [Fig. 3(D)].

The average Na^+, K^+-ATPase activity for each Pb treatment over the duration of the 28-day test ranged from 0.95 to 3.46 μmoles ADP/mg protein/h. Activity of the baseline mussels (3.00) and the day 0 control mussels (3.13) was not significantly different ($P = 0.76$). There was no significant change in activity of the control mussels over time compared to the baseline and day 0 control measurements. However, Na^+, K^+-ATPase activity decreased with increasing Pb exposure by day 28 (Fig. 4). On day 28, mussels from the 57 μg/L Pb treatment had activity that was significantly less than the day 28 control ($P = 0.033$), baseline ($P = 0.033$) and day 0 control mussels ($P = 0.046$).

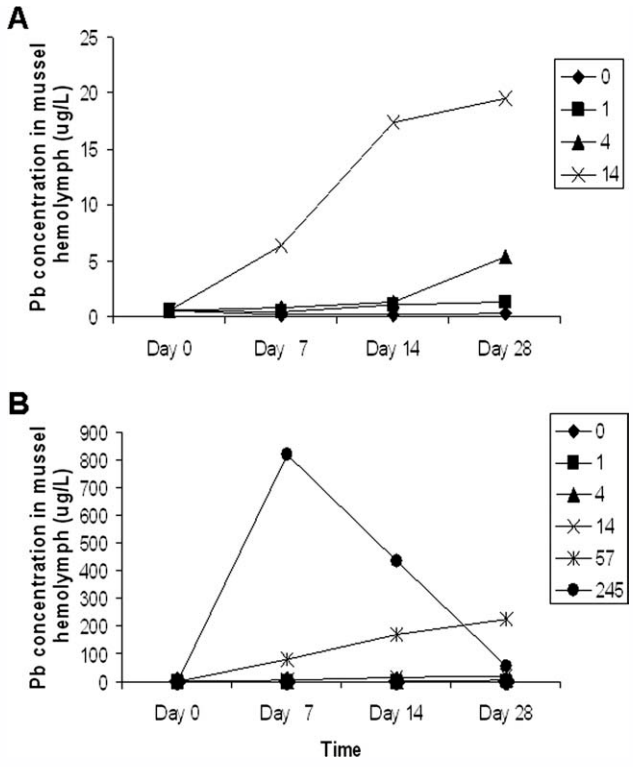

Fig. 2. The average hemolymph Pb concentration of mussels in the (A) low range (0–14 μg Pb/L) and (B) full range (0–245 μg Pb/L) of exposures over the 28-day study.

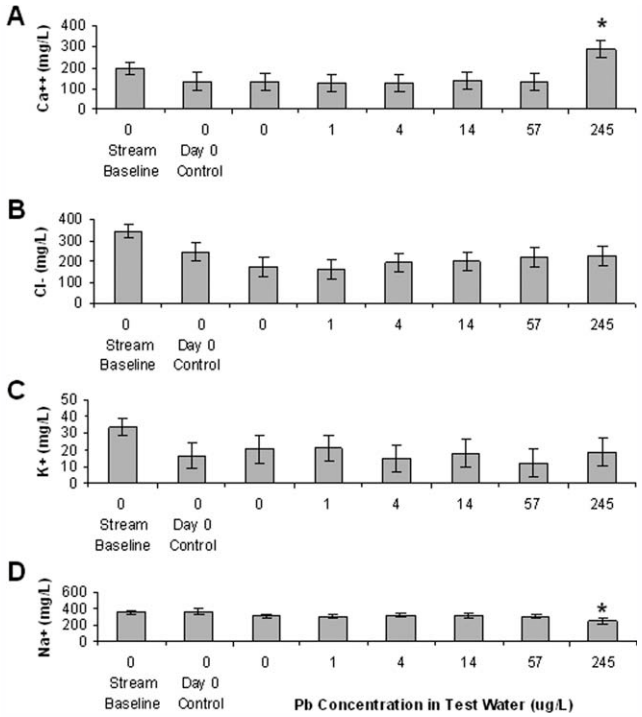

Fig. 3. Hemolymph ion concentrations, as mg/L, for each treatment group on day 28 for (A) Ca^{++}, (B) Cl^{-}, (C) K^{+}, and (D) Na^{+}. An asterisk denotes the values significantly different from the corresponding control.

The Na^{+}, K^{+}-ATPase activity in mussels from the 245 μg/L treatment on day 28 was significantly less than all other treatments at that time point, as well as significantly less ($P < 0.0001$) than the day 28 control, day 0 control and baseline mussels. The 245 μg/L Pb treatment significantly reduced Na^{+}, K^{+}-ATPase activity by 70% from the control by day 28. About 91 to 92% of the reduction in Na^{+}, K^{+}-ATPase activity was explained by Pb concentration in the test water [$R^2 = 0.91$; $P = 0.013$, Fig. 5(A)] and Pb concentration in mussel tissue [$R^2 = 0.92$; $P = 0.013$, Fig. 5(B)].

