

## ABSTRACT

MOSHER, SHAD. Biomarkers of Lead Exposure in the Freshwater Mussel *Elliptio complanata* for Assessing Transportation Related Impacts. (Under the direction of W. Gregory Cope.)

Native freshwater mussels (family Unionidae) are one of the most rapidly declining faunal groups in North America with about 70% of the nearly 300 species considered vulnerable to extinction, or already extinct. The aim of this research was to assess current lead (Pb) concentrations in native freshwater mussel tissue and sediment near bridge crossings in relation to traffic count, as well as to evaluate potential biomarkers in mussels for Pb exposure and effect. The first phase of this study involved examination of Pb concentrations in adult Eastern elliptio (*Elliptio complanata*) mussel tissue and sediment at 40 sites throughout North Carolina, ranging in average daily traffic count, land-use patterns and watershed characteristics. Data from these sites revealed that, on average, there was significantly greater Pb concentrations in mussels from sites with traffic count  $\geq 500$  vehicle crossings/day (vc/d) compared to mussels from sites with  $< 500$  vc/d. Two 28-d toxicity tests with Pb and adult Eastern elliptio mussels were conducted in the second phase of the study to compare the field-derived Pb bioaccumulation data to accumulation rates with known exposures, and to examine sublethal biomarkers of Pb exposure and toxicity. Endpoints for the tests included mussel survival, metal accumulation, shell length and weight. For the first test,  $\delta$ -aminolevulinic acid dehydratase (ALAD) inhibition was assessed as a biomarker of exposure and effect in mussel hemolymph. In the second test,  $\text{Na}^+, \text{K}^+$ -ATPase activity in mussel gill tissue was examined as a biomarker of exposure and effect, along with measurements of hemolymph ion concentrations. Pb was measured in mussel

hemolymph in both tests at concentrations near observed exposure concentrations, and at the greatest exposure concentration in the first test (maximum of 396  $\mu\text{g/L}$ , average of 251  $\mu\text{g/L}$ ), the hemolymph Pb concentration was 3 times the maximum amount present in test water. In contrast, the greatest exposure concentration in the second test (maximum of 458  $\mu\text{g/L}$ , average of 245  $\mu\text{g/L}$ ), had hemolymph concentrations only 0.12 times the maximum amount present in test water. This finding suggested a threshold effect maximum concentration for Pb exposure and hemolymph concentrations. The average Pb concentration in mussel tissue was strongly correlated with Pb exposure concentration ( $R^2 = 0.99$ ) at day 28 for both tests. The enzyme ALAD was not present in measurable concentrations in freshwater mussel hemolymph, gill, foot, mantle or visceral tissue. The  $\text{Na}^+, \text{K}^+$ -ATPase activity was found to be a strong biomarker of Pb exposure in freshwater mussel gill tissue (making up about 50% of the total ATPase present) using a K-free salt solution for detection, and was found to be negatively correlated with average tissue Pb concentration ( $R^2 = 0.82$ ) on day 28. Pb accumulated rapidly in mussel tissue during the first two weeks of the second exposure, with lesser accumulation during the final two weeks, with only the 121  $\mu\text{g/L}$  treatment group changing significantly. Because Pb was being removed from the test water throughout the duration of exposure, I conclude that the mussels were approaching equilibrium with the aqueous environment and were eliminating the Pb in lysosomes and granulocytes through pseudo-feces. This also points out that mussels may play an important role in the removal of contaminants such as Pb from the aquatic ecosystem, as they are critical in maintaining water quality by filtration. Although the major contribution of Pb to the environment from now-banned leaded gasoline remains evident in North Carolina, the

concentration measured in mussel tissue and sediment was not observed at levels that would cause immediate or short-term reductions in Eastern elliptio populations. Moreover, the sub-lethal effects of Pb on Na<sup>+</sup>,K<sup>+</sup>-ATPase activity, although a strong biomarker of exposure, appear not to be significantly affected at environmentally relevant concentrations of Pb. However, other species of freshwater mussels may be more sensitive to Pb contamination and further assessment is needed to verify the role Pb may be having in the overall declines in mussel species diversity being observed worldwide.

Biomarkers of Lead Exposure in the Freshwater Mussel *Elliptio complanata*  
For Assessing Transportation Related Impacts

by  
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## **DEDICATION**

I'd like to dedicate this work to my Dad, who, by example, sparked my interest in science, and gave me my love of, and desire for, the pursuit of knowledge. This is for you.

## BIOGRAPHY

Shad Mosher was born May 8<sup>th</sup>, 1981 in a little town called Chapel Hill, NC. He went to Carolina Friends School up and through high school where he acquired his love of the ocean, receiving his SCUBA license at the age of 15, and biology, taking every course offered including a Biochemistry class co-taught by both the Biology and Chemistry teachers. It was logical then that after graduating in 2000, he would attend UNC Wilmington receiving his Bachelor's in Marine Biology in 2004. He then had a little retreat from academia, where he worked for the North Carolina Public Interest Research Group (NC PIRG) trying to raise public awareness to the injustices being done to the environment. Next was a job as a field technician doing stream surveys of freshwater mussels. It was through this job where he came into contact with Dr. Greg Cope, who he started work as a lab technician for, leading into his adventures as a graduate student. It was during this time he met his wife, Tiffany, and everything seemed to fall into place. Shad successfully defended and met all requirements for graduation in August, 2008 and accepted a 1-2 year research position with NCSU for his advisor, Dr. Cope, working on emerging contaminants of freshwater mussels. Shad was quoted as saying "I couldn't be happier with where I am today, I just feel so lucky about everything in my life." What's next for the young scientist? "Who knows, it's been a busy couple of months, maybe a house." He and his wife Tiffany are set to close on their first home in the end of November, 2008.

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I would also like to thank my advisor and committee chair Dr. Greg Cope for his endless help and guidance (I could not have done this without him) and my committee members Dr. Damian Shea and Dr. Thomas Kwak. And thanks to Jason Mays for field collection, Tamara Pandolfo and Peter Lazaro for all their help with laboratory and field work, Consuelo Arellano for her statistical expertise, and Sharon Prochazka for her GIS work. Lastly I'd like to thank the North Carolina Department of Transportation for funding my research.

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## **BACKGROUND**

### ***Freshwater Mussels***

Native freshwater mussels, of the family Unionidae, may unfortunately be among the groups of aquatic organisms adversely affected by persistent, low-level exposure to lead (Pb) in our surface waters. The unionid genera are recognized to be among the most sensitive of families tested to specific contaminants [1]. They are filter- and deposit-feeding, long-lived (40-100 years) organisms that live burrowed in sediments of streams and rivers. Freshwater mussels are one of the most imperiled faunal groups in North America with about 70% of the nearly 300 native species considered vulnerable to extinction or already extinct [2-4]. This decline in mussel populations and diversity has been attributed to several factors including alterations to habitat from construction of dams and channel modification, siltation, introduction of exotic bivalve species and pollution from contaminants such as Pb [4, 5]. Preliminary data gathered by our laboratory during 2002 suggested that the difference in concentrations of lead upstream and downstream in native freshwater mussel tissue collected from streams adjacent to highways in the upper Neuse River Basin of North Carolina [6], were correlated with average daily traffic count when vehicle crossings per day were less than 4,000 (Figure 1). However, additional data from higher traffic areas are needed to verify this relation.

### ***Lead***

Lead contamination is a widespread, global problem. Even though emissions to the environment in North America have declined in the past two decades as a result of decreased

industrial discharges, as well as the ban of Pb from gasoline [7, 8], there is still considerable amounts left in the environment as well as that currently being released through mining, smelting, atmospheric deposition including coal combustion emissions, racing/boat/tractor fuels, hair-coloring products, plastics and vinyl, and lead-based paint [5, 7, 9-16]. One estimate is that of the 5.9 million metric tons of Pb used in gasoline throughout its history, 4-5 million metric tons were deposited as residue in the environment [12]. Because Pb is relatively immobile, it will continue to persist in the environment at areas where deposition occurred for many years. Of the lakes tested in North America, there were none found that had no contamination by Pb [15]. Atmospheric deposition is still a large source of cycling Pb, with one study estimating 90% of Pb in the soil of a boreal forest in Sweden being directly contributed from atmospheric deposition [17]. Many studies have shown greater levels of Pb in roadside sediments [12, 18-21] from historic transportation, with most of the Pb found in the small grain fraction (<63  $\mu\text{m}$ ), which is more likely to be re-suspended or eroded into rivers and streams adjacent to these roads [18, 19].

### ***Bioindicators***

Freshwater mussels are considered good sentinels for Pb contamination in aquatic ecosystems [22-25]. The freshwater mussel *Elliptio complanata* in particular meets many of the prerequisites for an ideal biomonitor of stable trace metals [26] in that they are sessile, relatively abundant, and large enough to provide sufficient tissue mass for analysis of the contaminant of interest. Moreover, they have been shown to tolerate a wide range of Pb contamination [27-30], allowing for laboratory studies, and are strong accumulators of Pb [30-32] with an established correlation between metal accumulation and the ambient

bioavailable concentration [32]. While one study found no change in Pb concentration in the mussel *Unio pictorum* after transplantation to a site near effluent from a wastewater treatment plant [33], the control mussels had high levels of Pb contamination (23 µg/g), suggesting they were not taken from an uncontaminated site. The half-life of Pb in mussel tissue has been shown to be relatively long with no observed loss over 370 days [34], and uptake results suggesting that there would be no effective loss from tissue over the lifetime of the mussel (~50 years), thus mussels transplanted from a contaminated site will continue to reflect the Pb levels of their original location. Therefore mussels used for biomonitoring should always be from an uncontaminated reference site, or from culturing facilities, in order to accurately detect increased uptake of the contaminants.

### ***Biomarkers***

Because unionids are such an imperiled fauna, it is critical to develop non-lethal biomarkers and sampling techniques when available. Hemolymph extraction has been shown to be a non-lethal sampling technique [35]. Therefore, I evaluated several possible biomarkers for Pb exposure and effect utilizing mussel hemolymph.

One of the classic biomarkers for Pb exposure in mammals, fish and some invertebrates is  $\delta$ -aminolevulinic acid dehydratase (ALAD) activity. ALAD is a critical enzyme in the pathway responsible for heme synthesis, the molecule that binds and transports oxygen in the blood of vertebrates and some invertebrates. ALAD converts aminolevulinic acid (ALA) to porphobilinogen (PBG). ALAD has been shown to be an excellent specific biomarker of Pb exposure, but not other metals, in many different species including humans [36], birds [37], frogs [38], fish [11, 39-45] and the gastropod *Biomphalaria glabrata* [46].

ALAD enzyme activity is most always negatively correlated with the amount of Pb accumulated by the organism. One of the aims of this study was to assess mussel hemolymph and tissue for ALAD as a potential non-lethal biomarker for Pb exposure.

The Na<sup>+</sup>,K<sup>+</sup>-ATPase, or sodium pump, is responsible for the co-transport of sodium ions out of, and potassium ions into the cell membrane in most eukaryotes, and helps in ionic regulation [47]. The activity of Na<sup>+</sup>,K<sup>+</sup>-ATPase has been shown to be significantly reduced [48-51] and correlated with Pb tissue concentration by noncompetitive inhibition. While no Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was detected in the mantle tissue of *Anodonta cataracta* [52], activity has been detected in *Anodonta cygnea* [53] and found in mantle and gills in freshwater and marine bivalves of the genera *Carunculina* [54, 55], *Lampsilis*, *Corbicula* and *Rangia* [54] and *Tapes* [56], albeit all had relatively low levels. We assessed gill tissue for Na<sup>+</sup>,K<sup>+</sup>-ATPase activity as a biomarker for Pb exposure in Eastern elliptio.

The other non-lethal biomarker we assessed was ion (Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>, and Ca<sup>2+</sup>) concentrations in mussel hemolymph. Even with high renal ion absorption resulting in an excretory fluid concentration half that of hemolymph [57], freshwater mussels are subject to high ion loss from this excretion. As a result, they have developed extremely low hemolymph osmolalities of 45-60 mOsm [54] (36 mOsm for the zebra mussel [58]) to reduce loss and expended energy for active ion uptake [59]. Lead is known to cause imbalances in Na<sup>+</sup> and Cl<sup>-</sup> levels in rainbow trout *Oncorhynchus mykiss* [48] attributed to reduced Na<sup>+</sup>,K<sup>+</sup>-ATPase activity, as well as glucose and ion disruption in rainbow trout *Salmo gairdneri* and whitefish *Coregonus spp.* [45, 60], ion disruption in crabs [61] and Na<sup>+</sup> imbalances in snails [62]. Because Pb has been shown to cause disruption to protein and glucosamine in unionids

[63], and other heavy metals such as Cd will cause osmotic imbalances in unionids [64], I hypothesized the disruption of ion transporting enzymes, hemolymph ion concentration and chemistry as a consequence of Pb exposure in Eastern elliptio.