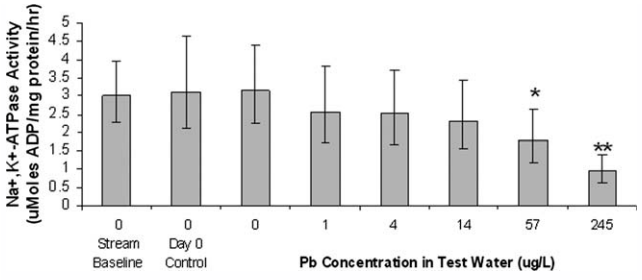

Fig. 4. The average Na^{+}, K^{+}-ATPase activity for each treatment group by day 28 of the study. Error bars represent 95% confidence intervals. Values that are significantly different from the control, as well as the baseline and day 0 values at $\alpha = 0.05$ are signified with a single asterisk (*), and at $\alpha = 0.0001$ with a double asterisk (**).

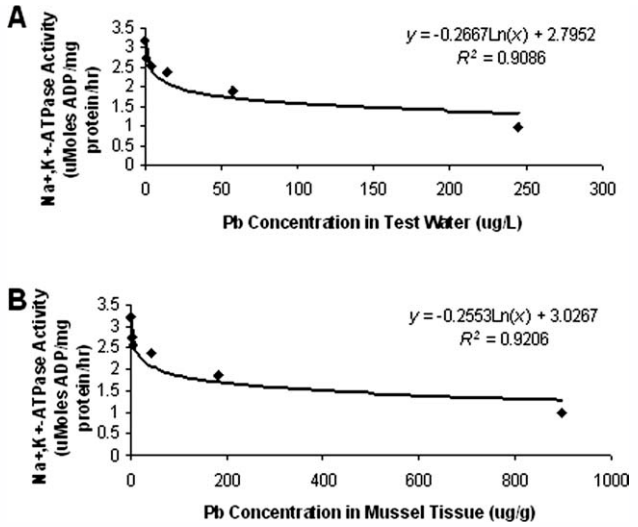

Fig. 5. Na^{+}, K^{+}-ATPase activity correlations with treatment group Pb exposure concentration (A) and tissue Pb concentration (B) at the end of the 28-day study.

DISCUSSION

Mussels accumulated significant quantities of Pb in their tissues by days 7 and 14, but no further significant increases were observed on day 28. This trend is in agreement with a study of Pb exposure in the marine bivalve *Crassostrea gigas* that found no further accumulation after 2 weeks of exposure (Amiard et al., 1995). The rate of Pb accumulation increased with exposure concentration, as has been shown in similar exposures with the zebra mussel *Dreissena polymorpha* (Kraak et al., 1994). The average tissue BAF of 2.66 for *Elliptio complanata* in this study was 7.6 times greater than the 0.35 BAF reported for zebra mussels (Zimmermann et al., 2002). Because tissue concentration was strongly correlated ($R^2 = 0.999$; $P < 0.0001$) with exposure concentration, and appeared to stabilize by day 14, this suggests that *Elliptio complanata*, as other bivalves have indicated (Metcalfe-Smith et al., 1996; Gundacker, 2000; Dobrowolski and Skowrońska, 2002; Yap et al., 2004), is a good sentinel for detection of recent Pb contamination.

Pb concentrations in mussel hemolymph were more variable than those in tissue; however, significant increases were observed with increasing exposure concentrations within treatment groups. Because Pb levels in the test water continued to be depleted throughout the study (i.e., taken up by the mussels as any adsorption to the walls of the glass aquaria would rapidly cease due to saturation of binding spots), Pb concentrations in hemolymph did not significantly increase from day 7, and Pb concentrations in tissue did not significantly increase after 2 weeks, we conclude that the mussels were actively eliminating Pb from their bodies. Storage of Pb in the shell (Wiesner et al., 2001;

Becker et al., 2005; Gillikin et al., 2005) was likely taking place (although not measured in our study), but the majority of the elimination was most likely in lysosomes or granulocytes associated with production of pseudofeces (Amiard et al., 1995; Marigómez et al., 2002). The fact that the greatest exposure concentration resulted in a rapid increase in hemolymph Pb, and subsequent reductions leading to the lowest BAF among the treatment groups by day 28, suggests a lag time between initial exposure and increased lysosomal production and size, as seen in the zebra mussel (*Dreissena polymorpha*) when exposed to Pb (Giamberini and Pihan, 1997), thereby allowing the mussels to better manage the transport and elimination of Pb.