### ***Uptake and Effects***

For aquatic organisms, the gills are a primary repository for Pb accumulation. This has been observed for snails [65], fish [66], and freshwater mussels as well. When mussels are exposed to heavy metals, uptake occurs mainly in the gill and mantle [22, 23, 30, 67] and observed effects have included changes in growth [67], filtration [28, 30, 67-69], enzyme activity [67] and behavior [67, 69]. An increase in lysosomal numbers and size [70] and DNA strand-breakage [71] has also been seen in mussels exposed to Pb, although lysosomes are more an indicator of general environmental stress also being affected by PAHs and PCBs, and DNA strand-breakage was only seen at the lowest exposure concentration indicating DNA repair was occurring at higher concentrations. Shell mass was reduced in the snail *Helix aspersa* exposed to Pb in its food [72], though exposure concentration was extremely high (500 µg/g Pb). In the marine bivalve *Crassostrea gigas*, it was shown that exposure to Pb from sediment caused ultrastructural damage to gill epithelium, but less severely than when exposed to Pb from water [27]. Ultrastructural damage included disruption of plasma membranes, loss of microvilli, loss of organelles, loss of cristae from mitochondria, and swelling of the nuclear envelope.

### ***Bioavailability***

The bioavailability of Pb in the aquatic environment is dependent on many factors. Aside from biological factors including species, age, size, growth rate and sex, which explain

a large portion of the variability among *Elliptio complanata* [25], there are many environmental factors affecting Pb bioavailability and uptake. Both the pH of the water and the amount of calcium sulfate ( $\text{CaSO}_4$ ) and dissolved organic carbon (DOC) present have affected the availability of Pb to fathead minnow [73], with variations from pH 7.4 increasing, and addition of  $\text{CaSO}_4$  or DOC decreasing toxicity. Two forms of Pb (lead acetate trihydrate and lead carbonate) were both found highly adsorbed onto organic matter in sediment [74]. When dissolved organic matter (DOM) is present, however, Pb accumulates and becomes more persistent, thereby increasing the level of exposure and thus raising the bioavailable amount [75]. Humic water increases the bioavailability of most metals compared with tap water [76] by forming bonds with functional groups of carboxylic acid, facilitating uptake. Other metals can also alter Pb bioavailability. Cadmium accumulation in the unionid *Pyganodon grandis* was found to be reduced proportionately with increasing concentrations of Cu, Zn, Pb and Ni [77].

### ***Accumulation***

While several studies have found little or no correlation between Pb accumulation in freshwater mussels and the amount present in the environment [31, 78], these studies used total Pb concentration of sediment in their comparisons. Because so many factors affect the bioavailability of Pb, it becomes necessary to adjust the total Pb concentration of the sediment with its modifying factors to understand how much Pb is actually available to the organism for uptake and accumulation. For instance, correlations were found with Pb concentrations between sucker fish and sunfish and the “organic-rich” sediment [79, 80]. In a study with *Elliptio complanata*, it was shown that Pb accumulation was reduced by the

presence of iron oxyhydroxides and organic matter, and that once sediment levels were “normalized” for organic carbon content [32], the correlation coefficient was increased with respect to Pb levels in mussel tissue. *Elliptio complanata* tissues, while having a larger amount of individual variability of trace metal concentrations than their shells [81], can still be used as a good sentinel of environmental concentrations of Pb. Because mussel growth is reduced in the presence of heavy metals [67] and therefore not accumulating elements at normal rates, shells have been suggested to be more an indication of availability and physiological exchange rates at times of growth, rather than current environmental concentrations [82].

### ***Storage and Removal***

When bivalves are exposed to lead, lysosomes are the key intracellular structures where lead storage takes place in the tissues [27, 83], and these lysosomes are then removed by cellular apex extrusion. This process explains the observed increase in lysosomal numbers and size [70]. Storage has also been seen in granular concretions [83-86]. The concentration of Pb in the tissue of zebra mussels has been shown to fluctuate throughout the year, increasing with metabolism in the summer months [87]. When zebra mussels are in the presence of waterborne Pb, they take it up through their inhalant siphon, where it is accumulated in the gills [83], and is processed through several pathways. Dissolved Pb can associate with both granulocytes and blood plasma ligands, where it is then transported to heart, kidney and digestive cells. Particulate Pb will travel to the stomach and enter digestive cells. Lead can be incorporated into the shell, and elimination occurs through the exhalant

siphon by one of three ways; with excretory concretions in urine, digestive cell lysosomes in feces, or granulocytes in urine and feces [83].

### ***Research Objectives***

The overall objectives of my research were two-fold, and were approached in a landscape-level and two laboratory studies. For the landscape-level study, I examined Pb concentrations in mussel tissue and sediment from 40 sites throughout North Carolina ranging in average daily traffic count and landscape use patterns in order to assess the current Pb contamination level, and to examine trends/correlations with traffic count. In the laboratory, I conducted two, 28 – day toxicity tests with adult Eastern elliptio mussels, to assess Pb uptake kinetics, compare the field-derived Pb bioaccumulation data to obtain a rate of accumulation, and to examine potential biomarkers of Pb exposure and effect.

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Figure

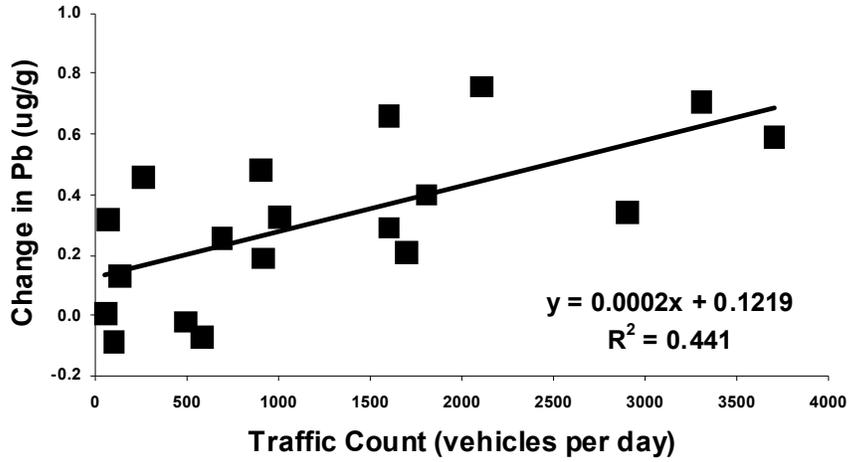


Figure 1. Preliminary data on correlation between traffic count and change in Pb among the freshwater mussel *Elliptio complanata*. (Data from Levine et al. 2005)

## CHAPTER 1

### **Assessing lead concentrations of sediment and freshwater mussels at road crossings from streams in central North Carolina**

#### **Abstract**

The aim of this study was to determine lead (Pb) accumulation and trends in the freshwater mussel *Elliptio complanata* near road crossings as an indicator of traffic-related impacts. A total of 40 sites throughout North Carolina were sampled that varied in land use patterns, geographic distribution, and traffic density. Three mussels and one sediment sample were taken both upstream and downstream of the crossing structure (within 50 to 100 meters) at each site and analyzed for Pb concentrations. Mussel Pb concentrations ranged from 0.52 – 13.83  $\mu\text{g/g}$  (dry weight), and sediment Pb concentrations ranged from 1.53 – 10.28  $\mu\text{g/g}$  (dry weight). There were no correlations between average mussel Pb concentration and traffic count, average sediment Pb concentration and traffic count, or between mussel and sediment Pb concentrations among the sites. There was, however, a significant difference ( $P \leq 0.0425$ ) between Pb concentrations from mussels collected from low traffic sites ( $< 500$  vehicle crossings/day (vc/d)) compared to higher traffic sites (500 – 60,000 vc/d), but not among the high traffic sites. My results indicate a trend of decreasing Pb concentrations in sediment with time, and I conclude that Pb concentration in sediment is no longer correlated with traffic count because historic inputs from leaded gasoline have ceased. However, because Pb in mussel tissue is not thought to decrease significantly over

its lifetime due to the extremely long half-life of Pb in the tissue, the adult freshwater mussel *Elliptio complanata* is a good integrator of Pb contamination over its lifetime.

### **Introduction**

Native freshwater mussels, of the family Unionidae, are filter- and deposit-feeding, long-lived (40-100 years) organisms that live burrowed in sediments of streams and rivers, and therefore may be among the groups of aquatic organisms adversely affected by persistent, low-level exposure to lead (Pb) in our surface waters. They are one of the most imperiled faunal groups in North America with about 70% of the nearly 300 native species considered vulnerable to extinction or already extinct [1-3], and with unionids recognized as one of the most sensitive families tested to specific contaminants [4], they are sentinels for assessing environmental conditions [5-8]. The freshwater mussel *Elliptio complanata* in particular meets many of the prerequisites for an ideal biomonitor of stable trace metals [9] in that they are sessile, relatively abundant, and large enough to provide sufficient tissue mass for analysis of the contaminant of interest. Moreover, they have been shown to tolerate a wide range of Pb contamination [10-13], allowing for laboratory studies, and are strong accumulators of Pb [13-15] with an established correlation between metal accumulation and the ambient bioavailable concentration [15]. One study, however, found no change in Pb concentration in the mussel *Unio pictorum* after transplantation to a site near effluent from a wastewater treatment plant [16], but the control mussels had high levels of Pb contamination (23 µg/g), suggesting they were not taken from an uncontaminated site. The half-life of Pb in mussel tissue has been shown to be relatively long (no observed loss from tissue after a year,

suggesting no significant loss over the lifetime of the mussel) [17], thus mussels transplanted from a contaminated site will continue to reflect the Pb levels of their original location. Therefore mussels used for biomonitoring should always be from an uncontaminated reference site in order to accurately detect increased uptake of the contaminants. As freshwater mussel populations and diversity have declined steadily since the mid-1800's being attributed to several factors including alterations to habitat from construction of dams and channel modification, siltation, introduction of exotic bivalve species and pollution from contaminants such as Pb [3, 18], it is critical to assess the level of effect traffic related stressors are having on mussel health.

Lead contamination is a widespread, global problem. Even though emissions to the environment in North America have declined in the past two decades as a result of decreased industrial discharges, as well as the ban of Pb from gasoline [19, 20], considerable amounts remain in the environment as well as those currently being released through mining, smelting, atmospheric deposition including coal combustion emissions, racing/boat/tractor fuels, hair-coloring products, plastics and vinyl, and lead-based paint [18, 19, 21-28]. One estimate is that of the 5.9 million metric tons of Pb used in gasoline throughout its history, 4-5 million metric tons were deposited as residue in the environment [24]. Because Pb is relatively immobile, it will continue to persist in the environment in areas where deposition took place for many years. Of the lakes tested in North America, there were none found that had no contamination by Pb [27]. Atmospheric deposition is still a large source of cycling Pb, with one study estimating 90% of Pb in the soil of a boreal forest in Sweden being directly contributed from atmospheric deposition [29]. Many studies have shown greater

levels of Pb in roadside sediments [24, 30-33] with most of the Pb found in the small grain fraction (<63  $\mu\text{m}$ ), which is more likely to be re-suspended or eroded into rivers and streams adjacent to these roads [30, 31].

Preliminary data gathered by our laboratory in 2002 suggested that the difference in concentrations of lead upstream and downstream in native freshwater mussel tissue collected from streams adjacent to highways in the upper Neuse River Basin of North Carolina [34], were correlated with average daily traffic count when vehicle crossings per day were less than 4,000 (Figure 1). However, additional data from higher traffic areas are needed to verify this relation. In this study, I examine Pb concentrations in mussel tissue and sediment from 40 sites throughout North Carolina ranging in average daily traffic count and landscape use patterns, to better characterize the current stage of Pb contamination in North Carolina's streams and its bioavailability to the freshwater mussel *Elliptio complanata*.

## **Materials and Methods**

### ***Site Locations***

Samples of mussel soft tissue and sediment were collected from 40 sites (Figure 2). Sites were chosen based on land use patterns, geographic distribution, and a well distributed range of traffic density among sites. All sites were selected after verification of the presence of the target species at sites that met the previously stated criteria. At each site, three mussels and one sediment sample were obtained, both upstream and downstream of the crossing structure. Sampling locations at a site were chosen from within an area of stream greater than 50 m but less than 150 m from the crossing structure with consideration given to

selecting sampling locations that represented the typical suitable habitat from that stream section. Mussels were selected at random and represented the average size of adult individuals from the site. Mussels averaged 46.3 grams in weight (ranging from 18.8 – 124.5) and 71.3 mm in length (ranging from 53.0 – 97.5). Surficial (uppermost 5 cm) sediment samples were collected with a stainless steel scoop from within areas inhabited by the mussels that contained fine particulate matter.

### ***Analytical Procedures***

Mussel and sediment samples were prepared and analyzed by standard methods at Research Triangle Institute (RTP, NC) for levels of the elements Pb, Hg, Cd, Pt, Pd, and Rh, with Pb being the primary target of interest in this study. Mussel tissues were 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 mass spectrometry (Thermo Element 2 Magnetic Sector ICP-MS). The average percent recovery of spiked samples (n = 7) was 88%. The recovery of Pb from spiked sediment samples averaged 104%. Detectable levels of Pb were confirmed in all mussel tissue and sediment samples.

Sediment samples were then sent to the Soil Science Department at NCSU for total carbon (TC) analysis. Samples were frozen and water was sublimated by freeze drying for 5 days in a Virtis 1200XL freezemobile. Dried samples were sieved to < 2 mm using a stainless steel mesh screen. The < 2 mm fraction was well mixed and a sub-sample of approximately 1 g was removed. This sub-sample was ground to < 250 µm using an agate

motar and pestle. A portion (24 to 27 mg) of each sub-sample was weighed into tin capsules and analyzed for TC (%) on a Perkin Elmer 2400 CHN Elemental Analyzer. In the presence of excess oxygen and combustion reagents, samples were combusted completely and reduced to elemental gases such as CO<sub>2</sub>, H<sub>2</sub>O, N<sub>2</sub>, and SO<sub>2</sub> which were further mixed and separated. Temperatures in the combustion and reduction chamber were 925 °C and 640 °C, respectively. For quality control, two control soils were measured after every 22 samples.

### ***Statistical Analysis***

Variations in average Pb concentration in mussels and sediment at each site were analyzed following a generalized linear model with traffic count (traffic count groups) considered as the fixed-effect factor and mussels or sediment considered as the random factor (repetitions), allowing for heterogeneous residual variances, with residual variances estimated separately for each traffic count group. Analyses were performed with Proc MIXED procedure in SAS v9.1.3 (SAS, Cary, NC).

Regression of Pb differences in sediment upstream and downstream on traffic count was performed using the SAS procedure PROC REG, to analyze whether there was a linear relationship. Similar analyses were conducted to analyze the linear relationship between differences in mussel upstream and downstream with traffic count, mussel averages with traffic count, and sediment averages with traffic count. Statistical significance was determined with an  $\alpha = 0.05$ .

## Results

For mussel tissue samples, the average difference between downstream and upstream Pb concentrations, while not significant due to high variability, was positive with downstream mussels having 0.27  $\mu\text{g/g}$  (12%) more Pb than upstream mussels. The Pb concentrations were averaged for the three upstream and three downstream mussels for site comparisons, and listed in order of increasing traffic count (Table 1). Samples from the 390 vc/d and 1700 vc/d sites were judged to be outliers and were not used in site comparisons. When the average Pb concentration in mussels was compared to the traffic density at each site (Figure 3), there was no discernable relation ( $R^2 = 0.03$ ;  $P = 0.41$ ). However, when the sites were partitioned into groups with vehicle crossings per day of 0-49, 50-499, 500-999, 1000-4999, 5k-10k, and 10k-60k (Figure 4), there was a trend of greater Pb in mussel tissue with increasing traffic count up to the 500-999 group, but this did not increase further with the higher traffic groups. The groups of 500-999 and up were significantly ( $P \leq 0.0071$ ) greater than the 0-49 group, and significantly ( $P \leq 0.0425$ ) greater than the 50-499 group.

The measured Pb concentrations in sediment were averaged for each site with the difference between downstream and upstream (Table 2) and again the 390 and 1700 vc/d sites were excluded for calculations. On average, the downstream sediment samples had 0.62  $\mu\text{g/g}$  (16%) more Pb than samples taken from upstream and 2  $\mu\text{g/g}$  (43%) more for sites with over 20,000 vehicle crossings per day, though there was too much variability for these results to be statistically significant. There was no correlation found when comparing the average sediment Pb concentrations ( $P = 0.56$ ) to traffic count (Figure 5). Nor was there any

correlation when concentrations of Pb in mussel tissue and the concentrations of Pb in the sediment were compared at the sites (Figure 6).

When the sediment Pb concentration is adjusted for the total carbon (TC) content (in %) for each site (Figure 7), the 1000-4999 vc/d group becomes significantly ( $P = 0.039$ ) larger than the control 0-49 vc/d group compared to the non-adjusted sediment Pb (Figure 8), but none of the other groups are significantly different. In addition, no trend is present ( $P = 0.623$ ) when comparing the adjusted sediment Pb for each site with traffic count. Nor is there an increase in correlation between mussel Pb and adjusted sediment Pb ( $P = 0.635$ ) following normalization with TC content.

## **Discussion**

From comparisons of mussel tissue Pb concentration with traffic density (Figure 4), I found that there was significantly ( $P \leq 0.0425$ ) greater Pb in mussels from sites with higher traffic counts than from reference sites, up to the 500-999 vehicle crossings per day category. The lack of relation from the 500-999 category and greater, however, suggests that while Pb from historic leaded gasoline use is still present in roadside soil and stream sediment, the reduction in use of leaded gasoline in the past 38 years since the phase out started, and past 12 years since the ban, has resulted in a wide range of Pb concentration in sediment at each site that is unrelated to current traffic density and patterns. The existing Pb is continuing to be processed by the specific hydrology and ecology of the individual streams [19]. For example, faster flowing streams tend to have greater sediment movement and Pb loss due to erosion, than in slower flowing streams [35], or in slower moving sections of the stream such

as pools (as opposed to riffles or runs) [36] with depositional habitats. In addition, highly erosional sediment with high sand and low organic content could also help explain the lack of correlation between sediment Pb concentrations and traffic count, as well as mussel Pb concentration and traffic count. Because it has been shown that adjusting for the organic carbon content of sediment can increase the correlation between mussel and sediment Pb concentrations [15], sediment Pb was adjusted for TC concentration. However, no observable increase in the correlation was observed. This suggests that the lack of correlation between mussel tissue and sediment is more likely an indication that the measured Pb in mussels is from historic inputs, rather than that our analysis of the sediment does not represent the bioavailable portion of Pb. From these results, I conclude that while sediment Pb concentration is no longer correlated with traffic count, the adult freshwater mussel *Elliptio complanata* is a good sentinel of Pb contamination over its lifetime as they maintain Pb tissue concentrations from earlier contamination which may no longer be present.

When the Pb concentrations in mussel tissue from this study are compared to concentrations from earlier research in North Carolina [34], the average concentrations of the higher traffic groups (500 – 60k vc/d) in this study were greater than those found at the previous study's sites with traffic count < 4,000 vc/d. Also, our reference group with 0-49 vc/d was comparable to the low end of that study's range with similar traffic counts. A mussel species *Elliptio dilatata* found in the Midwestern US and related to the one studied here was found to have an average of 1.6 µg/g of Pb in their tissue at reference sites [18], which was around the lower range for our high traffic groups. With those levels of Pb in

*Elliptio sp.*, and zebra mussels from the Great Lakes found to be around 2 µg/g [37], our findings suggest an average range for Pb in freshwater mussels of 1 – 2 µg/g, depending on historical land use. The Pb concentrations in mussel tissue from the high traffic groups in this study was found to be twice that of those from *Elliptio buckleyi* in a recent study near Istanbul, Turkey [38] which were considered to be threatened by pollution. Sediment Pb concentrations in this study were also similar to those found in 2003 (1.45 µg/g) in Ranco Bay, Italy [39] which was an historically polluted area.

Lead concentrations in freshwater sediments from throughout the US have decreased from 1970 – 2001 [40] since the phase-out and ban of leaded gasoline. According to EPA's STORET database, the average Pb concentrations in stream sediment were reduced in Mecklenburg County, NC from 16 µg/g in 1979 (which was very similar to the average in NC at the time of 16.6 µg/g) to 10 µg/g by 2003. The average sediment Pb concentration from the 40 sites in NC in this study (2007 – 2008) was 3.9 µg/g. My results point toward the continuation of decreasing Pb concentration in river and stream sediment over time since the reduction and ban of leaded gasoline.

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## Tables

**Table 1. The average lead concentration in mussels upstream and downstream of road crossing structures in North Carolina (n=three mussels at each upstream and downstream location).**

vc/d	Mean lead concentrations in Mussels ( $\mu\text{g/g}$ dry wt)			
	Upstream	Downstream	Difference	Site Mean (n=6)
0	0.65	0.58	-0.07	0.62
30	0.49	0.55	0.06	0.52
390	10.85	11.47	0.61	11.16
430	0.91	1.26	0.34	1.08
470	0.56	0.67	0.12	0.61
500	0.90	1.02	0.12	0.96
500	1.91	1.80	-0.10	1.85
500	2.20	2.91	0.70	2.55
500	1.90	2.02	0.12	1.96
540	1.20	1.99	0.79	1.59
610	1.35	1.64	0.29	1.50
610	1.16	1.37	0.20	1.27
650	1.25	1.28	0.03	1.26
680	0.74	0.96	0.21	0.85
680	1.69	1.47	-0.22	1.58
935	4.54	4.31	-0.23	4.42
1,200	0.46	0.83	0.37	0.65
1,400	0.50	0.90	0.40	0.70
1,600	0.83	1.21	0.37	1.02
1,700	12.45	15.21	2.76	13.83
1,800	1.24	1.57	0.33	1.41
1,800	0.57	0.47	-0.09	0.52
2,000	3.38	3.25	-0.13	3.32
2,200	0.77	0.80	0.03	0.78
2,300	1.82	3.09	1.27	2.46
2,400	1.58	3.04	1.47	2.31
2,400	1.62	2.19	0.58	1.91
2,800	2.40	3.66	1.26	3.03
3,300	1.84	0.95	-0.89	1.39
5,700	0.86	1.21	0.35	1.03
5,850	2.42	2.85	0.43	2.63
9,600	2.68	2.08	-0.60	2.38
12,000	0.67	0.43	-0.24	0.55
24,000	2.50	1.85	-0.66	2.18
28,000	1.78	2.42	0.64	2.10
43,000	1.41	2.14	0.73	1.77
58,000	2.43	1.14	-1.29	1.78

**Table 2. The average lead concentration in sediment for each site, with the difference between downstream and upstream.**

vc/d	Mean lead concentration in Sediment ( $\mu\text{g/g}$ dry wt)	
	Sediment	Difference
0	3.86	N/A
30	2.89	0.38
390	9.81	2.32
430	3.95	-0.33
470	5.15	1.69
500	4.58	-3.65
500	5.77	-0.71
500	2.60	N/A
500	1.78	0.76
540	4.96	2.29
610	2.92	0.47
610	3.02	1.37
650	6.83	-1.49
680	5.38	0.25
680	4.32	2.14
935	1.89	0.93
1,200	3.62	1.54
1,400	2.10	-0.46
1,600	3.02	0.89
1,700	4.58	-1.18
1,800	5.93	0.78
1,800	1.97	N/A
2,000	3.79	1.39
2,200	4.68	2.32
2,300	1.99	1.46
2,400	2.50	N/A
2,400	4.92	-1.19
2,800	4.66	-1.35
3,300	2.87	-1.59
5,700	4.25	2.36
5,850	2.83	1.87
9,600	1.53	0.60
12,000	4.76	-1.13
24,000	1.54	0.10
28,000	3.19	1.44
43,000	10.28	7.18
58,000	5.40	-0.84

## Figures

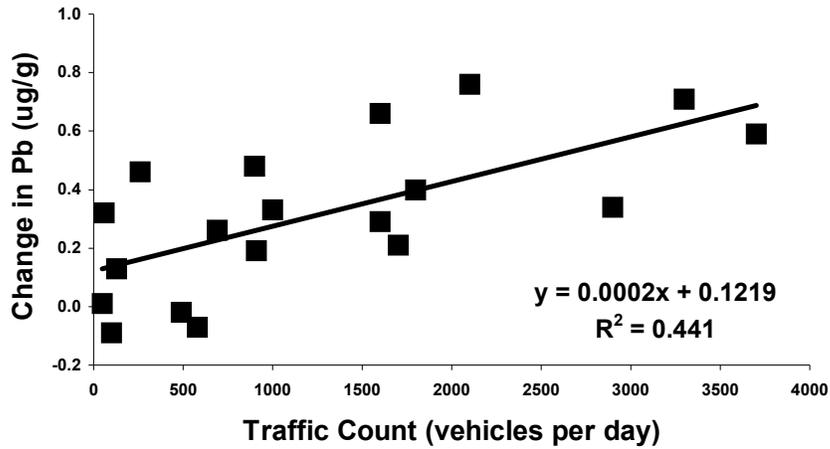


Figure 1. Preliminary data on correlation between traffic count and change in Pb among the freshwater mussel *Elliptio complanata*. (Data from Levine et al. 2005)

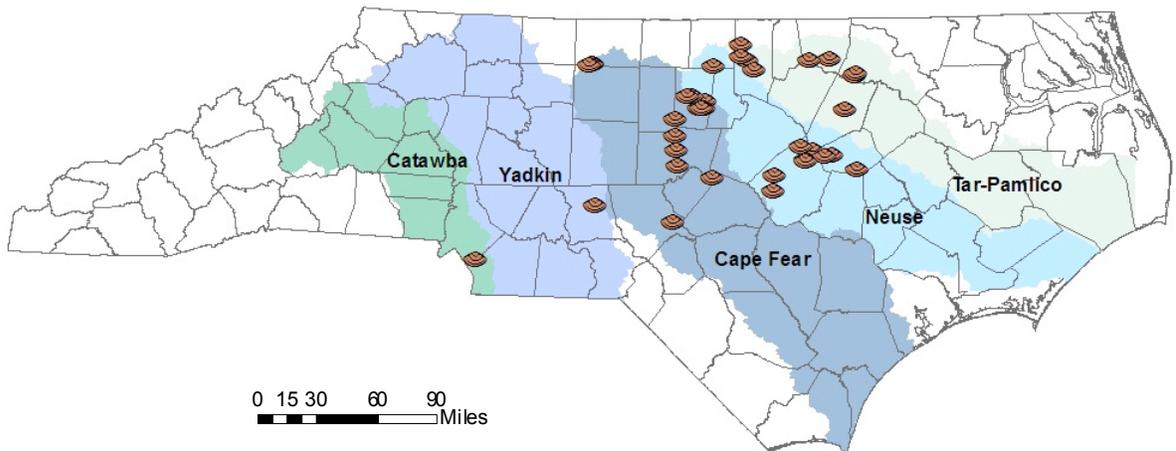


Figure 2. Site locations where mussel and sediment samples were taken within North Carolina.