The baseline hemolymph average of 195 mg Ca^{2+}/L for this experiment was similar to the median of 175 mg Ca^{2+}/L from other observations with *Elliptio complanata* (Gustafson et al., 2005b) and well within the 95% confidence interval (CI) of 131 to 237 mg Ca^{2+}/L as derived by Gustafson et al. (2005b). The 245 μg/L Pb treatment resulted in an average Ca^{2+} concentration on day 28 significantly greater ($P < 0.001$) than all other treatments, and the upper CI (Gustafson et al., 2005b). Interestingly, this increase was observed only in the greatest Pb treatment, which was in contrast to an apparent initial decrease in Ca^{2+} concentrations in hemolymph when mussels were acclimated from natural stream water at a hardness of 25 mg/L (baseline mussels) to ASTM soft water with a hardness of 42 mg/L in the laboratory (day 0 controls) environment.

From the individual and combined results of the hemolymph ion analysis, we conclude that the adverse effects of Pb on ion concentrations occurred at concentrations greater than those that mussels would routinely encounter in the environment. However, the reduced Na^+ concentration in mussel hemolymph at the greatest exposure concentration by day 28 was expected because Na^+, K^+-ATPase activity was shown to be reduced to 30% of the controls. Moreover, because 3 Na^+ ions are exchanged for 2 K^+ ions, we would expect to see changes in ion concentration from Na^+ before it is observed for K^+. Increased Ca^{2+} in hemolymph at the greatest Pb exposure is unexplained, but may be a direct result of the mussels' attempting to protect their tissues from Pb uptake and toxicity. The presence of Ca^{2+} could reduce Pb uptake and associated toxicity by competing for the same uptake sites (Grosell et al., 2006b). Therefore, if the mussels were to remobilize Ca^{2+} from their shell, as has been observed (Wheeler et al., 1975; Hudson, 1992; Nair and Robinson, 1998), they could incorporate Pb into the shell while generating a Ca^{2+} buffer, thereby reducing Pb levels, uptake, and toxicity to the tissues.

By day 28 of the experiment, Na^+, K^+-ATPase activity was significantly reduced in mussel gill tissue with increasing concentrations of Pb in the test water. The mussels at the lowest exposure concentration of 1 μg/L of Pb had an average Na^+, K^+-ATPase activity 18% less than the controls, and because Na^+, K^+-ATPase is responsible for maintaining Na^+, K^+ transmembrane gradients, it has been suggested that its inhibition may lead to gill tissue damage (Sancho et al., 1997). In addition, disruption of Na^+ levels in the cell would affect Na^+/H^+ exchange altering pH levels, and thereby affecting shell formation and dissolution (Byrne and Dietz, 1997) along with Ca^{2+} and HCO_3^- levels. Because Pb competes for Ca^{2+} uptake sites (Rogers et al., 2005), its presence would likely affect ion concentrations and shell formation and dissolution.

The activity of Na^+, K^+-ATPase in gill tissue of mussels exposed to Pb in this study was adversely affected at the two greatest test concentrations; however, the variation in activity among individual *Elliptio complanata* (as demonstrated by the relatively wide CI) warrants additional research. Based on data from a companion study, we conclude that current measured environmental concentrations of Pb in sediment averaging 3.9 μg/g dry weight and *Elliptio complanata* tissue concentrations averaging 1.6 μg/g dry weight (Mosher, 2008) may be sufficient to cause reductions of Na^+, K^+-ATPase activity. However, because gill dissection is not a nonlethal sampling technique, the use of Na^+, K^+-ATPase activity would not be a tool suitable for use with threatened and endangered species of mussels. The use of a Pb-specific biomarker such as δ-aminolevulinic acid dehydratase (ALAD) activity, which has recently been demonstrated in tissues of the Asian clam *Corbicula fluminea* (Company et al., 2008) that can be sampled and analyzed nonlethally (e.g., in hemolymph or tissue biopsy plugs) may be an improved alternative to Na^+, K^+-ATPase activity in assessing Pb exposure in native mussels.

CONCLUSION

Overall, ion concentrations in hemolymph of the freshwater mussel *Elliptio complanata* did not appear sensitive enough to reliably indicate adverse effects of Pb at low-level exposures (only a significant alteration of $Ca^{2+}$and Na^+ at the highest test concentration of 245 μg/L) by the end of the 28-day test. The activity of Na^+, K^+-ATPase was adversely affected by Pb at the two greatest exposure concentrations of 57 and 245 μg/L, but because of its required lethal sampling of gill tissue, and high individual variation in mussels, further research is recommended on this and other more specific endpoints of Pb exposure (e.g., ALAD activity) that may be sampled and analyzed in a nonlethal manner. Moreover, because Eastern Elliptio were found to reach an equilibrium with exposure levels and tissue concentration relatively quickly (about 2 weeks), this suggests useful application of these more common and widespread unionid mussels for assessing environmental quality for those species that are rare, threatened, or endangered.

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