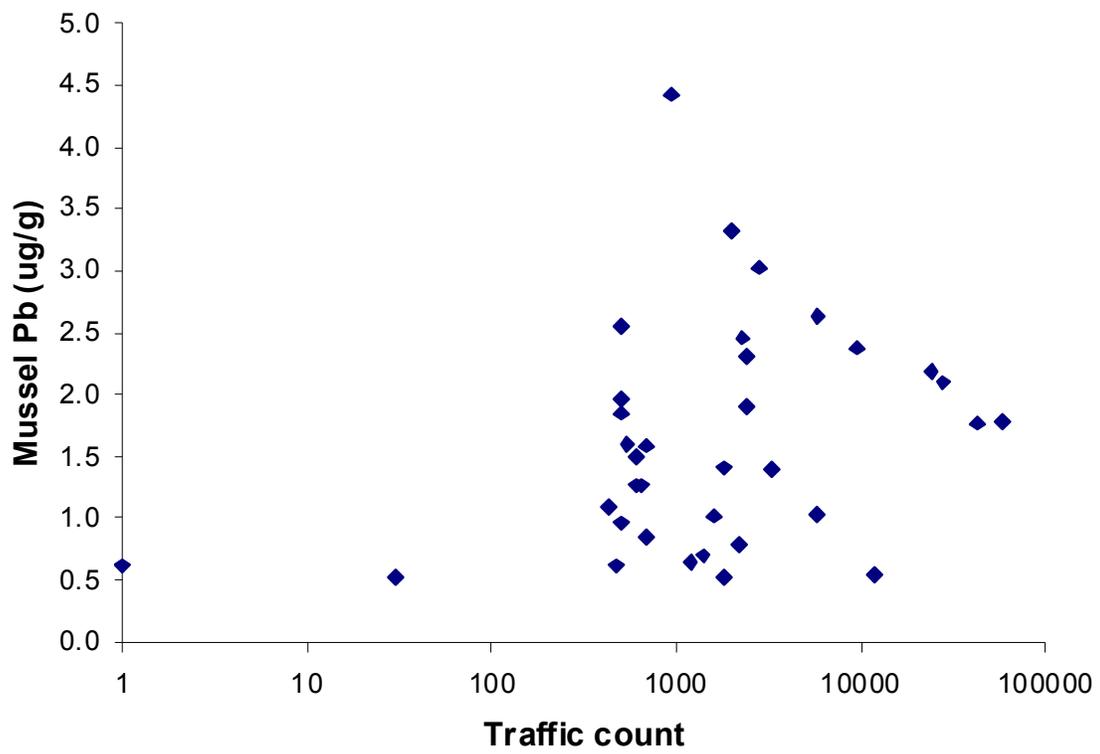
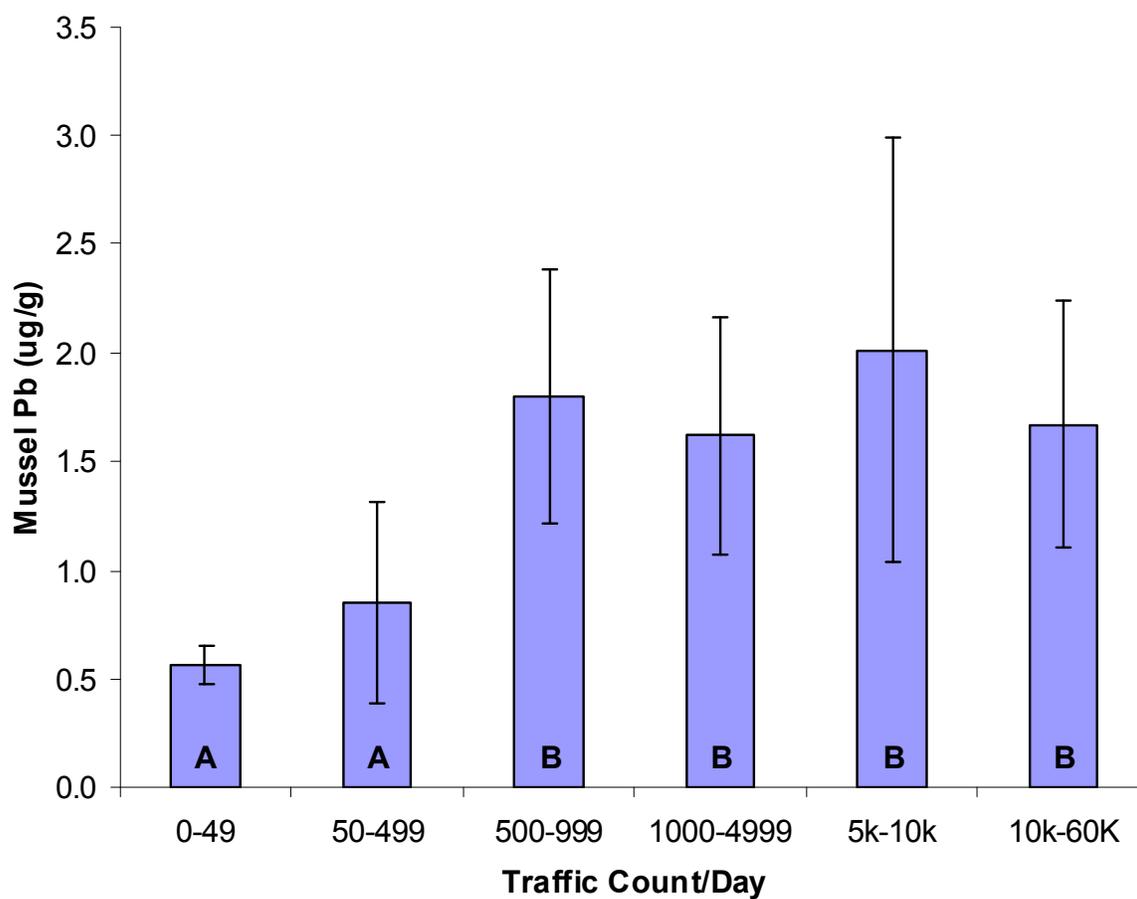


Figure 3. The average Pb concentration of mussels for each site with the corresponding traffic count.



**Figure 4. The average mussel Pb concentration per traffic group, with error bars representing 95% confidence intervals. Means with different letters are significantly different.**

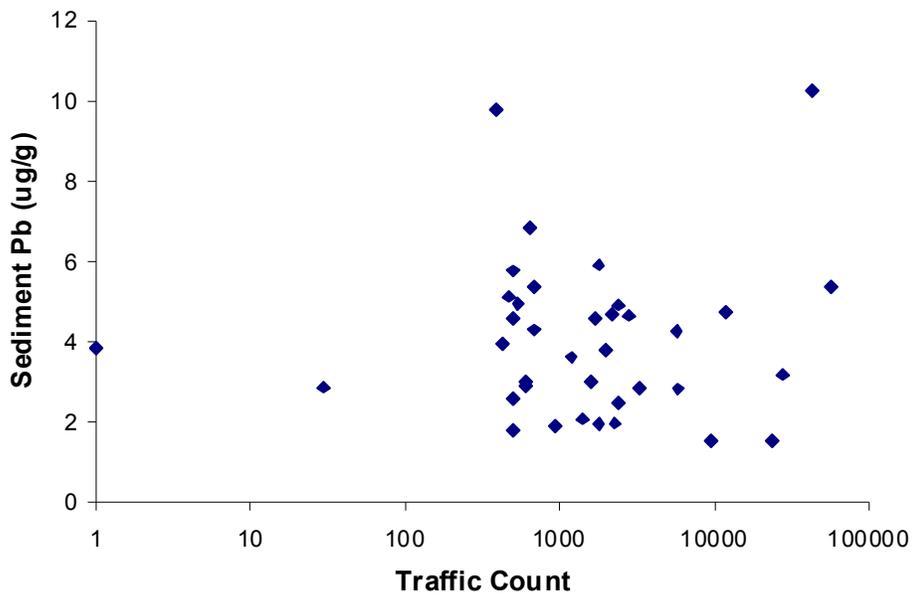


Figure 5. Scatter plot of sediment Pb concentrations with traffic count.

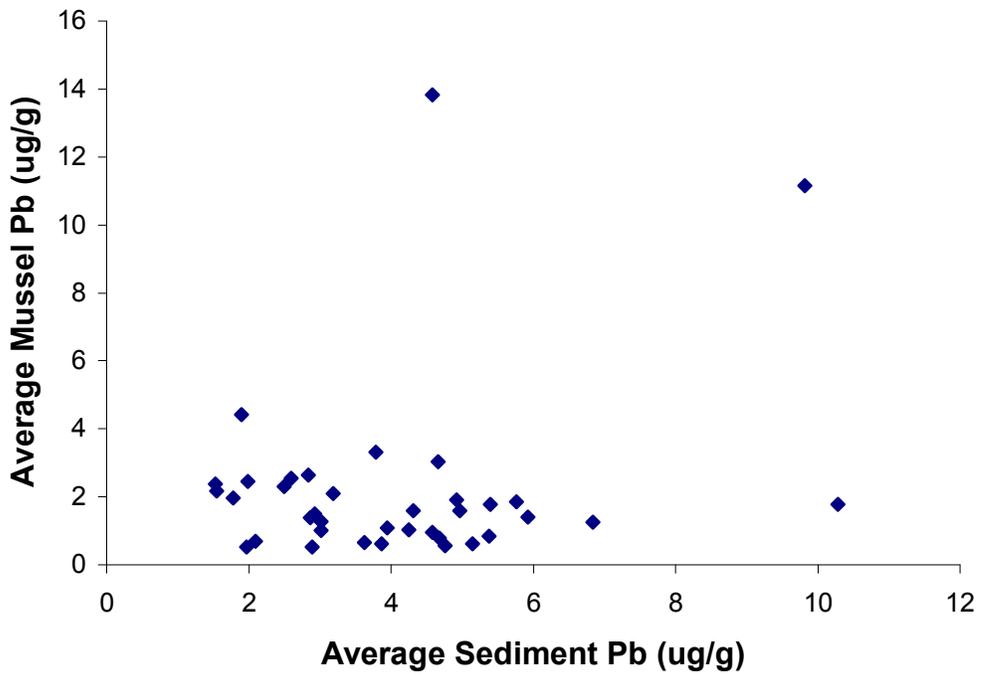
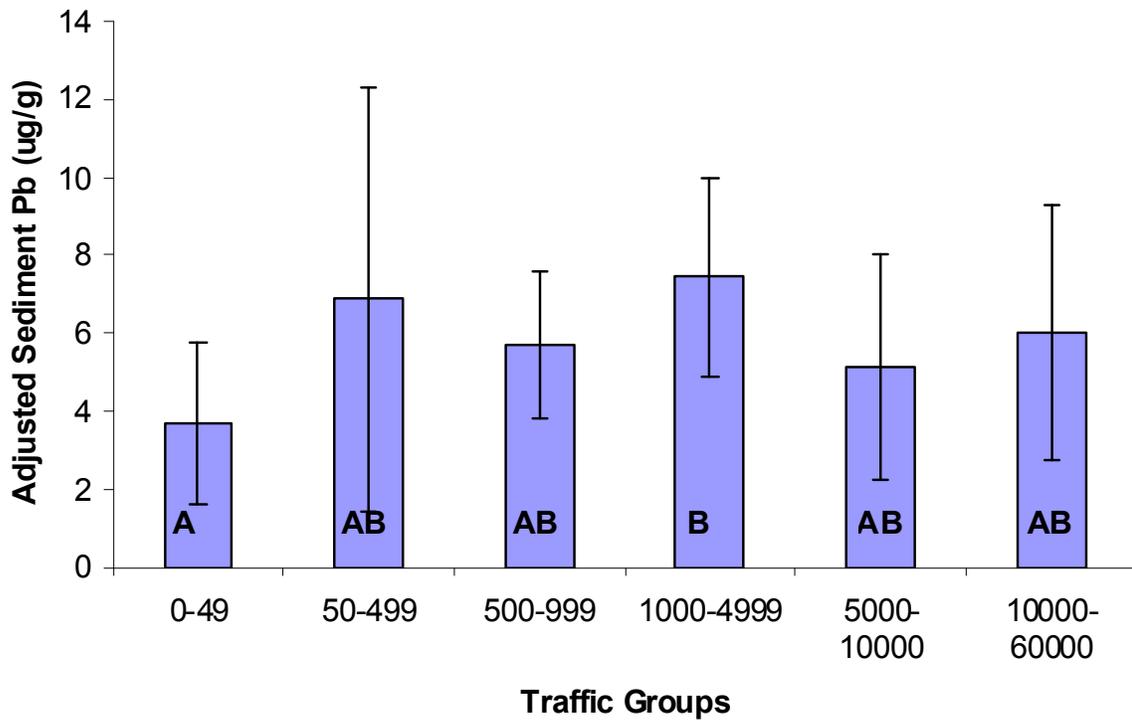


Figure 6. Average mussel Pb concentration at each site with average sediment Pb concentration.



**Figure 7. The average sediment Pb levels for each traffic group adjusted for the total carbon (TC) content. Error bars represent 95% confidence intervals.**

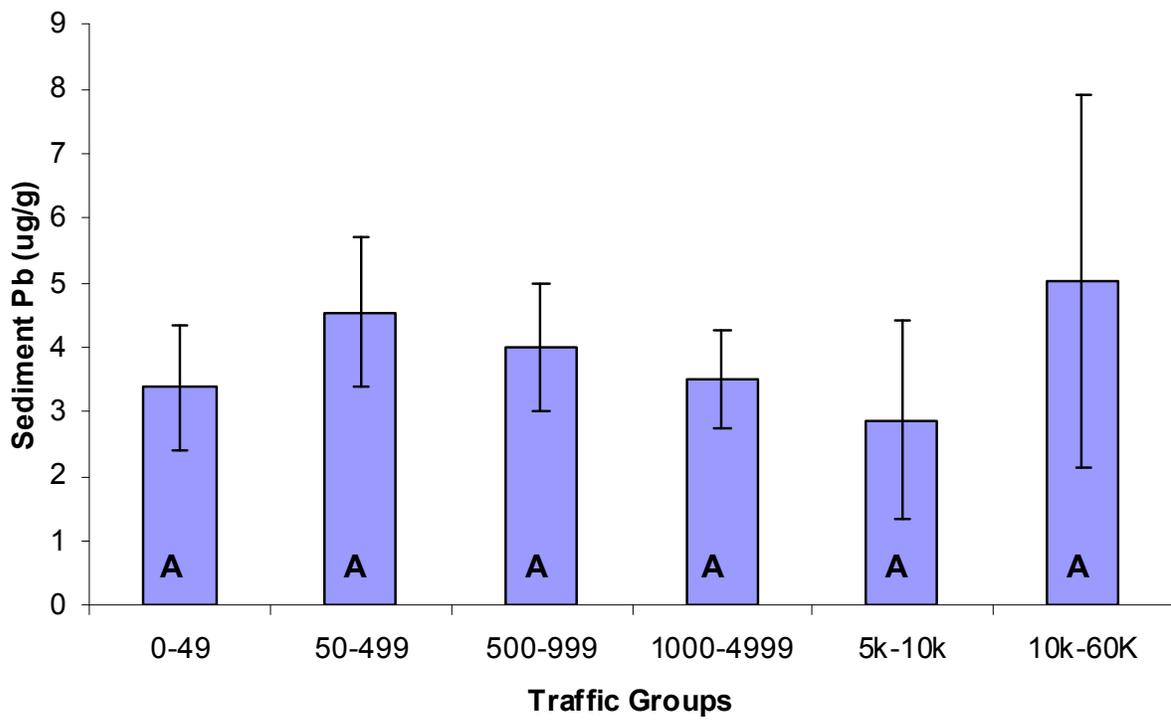


Figure 8. Average concentration of Pb in sediment for each traffic group. Error bars represent 95% confidence intervals.

## CHAPTER 2

### **Assessing accumulation and sublethal effects of lead in a freshwater mussel**

#### **Abstract**

I conducted a 28 day laboratory test with Eastern elliptio (*Elliptio complanata*) to determine uptake kinetics and assess several potential biomarkers of lead (Pb) exposure and effect. Mussels were sampled from a relatively uncontaminated reference site, and exposed to eight concentrations of Pb (as lead nitrate) ranging from 1 to 251  $\mu\text{g/L}$ , as a static renewal test. Three potential biomarkers of Pb exposure and effect were assessed within the mussel hemolymph, including ALAD activity, ion concentrations/ratios, and Pb concentrations. No ALAD activity was found at any time point within the hemolymph of *Elliptio complanata*, while it was both present and fully inhibited by 66  $\mu\text{g/L}$  of Pb in simultaneously exposed fathead minnows used as a positive control. No correlations existed between ion concentrations in hemolymph of repeatedly sampled mussels and their Pb exposure concentration. However, for a subsample of non-repeatedly sampled mussels,  $\text{Ca}^{2+}$  levels were reduced below the 95% CI of the baseline range for  $\text{Ca}^{2+}$  in low Pb exposures ( $\leq 3$   $\mu\text{g/L}$ ), and above it for high lead exposures (11, 66 and 251  $\mu\text{g/L}$ ), indicating potential adverse effects of Pb on  $\text{Ca}^{2+}$  levels within the hemolymph. Concentrations of Pb in mussel tissue on day 28 were strongly correlated with exposure concentrations. Concentrations of Pb increased in the hemolymph of all mussels exposed to Pb, with several different trends depending on the level of exposure; for concentrations  $\leq 11$   $\mu\text{g/L}$  hemolymph accumulated

up to 3.6 times the exposure concentration with a plateau at day 14 for the 11 µg/L treatment, exposures of 26 and 66 µg/L caused a plateau on day 14 at 1.4 and 1.2 times the exposure concentration, and the greatest exposure concentration of 251 µg/L did not plateau but accumulated Pb rapidly and linearly to five times the exposure concentration by day 28. Overall, I found that ALAD proved to be an unsuitable biomarker for Pb exposure in the freshwater mussel *Elliptio complanata*, Ca<sup>2+</sup> levels in hemolymph could potentially be adversely effected by Pb exposure in non-repeatedly sampled mussels, although further assessment is needed to confirm this relationship, and Pb levels measured in hemolymph of exposed mussels suggested regulation of the heavy metal up to levels as high as 66 µg/L, whereas concentrations in tissue proved to be strongly correlated ( $R^2 = 0.98$ ;  $P < 0.05$ ) to exposure concentration. Thus freshwater mussels appear to accumulate Pb with a concentration dependent manner and start actively regulating Pb uptake by day 14.

## **Introduction**

Native freshwater mussels, of the family Unionidae, are filter- and deposit-feeding, long-lived (40-100 years) organisms that live burrowed in sediments of streams and rivers, and therefore may be among the groups of aquatic organisms adversely affected by persistent, low-level exposure to lead (Pb) in our surface waters. They are one of the most imperiled faunal groups in North America with about 70% of the nearly 300 native species considered vulnerable to extinction or already extinct [1-3], and with unionids recognized as one of the most sensitive families tested to specific contaminants [4], they are good sentinels for assessing environmental conditions [5-8]. Freshwater mussel populations and diversity

have declined steadily since the mid-1800's which has been attributed to several factors including alterations to habitat from construction of dams and channel modification, siltation, introduction of exotic bivalve species and pollution from contaminants such as lead (Pb) [3, 9]. Lead contamination is a global problem, and many studies have shown greater levels of Pb in roadside sediments [10-14] with most of the Pb found in the small grain fraction (< 63  $\mu\text{m}$ ), which is more likely to be re-suspended or eroded into rivers and streams adjacent to these roads [11, 12].

Because unionids are such an imperiled fauna, it is critical to develop non-lethal biomarkers and sampling techniques when available. Hemolymph extraction has been shown to be a non-lethal sampling technique [15]. Therefore, in this study I evaluated several possible biomarkers for Pb exposure and effect utilizing mussel hemolymph. One of the classic biomarkers for Pb exposure in mammals, fish and some invertebrates is  $\delta$ -aminolevulinic acid dehydratase (ALAD) activity. ALAD is a critical enzyme in the pathway responsible for heme synthesis, the molecule which binds and transports oxygen in the blood of vertebrates and some invertebrates. ALAD converts aminolevulinic acid (ALA) to porphobilinogen (PBG). ALAD has been shown to be an excellent specific biomarker of Pb exposure, but not other metals, in many different species including humans [16], birds [17], frogs [18], fish [19-26] and the gastropod *Biomphalaria glabrata* [27]. ALAD enzyme activity is most always negatively correlated with the amount of Pb accumulated by the organism. One of the aims of this study was to assess mussel hemolymph and tissue for ALAD as a potential non-lethal biomarker for Pb exposure. The other non-lethal biomarker I assessed was ion ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ , and  $\text{Ca}^{2+}$ ) concentrations in mussel hemolymph. Even with

high renal ion absorption resulting in an excretory fluid concentration half that of hemolymph [28], freshwater mussels are subject to high ion loss from this excretion. As a result, they have developed extremely low hemolymph osmolalities of 45-60 mOsm [29] (36 mOsm for the zebra mussel [30]) to reduce loss and expended energy for active ion uptake [31]. Because Pb has been shown to cause disruption to protein and glucosamine in unionids [32], and other heavy metals such as Cd will cause osmotic imbalances in unionids [33], I hypothesized the disruption of ion transporting enzymes, hemolymph ion concentration and chemistry as a consequence of Pb exposure in Eastern elliptio.

The objectives of this study were to determine bioaccumulation of dissolved Pb by the freshwater mussel *Elliptio complanata*, assess several potential biomarkers for Pb exposure and effect, and compare the findings with results from mussels collected from stream locations.

## **Materials and Methods**

### ***Collection, Transport, and Holding of Mussels***

Laboratory methods followed the ASTM's guidelines for conducting laboratory toxicity tests with freshwater mussels [34] with modifications for mussel adult testing. For the 28 day lead exposure study, I collected 50 adult Eastern elliptio mussels from a relatively uncontaminated section of the Eno River, near Hillsborough, North Carolina. Mussels averaged 77.4 millimeters (mm) in length ranging from 67.5 to 87.9 mm, and 68.5 grams (g) in weight ranging from 40.4 to 97.9 g. Mussels were sampled more than 100 meters upstream from the highway crossing and placed in ice chests and covered with damp, cool

dive bags to prevent desiccation. The mussels were transported directly to the laboratory (30 minutes transport time) where 45 of the mussels were scrubbed with a soft-bristle brush, rinsed with deionized water, and placed into individual three liter glass jars. The jars contained two L of ASTM soft water [35] that was gently aerated by a central aeration unit (Sweet Water Air Pump SL24 Aquatic Eco-Systems, Inc., Apopka, FL, USA) as pictured (Figure 1). Standard methods [36] were used for all measurements of water-quality parameters. All test water was measured for pH with a Beckman Model  $\Phi$  240 (Beckman Instruments, Fullerton, CA, USA) calibrated meter. Alkalinity was determined by titration of 0.02 N H<sub>2</sub>SO<sub>4</sub> to pH 4.5 and hardness by titration with 0.01 M ethylenediaminetetra-acetic acid (EDTA).

### ***Experimental Procedures***

Alkalinity, hardness and pH were all tested before the test initiation, and for quality assurance of every water batch throughout the study. The remaining five mussels were weighed and measured, gently pried open, had ~ 1 mL of hemolymph extracted from the anterior adductor muscle, and were then bagged and stored frozen (-20°C) for Pb analysis. The hemolymph was divided into three cryotubes with two frozen at -80°C for ion and ALAD analysis and the third at -20°C for Pb analysis. These five mussels constitute the baseline concentrations. Four days later blood was collected from 11 fathead minnows to generate baseline levels for ALAD activity. Fathead minnows were selected as the fish species for a positive control in the ALAD analysis because they have been shown in the literature to provide consistent responses to Pb exposure. The size of fish was kept similar to

provide sufficient blood volume for ALAD analysis. Heparinized razors, glass pipettes and cryotubes were used during blood collection to minimize clotting.

The 45 test mussels were acclimated to test conditions for 72 hours prior to initiation. Six jars were also set up for fathead minnows, with five per jar, for an ALAD positive inhibition control test. Three jars with control fish and three jars with 125 µg/L lead exposed fish. Prior to the start of the test, the mussel in each jar was fed 20 mL of a suspension containing 2 mL of Instant Algae<sup>®</sup> Shellfish Diet and 1 mL *Nannochloropsis* concentrate (Reed Mariculture, Campbell, CA, USA) in one L of deionized water. Fish were fed at the same time as the mussels with TetraMin<sup>®</sup> tropical fish flakes. After two hours to allow for feeding, mussels were individually removed from their jar, gently pried open, and a 25 gauge syringe was used to withdraw 0.25 mL of hemolymph from the anterior adductor muscle of each and they were then immediately returned to their jars. Hemolymph was taken and pooled from the first four replicates of each concentration, including the control, to achieve 1 mL total volume. Hemolymph was then divided into aliquots of 0.25 mL for ion (Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>, Ca<sup>2+</sup>) analysis, 0.25 mL for ALAD analysis (both stored at -80°C) and 0.5 mL for Pb analysis stored at -20°C. The fifth and final replicate for each concentration was not sampled until the end of the experiment as a control for repeated weekly hemolymph sampling. Each jar was then siphoned and renewed (~ 90%) with fresh ASTM soft water. Jars were then spiked with Pb from a concentrated stock solution (1,000 mg/L) prepared from lead nitrate to generate a final Pb concentration in the jars of 0, 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125 and 500 µg/L.

Water and toxicant renewals were conducted three times per week. Before each renewal the mussel in each jar was fed and allowed to siphon for two hours. Sampling of hemolymph from the first four replicates of each treatment was conducted weekly until the end of the exposure on days 7, 14, 21 and 28. Water chemistry was measured with a calibrated multi-probe (YSI Model 556 MPS, Yellow Springs Instruments, Yellow Springs, OH, USA) at 48 and 72 hour time points for controls, 31.25 and 500 µg/L Pb spiked jars for quality assurance. Water samples were taken, 5 mL from three jars per concentration, at 0, 48 and 72 hour time points for Pb concentration verification, and stored preserved (75 µL of concentrated trace metal grade nitric acid) for analysis.

Physiochemical characteristics of water in the test jars averaged 21.0°C (range 20.9 – 21.29) for temperature, dissolved oxygen 8.3 mg/L (range 7.9 – 8.7), pH 8.0 (range 7.8 – 8.1), alkalinity 30 mg/L as CaCO<sub>3</sub> (range 28 – 32), and hardness 42 mg/L (range 40 – 44).

### ***ALAD Methods***

A traditional biomarker for lead exposure in mammals and fish, δ-aminolevulinic acid dehydratase (ALAD), is a critical enzyme in the pathway responsible for heme synthesis [19, 24]. The presence of lead inhibits ALAD from forming porphobilinogen (PBG) from two δ-aminolevulinic acid (ALA) molecules. Heme is responsible for binding to, and carrying oxygen molecules in the blood, and transporting them through the body.

It has been shown that ALAD is present in some gastropod tissues and hemolymph, therefore, I conducted a parallel exposure of *Elliptio complanata* and fathead minnows (as a positive control) to evaluate the presence of ALAD in freshwater mussel hemolymph as a potential biomarker of Pb exposure.

The ALAD method was modified from that of Schmitt, et al. [20] for mussel hemolymph and tissue using a microplate assay (Appendix 1). Mussel tissue was sonicated to minimize clotting. In detail, mussel hemolymph and fish blood samples were removed from the -80°C freezer and placed in a 4°C refrigerator to thaw, along with one cryotube of the PBG stock solution (221 µM PBG). Six centrifuge tubes were labeled for each sample: blank A, B and C, and ALA A, B and C. To each blank tube, 25 µL of assay buffer (0.2% Triton X-100 in 0.1M phosphate buffer (pH 6.2)) was added. To each ALA tube, 25 µL of ALA buffer (670 µg ALA·HCl/mL) was added. 75 µL of assay buffer were added to six of the seven PBG standard curve tubes, along with the controls. Blood samples were pipetted and weighed, and equal volume (10 µg = 10 µL) of deionized water was added. Blood dilutions and hemolymph samples were then both sonicated for 10 minutes. PBG serial dilution was prepared from the thawed stock solution, 150 µL was pipetted into the empty tube, and a 1:1 serial dilution of the seven tubes were prepared transferring 75 µL at a time and vortexing. Then 50 µL of each sonicated sample was added per tube for that sample, vortexed for five seconds, and incubated for one hour in a 37°C water bath. The modified Ehrlich's reagent was prepared by weighing out and mixing the appropriate amount of p-dimethylamino benzaldehyde to Ehrlich's reagent (e.g. 0.545 g to 30 mL Ehrlich's reagent) for the number of samples being run per batch. The Fusion™ Universal Microplate Analyzer (A153600 Meriden, CT) was allowed to warm up for at least one hour before analysis. After removing the samples from the water bath, the reaction was terminated by the addition of 200 µL stop solution (TCA/n-ethylmaleimide solution) to each tube. Samples were vortexed and centrifuged at 1,000 x g for 10 minutes. A 100 µL of supernatant of each sample was

pipetted into a 96-well plate, and 100  $\mu\text{L}$  modified Ehrlich's reagent was added to each well. The plate was placed on a plate shaker for 15 minutes of color development, and the absorbance was read on the plate reader at 540 nm.

### ***Analytical Procedures***

Mussels were prepared and analyzed at Research Triangle Institute (Research Triangle Park, NC) for Pb concentrations. Mussel tissues were 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 mass spectrometry (Thermo Element 2 Magnetic Sector ICP-MS). The average percent recovery of Pb from spiked mussel tissue samples was 101%, and ranged from 99 – 103%.

## **Results**

The test water was found to have an average hardness of 42 mg/L during the study with a range from 40 to 44, and an average alkalinity of 30 mg/L ranging from 28 to 32. The average measured Pb concentration in samples of test water ( $n = 3$ ) at time 0 and just after each renewal was 73% of the target concentration (**Table 1**). Mean measured exposure concentrations immediately following spiking were 2, 3, 5, 12, 19, 48, 101 and 396  $\mu\text{g/L}$ . After the 48 hour time intervals post renewal, the lower concentrations of 1.95 and 3.9  $\mu\text{g/L}$  were below detection limit, 7.8 to 62.5  $\mu\text{g/L}$  were 8% of target, and the higher concentrations of 125 and 500  $\mu\text{g/L}$  were about 25% of target. The concentrations at 72 hours post renewal

were similar to 48 hours with 7.8 µg/L or less being below detection limit, 15 to 62.5 µg/L at about 7% of target, and 125 and 500 µg/L at about 19% of target. This measured trend in depletion of toxicant concentration suggests that mussels were filtering Pb out of the water very rapidly, even towards the end of the 28 day study. The average daily exposure concentration was calculated as the weekly mean of three renewals, three 48 hour time points and one 72 hour time point. These values were found to be 1, 1, 3, 6, 11, 26, 66 and 251 µg/L Pb, and were used as the treatment groups.

The average Pb concentration in mussel tissue at the end of the study (Table 2) was strongly correlated to exposure concentration at lower environmental levels (Figure 2), and at the higher exposure concentration (Figure 3), with an  $R^2 = 0.98$  and  $0.98$  respectively.

Concentrations of Pb in hemolymph of the repeatedly sampled replicate 1 – 4 mussels, which were pooled per treatment group to obtain sufficient volume (Table 3), had several different trends over the 28 day study depending on their level of Pb exposure. These results are summarized for measured exposures of 0 – 66 µg/L (Figure 4) and for all ranges up to 251 µg/L (Figure 5). For Pb exposures of  $\leq 6$  µg/L, mussels showed slowly increasing concentrations of Pb in their hemolymph over time, never exceeding three times their exposure concentration. For exposures of 11 – 66 µg/L, concentrations plateau around day 14 with the 11 µg/L treatment group at 3.6 times its exposure concentration and the 26 and 66 µg/L exposures at 1.4 and 1.2 times exposure concentrations, respectively. However, for the greatest exposure concentration of 251 µg/L, hemolymph concentrations never plateau but appeared to bioconcentrate with rapid, linear accumulation, as shown by the best fit line with an  $R^2 = 0.98$ , to five times the exposure concentration. The replicate 5 mussels had

similar Pb hemolymph concentrations as their corresponding treatment group replicates 1 through 4 mussels on day 28, except for the greatest exposure which had a hemolymph concentration above 1700 µg/L. This was over 500 µg Pb/L above the concentration of the repeatedly sampled mussels in that treatment group.

Results from the ALAD activity inhibition assay (Figure 6) showed ALAD activity to be both present, and fully inhibited by the 66 µg/L Pb exposure from day 7 on, in the positive control fathead minnows. However, no ALAD activity was detected at any time point in the mussels, either in hemolymph or gill, mantle, foot and visceral tissue. Had there been any activity, I would have expected the mussel control to be similar to the fathead minnow control treatment.

There were no discernable correlations with Pb exposure and ion levels in the hemolymph of *Elliptio complanata* when evaluating the four replicates of repeatedly sampled mussels. However, Ca<sup>2+</sup> levels in hemolymph from the non-repeatedly sampled replicate 5 mussels (Figure 7) were found to be below the lower 95% confidence interval (CI) of 12.85 mg/dL, which was derived from our five baseline mussels, for low Pb exposures of 1 – 3 µg/L, and above the CI (16.23 mg/dL) for high Pb exposures of 11, 66 and 251 µg/L. Because the control treatment remained within the CI, this suggests a potential adverse affect by Pb. When comparing the 95% CI reference values for Ca<sup>2+</sup> levels in *Elliptio complanata* (13.1 – 23.7 mg/dL) generated by Gustafson, et al. [37], the first three Pb exposures in this test caused a decrease below this lower limit value, and the remaining treatments were still within range.

## Discussion

Because the levels of Pb in the exposure water declined so rapidly after each renewal over the study, increased in the hemolymph, and because concentrations of Pb in mussel tissue were so strongly correlated ( $R^2 = 0.98$ ;  $P < 0.05$ ) with exposure concentration, I conclude that freshwater mussels accumulate dissolved Pb extremely rapidly by ventilation. The fact that the middle exposures of Pb resulted in a plateau of Pb concentration in the hemolymph by day 14 while the greatest exposure resulted in rapid accumulation, suggests some type of metabolic regulation is occurring in the mussel. Because the levels of Pb in the test water were being depleted just as quickly by the end of the experiment as in the beginning, it is not likely that the mussels reduced uptake appreciably over time. This suggests that either the mussels started transporting the lead from hemolymph into tissue and/or shell, or they started eliminating it more efficiently in lysosomes through urine and pseudo-feces [38, 39].

Calcium levels in hemolymph of the non-repeatedly sampled replicate 5 mussels appeared to be adversely affected (changes in concentration could affect pH and result in reduced shell formation) by Pb exposure, however because this observation was based on a single mussel per concentration, this relation is uncertain without further assessment. While no trends were determined with the ion levels in hemolymph of the mussels repeatedly sampled, this may have been due to the damaging effects of repeated puncturing to the anterior adductor muscle during sampling than to the exposure of Pb. Even though repeated hemolymph sampling of three times over seven months is non-lethal [15], the sampling of five times over one month may have been causing additional stress, as well as possibly

allowing direct transport of ions into and out of the adductor muscle via the tracts left by the 25 gauge needle. By the end of the experiment, some of the adductor muscles had large holes in the side from tearing, as a result of weakening from multiple (five) punctures with little time for recovery. I conclude that the five sampling periods of the test mussels for hemolymph within 28 days was too aggressive, causing irreparable damage in some cases, and therefore any trends in ion levels with Pb concentrations could easily have been overshadowed. Another experimental design, allowing greater numbers of non-repeatedly sampled mussels to be analyzed for statistical verification, is required to determine if  $\text{Ca}^{2+}$  is adversely affected by Pb exposure. There is mechanistic plausibility for Ca-Pb interactions [40], but more data are needed to validate this relation.

Lastly, my results indicate that ALAD does not appear to be a suitable biomarker of Pb exposure in the freshwater mussel *Elliptio complanata*. Mollusks can contain either hemocyanin or hemoglobin for oxygen transportation within the hemolymph, or no respiratory proteins at all [41, 42] depending on the genera. And while the absence of iron hemoglobin in hemolymph does not necessarily negate its presence in the tissue of bivalves [42], I found no evidence of ALAD activity in *Elliptio complanata* gill, mantle, foot or visceral tissue. In this study, the mussels appeared to contain no iron-based hemoglobin in their hemolymph or tissues, thereby transporting oxygen either with the copper-based hemocyanin or dissolved directly in the fluid.

In the application to a biomonitoring assay,  $\text{Ca}^{2+}$  levels might be useful in determining the overall status of a mussel population, given enough individuals were sampled to reduce variability. However more work is needed in assessing  $\text{Ca}^{2+}$

concentrations in response to various stressors before such a monitoring assessment could be made.

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## Tables

**Table 1. Measured water Pb concentrations immediately following renewals (T0), 48 hours after renewal before next renewal (T48), and 72 hours after renewal before next renewal (T72), including percentages of the target concentration. The average daily exposure concentration over the 28 days is given as the weekly mean of three T0, three T48 and one T72.**

Target Pb Concentration (ug/L)	Mean Measured Concentration (ug/L)						Average Daily Exposure
	T0 (*n=3)	% of Target	T48 (*n=2)	% of Target	T72 (*n=3)	% of Target	
<b>Control</b>	0.0	n/a	0.0	n/a	0.0	n/a	0.0
<b>2</b>	1.8	90.2	0.0	0.0	0.0	0.0	0.9
<b>4</b>	2.6	65.5	0.0	0.0	0.0	0.0	1.3
<b>8</b>	5.2	65.2	1.7	20.7	0.0	0.0	3.2
<b>16</b>	12.2	76.2	0.5	3.2	1.0	6.4	6.4
<b>31</b>	19.2	61.9	1.2	4.0	2.8	9.0	10.5
<b>63</b>	48.4	76.8	3.2	5.2	4.0	6.3	25.9
<b>125</b>	101.0	80.8	33.4	26.7	27.8	22.3	66.3
<b>500</b>	395.9	79.2	121.1	24.2	75.2	15.0	250.8

\* Each 'n' represents 3 samples pooled.

**Table 2. The average tissue Pb concentration and standard deviations for the Baseline mussels and each exposure concentration at the end of the 28 day study.**

Treatment Concentration (ug/L)	Average Tissue Pb	
	Dry weight (ug/g)	Stnd. Dev.
Baseline (0)	0.147	0.036
Control (0)	0.288	0.086
1	1.461	0.348
1	2.997	0.882
3	5.461	1.104
6	9.675	2.627
11	25.903	7.771
26	62.735	8.713
66	117.800	25.506
251	857.829	231.585

**Table 3. The pooled hemolymph Pb concentration as µg/L. The stated " \* " Day 28 represents the individual hemolymph Pb concentration of the non-sampled replicate 5 mussels.**

Treatment Concentration (ug/L)	Day of Exposure					
	Day 0	Day 7	Day 14	Day 21	Day 28	*Day 28
Control (0)	<DL	<DL	<DL	0.448	<DL	<DL
1	<DL	1.18	1.03	1.78	1.40	0.921
1	0.553	11.0	0.584	0.706	1.22	5.37
3	<DL	0.723	2.61	3.55	8.13	4.86
6	<DL	2.02	5.57	8.12	19.6	34.9
11	0.632	4.04	35.1	46.3	37.6	50.1
26	<DL	25.1	34.2	14.4	58.8	17.3
66	<DL	43.8	75.4	66.9	86.3	61.9
251	4.46	419	740	1059	1243	1773

<DL = below detection limit

## Figures



Figure 1. Jar setup with aeration for 28 day Pb exposure study.

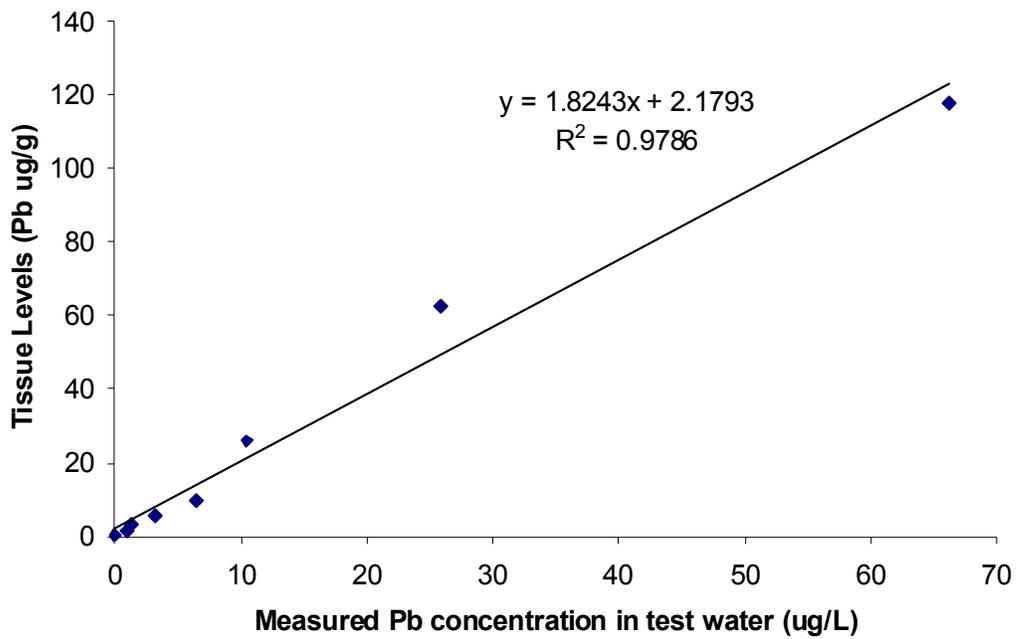


Figure 2. Correlation between Pb concentrations in mussels and their exposure concentrations, from the control to 66  $\mu\text{g/L}$ .

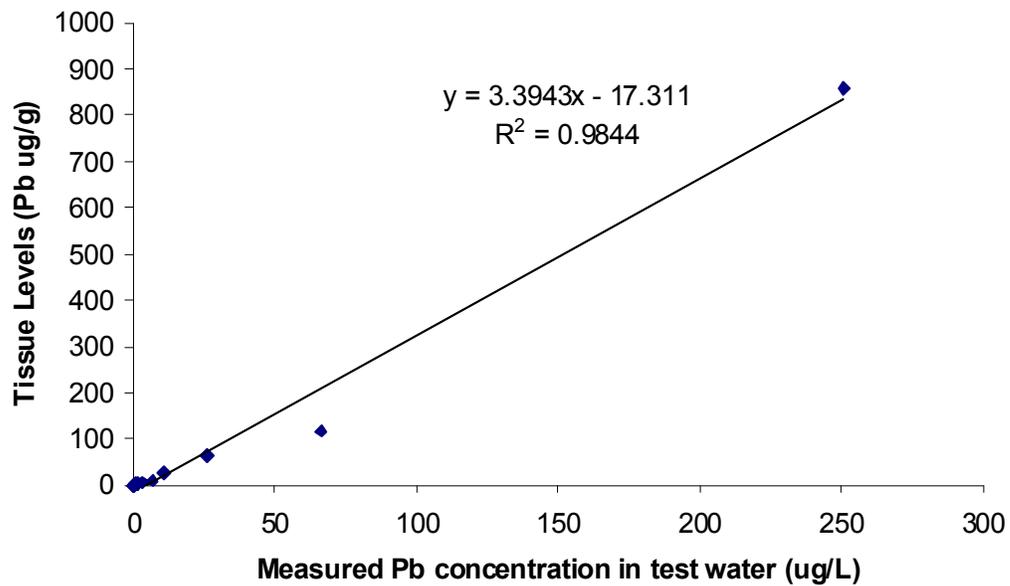


Figure 3. Correlation between Pb concentrations in mussels and their exposure concentrations, from the control to 251  $\mu\text{g/L}$ .

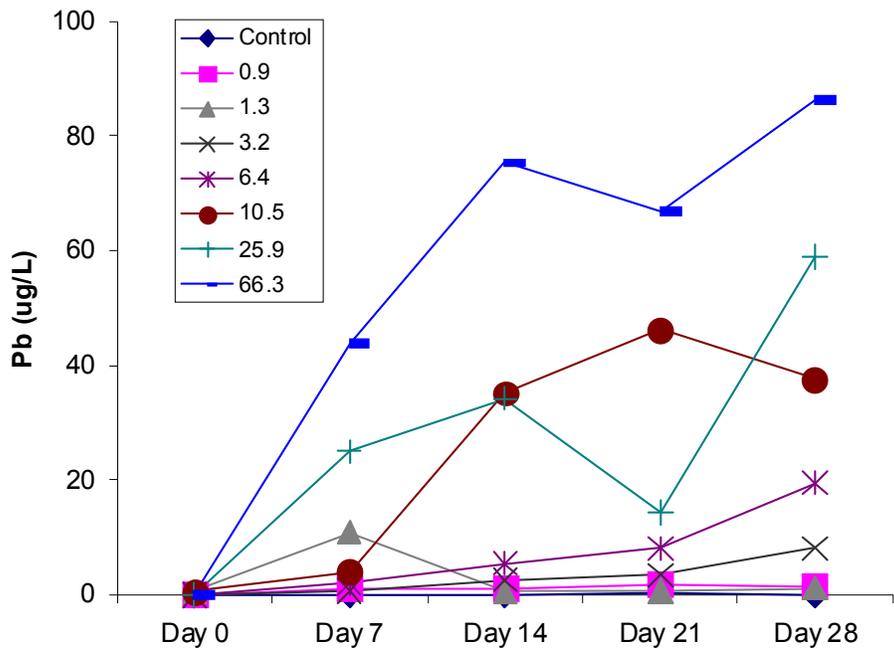


Figure 4. The hemolymph Pb concentrations over the 28 day study, as they relate to each treatment group excluding the 251  $\mu\text{g/L}$  treatment.

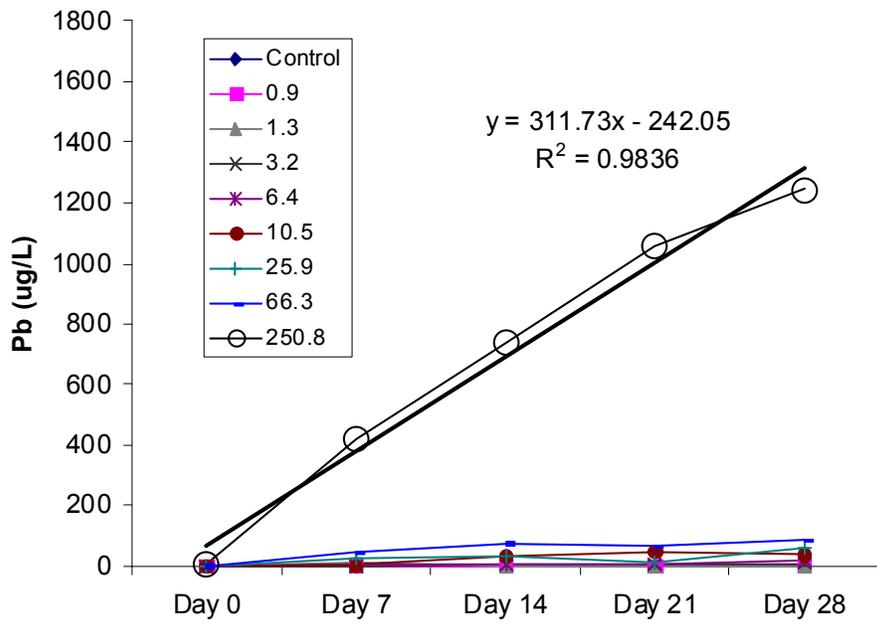


Figure 5. The hemolymph Pb concentrations over the 28 day study, as they relate to each treatment group including the 251  $\mu\text{g/L}$  treatment.

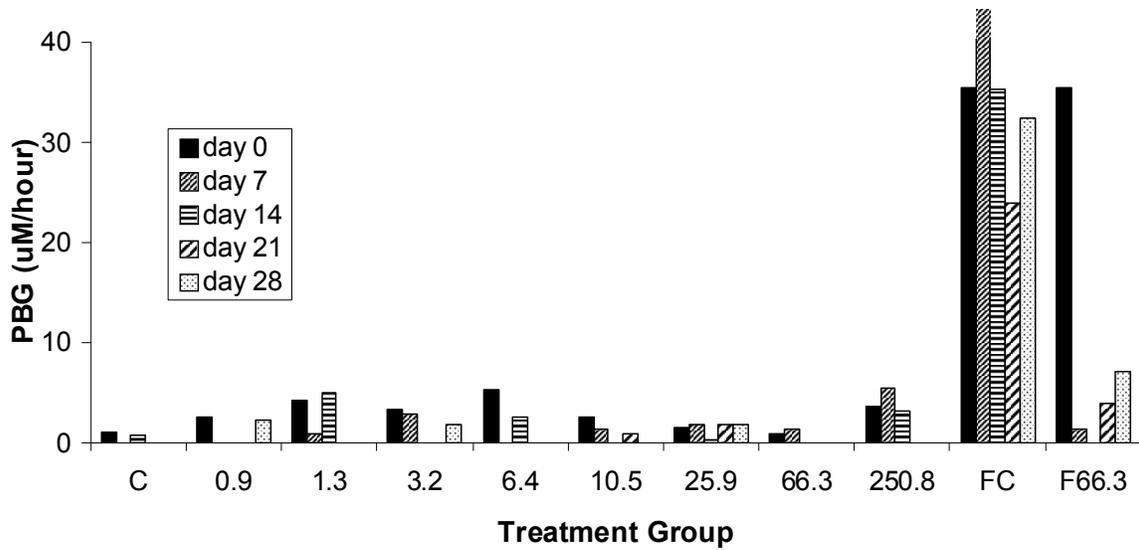


Figure 6. Results of the Pb ALAD activity inhibition assay for each mussel treatment group, including the fathead minnow control and 66 µg/L group, over the 28 day exposure.

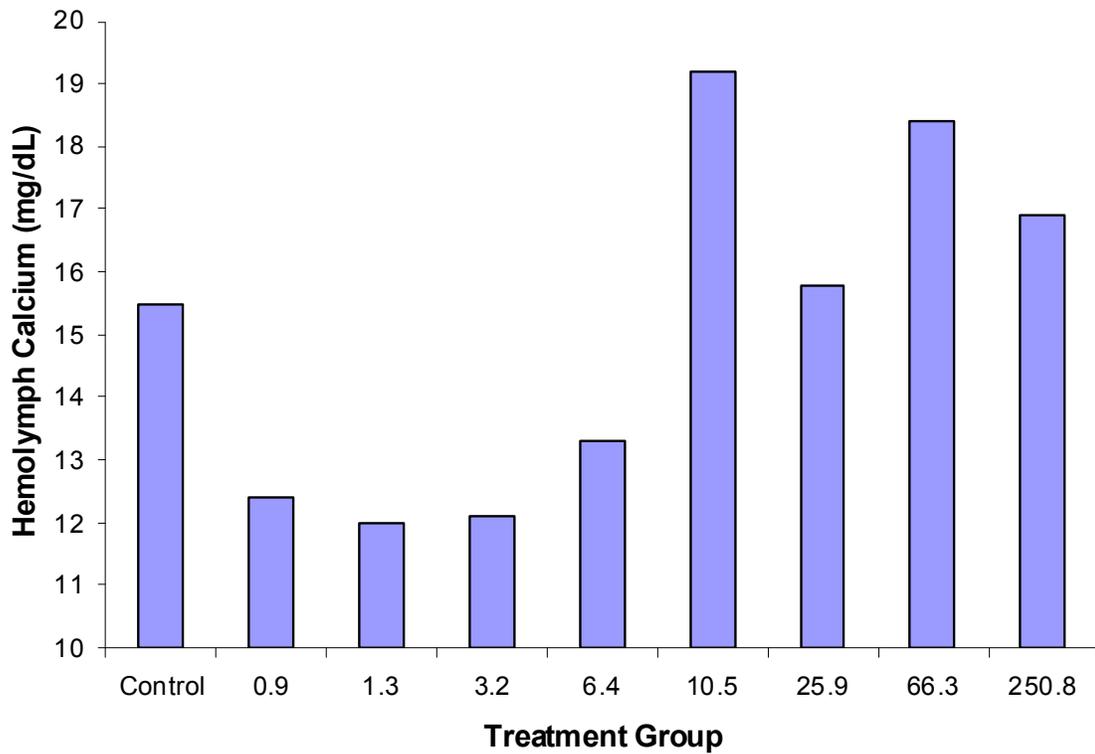


Figure 7. Calcium levels in hemolymph from the non-repeatedly sampled replicate 5 mussels, each treatment represents one mussel.

## APPENDIX 1

### **Microplate Assay for $\delta$ -Aminolevulinic Acid Dehydratase (ALA-D) in Frozen Blood Reagents (calculated for approximately 100 samples):**

#### 0.1 M Phosphate buffer:

Solution A – Dissolve 2.78 g sodium phosphate monobasic in 100 mL H<sub>2</sub>O.

Solution B – Dissolve 5.36 g sodium phosphate dibasic in 100 mL H<sub>2</sub>O.

Combine:     81.5 mL solution A,  
              18.5 mL solution B  
              70 mL H<sub>2</sub>O.

Adjust pH to 6.2 and bring to final volume of 200 mL with H<sub>2</sub>O. Store at 4°C.

#### Assay Buffer (0.2% Triton X-100 in 0.1M phosphate buffer (pH 6.2)):

Caution: Contact lenses should not be worn when handling triton X-100.

Dilute 100  $\mu$ L Triton X-100 to 50 mL with phosphate buffer. Store at room temperature.

#### ALA Buffer (670 $\mu$ g ALA·HCl/mL) :

Dissolve 10 mg ALA in 15 ml of Assay Buffer. Store at 4°C for a maximum of 2 days.

#### Stop Solution (TCA/n-Ethylmaleimide Solution):

Caution: Prepare in hood. n-Ethylmaleimide is poisonous. Dissolve 4.0 g trichloroacetic acid (TCA) and 2.7 g n-Ethylmaleimide in 100 mL H<sub>2</sub>O. Store in labeled dark glass container under the hood at room temperature.

#### Ehrlich's Reagent:

To prevent injury, goggles or face shield, gloves, and apron should be worn when handling perchloric acid. When diluting perchloric acid (or any other acid), always add ACID TO WATER, not the reverse. Prepare under the hood.

Combine:     3 mL distilled H<sub>2</sub>O  
              42 mL glacial acetic acid  
              10 mL 70% perchloric acid

\*1.0 g p-dimethylamino benzaldehyde (add right before assay)

Makes 55 mL reagent (without p-dimethylamino benzaldehyde). Store in a clearly labeled dark glass bottle. The day of the assay, pipette out the amount of reagent needed and add the appropriate amount of p-dimethylamino benzaldehyde (e.g. add 0.545 g to 30 mL Ehrlich's reagent).

Porphobilinogen Standard:

*Long Term Stock Solution:*

Prepare a 0.5 mg/mL PBG standard stock solution in Assay Buffer by adding 5 mg PBG to Assay Buffer. Check the concentration of the solution on the spectrophotometer using a molar extinction coefficient of  $6.2 \times 10^4$ . Aliquot 300  $\mu$ L of this standard into 33 cryotubes and store in foil-wrapped cryocase at  $-80^\circ\text{C}$ .

*Weekly Stock Solution:*

Dilute the *Long Term Stock Solution* for use in the standard curve as follows: Dissolve 50  $\mu$ L of the *Long Term Stock Solution* in 500  $\mu$ L Assay Buffer (yields 221  $\mu$ M PBG).

*Standard Curve Serial Dilution:*

Thaw one cryotube of the PBG *Weekly Stock Solution* (221  $\mu$ M PBG). Transfer 150  $\mu$ L to a 1 mL snap-cap centrifuge tube. Prepare a 1:1 dilution series of 7 tubes in Assay Buffer transferring 75  $\mu$ L volumes at a time. Proceed to step 7 in the Methods section.

Methods:

- Preparation: Thaw blood and Hemolymph samples, along with a PBG weekly stock solution tube, in the fridge at  $4^\circ\text{C}$  for at least 1 hour. Turn on the  $37^\circ\text{C}$  water bath. Prepare reagent amounts of ALA Buffer sufficient for the number of samples to be processed.

- Label six 1 mL snap-cap microcentrifuge tubes for each blood/hemolymph sample:

Sample #1	Sample #1
Blank	ALA
Rep A	Rep A

Samples are tested in triplicate, therefore label as Rep A, B, and C.

- To the blank replicate tubes, add 25  $\mu$ L Assay Buffer.  
To the ALA replicate tubes, add 25  $\mu$ L ALA Buffer.  
To the Control replicate tubes, add 75  $\mu$ L Assay Buffer.  
To the PBG Standard Curve Serial Dilution tube #1, add 75  $\mu$ L Assay Buffer.
- To avoid any interference of clotted blood in the assay, sonicate and dilute blood samples as follows: On a balance, pipette out approximately 150  $\mu$ L of blood into a 1.8 mL cryotube. Record the mass. Add an equal volume of ice cold  $\text{dH}_2\text{O}$  to the sample (e.g. if the blood samples has a mass of 148 mg, add 148  $\mu$ L of  $\text{dH}_2\text{O}$ ). Samples are then sonicated with hemolymph for 10 minutes to limit clotting.

Add 50  $\mu\text{L}$  of sonicated blood/hemolymph sample to each set of six tubes for that sample, and prepare PBG serial dilution with thawed weekly stock as described above. (See Reagents F. section).

- Vortex each tube for 5 seconds, then incubate in the water bath at  $37^{\circ}\text{C}$  for 1 hour.
- Prepare the modified Ehrlich's reagent by weighing out and mixing the appropriate amount of p-dimethylamino benzaldehyde to Ehrlich's reagent.
- Remove samples from the  $37^{\circ}\text{C}$  water bath. Terminate the reaction with the addition of 200  $\mu\text{L}$  of Stop Solution then centrifuge at 1000 x g for ten minutes.
- Turn on Plate Reader to allow light to warm up for one hour before reading.
- Transfer 100  $\mu\text{L}$  of the supernatant from the PBG standards and the samples to a 96-well plate in the order specified in the microplate assay layout sheet.
- Add 100  $\mu\text{L}$  Ehrlich's reagent to each well. Place on plate shaker for 15 min of color development. Read absorbance at 540 nm.

## CHAPTER 3

### **Evaluation of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity and hemolymph ion concentrations as biomarkers of Pb exposure in freshwater mussels**

#### **Abstract**

I conducted a 28 day laboratory test with Eastern elliptio mussels (*Elliptio complanata*) to determine uptake kinetics and assess several potential biomarkers of lead (Pb) exposure. Test mussels were sampled from a relatively uncontaminated reference site, and exposed to five concentrations of Pb (as lead nitrate) ranging from an average of 1 to 245 µg/L, as a static renewal test. There were nine mussels (replicates) per treatment concentration, allowing three mussels to be sampled at day 7, 14 and 28 for tissue Pb concentrations, hemolymph Pb compartmentalization/elimination analysis, and ion concentrations in hemolymph. Ion and Pb analyses were conducted on samples of hemolymph and compared to exposure concentration. A potential biomarker for Pb exposure, Na<sup>+</sup>,K<sup>+</sup>-ATPase activity, was assessed in gill tissue for each mussel. Mussels removed Pb from the water rapidly, with concentrations accumulating in tissues at an exposure-dependent rate for the first two weeks of exposure, with no significant increase from two to four weeks. Mussel tissue Pb concentrations ranged from 0.34 to 897.85 µg/g dry weight, were strongly correlated with Pb in test water at every time point and did not significantly increase after day 14. This finding supports *Elliptio complanata* as being a good sentinel for waterborne Pb contamination. Hemolymph Pb concentration was variable

and dependent on exposure concentration, with no changes over time after day 7, except for the greatest exposure concentration which showed significant reductions in Pb by four weeks. This suggests a threshold effect for hemolymph Pb concentrations around 1000 µg/L, where it is eliminated or bound more efficiently. Because Pb continued to be filtered out of the water throughout the experiment, while concentrations remained the same in tissue and actually reduced in hemolymph at the high exposure, I hypothesize that the mussels were actively eliminating the Pb from their bodies through lysosomes or granulocytes associated with pseudo-feces or depositing/storing it in the shell. The Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in the gill tissue of mussels was significantly reduced by Pb on day 7 and 28 of the exposure, with a high correlation to exposure concentration ( R<sup>2</sup> = 0.82; P = 0.0131, at day 28). Ion results correlated as expected with Na<sup>+</sup>,K<sup>+</sup>-ATPase activity, with reduced hemolymph Na<sup>+</sup> at the greatest exposure, while enzyme activity was at 30% of controls. Hemolymph Ca<sup>2+</sup> was significantly increased at the greatest Pb exposure and could be due to re-mobilization from the shell in an attempt to buffer the hemolymph against Pb uptake and toxicity. I conclude that Na<sup>+</sup>,K<sup>+</sup>-ATPase activity has potential as a biomarker of Pb exposure in mussels. However it was variable at the lower test concentrations and additional research is warranted over this range of concentrations.

### **Introduction**

Freshwater mussels are considered to be good sentinels for Pb contamination in aquatic ecosystems [1-4]. The freshwater mussel *Elliptio complanata* in particular meets many of the prerequisites for an ideal biomonitor of stable trace metals [5] in that they are

sessile, relatively abundant, and large enough to provide sufficient tissue mass for analysis of the contaminant of interest. Moreover, they have been shown to tolerate a wide range of Pb contamination [6-9], allowing for laboratory studies, and are strong accumulators of Pb [9-11] with an established correlation between metal accumulation and the ambient bioavailable concentration [11]. Because unionids are such an imperiled fauna, it is critical to develop non-lethal biomarkers and sampling techniques when available. Hemolymph extraction has been shown to be a non-lethal sampling technique [12]. Therefore, in this study I evaluated several possible biomarkers for Pb exposure and effect utilizing mussel hemolymph.

The two non-lethal biomarkers I assessed were direct Pb concentrations, as well as ion ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ , and  $\text{Ca}^{2+}$ ) concentrations in mussel hemolymph. Even with high renal ion absorption resulting in an excretory fluid concentration half that of hemolymph [13], freshwater mussels are subject to high ion loss from this excretion. As a result, they have developed extremely low hemolymph osmolalities of 45 – 60 mOsm [14] (36 mOsm for the zebra mussel [15]) to reduce loss and expended energy for active ion uptake [16]. Lead is known to cause imbalances in  $\text{Na}^+$  and  $\text{Cl}^-$  levels in rainbow trout [17] attributed to reduced  $\text{Na}^+$ , $\text{K}^+$ -ATPase activity, as well as glucose and ion disruption in rainbow trout and whitefish [18, 19], ion disruption in crabs [20] and  $\text{Na}^+$  imbalances in snails [21]. Because Pb has been shown to cause disruption to protein and glucosamine in unionids [22], and other heavy metals such as Cd will cause osmotic imbalances in unionids [23], I hypothesized the disruption of ion transporting enzymes, hemolymph ion concentration and chemistry as a consequence of Pb exposure in Eastern elliptio.

$\text{Na}^+, \text{K}^+$ -ATPase, or the sodium pump, is responsible for the co-transport of sodium ions out of, and potassium ions into the cell membrane in most eukaryotes, and helps in ionic regulation [24]. The activity of  $\text{Na}^+, \text{K}^+$ -ATPase has been shown to be significantly reduced [17, 25-27] and correlated with Pb tissue concentration by noncompetitive inhibition. While no  $\text{Na}^+, \text{K}^+$ -ATPase activity was detected in the mantle tissue of *Anodonta cataracta* [28], activity has been detected in *Anodonta cygnea* [29] and found in mantle and gills in freshwater and marine bivalves of the genera *Carunculina* [14, 30], *Lampsilis*, *Corbicula* and *Rangia* [14] and *Tapes* [31], albeit all had relatively low levels. We assessed gill tissue for  $\text{Na}^+, \text{K}^+$ -ATPase activity as a biomarker for Pb exposure in Eastern elliptio.

The objectives of this study were to determine bioaccumulation of dissolved Pb by the freshwater mussel *Elliptio complanata* and assess several potential biomarkers for Pb exposure and effect.

## **Materials and Methods**

### ***Collection, Transport, and Holding of Mussels***

Laboratory methods followed the ASTM's guidelines for conducting laboratory toxicity tests with freshwater mussels [32] with modifications for testing adult mussels. For the 28 day study, I collected 64 Eastern elliptio mussels from a relatively uncontaminated section of the Eno River near Hillsborough, North Carolina. Mussels were, on average, 77.7 millimeters (mm) in length ranging from 65.2 to 97.8 mm, and 55.2 grams (g) in wet weight ranging from 30.6 to 91.8 g. Mussels were sampled more than 100 meters upstream from the highway crossing and placed in ice chests and covered with damp, cool dive bags to prevent

desiccation. Mussels were transported directly to the laboratory (30 minutes transportation time) where 58 mussels were scrubbed with a soft-bristle brush, rinsed with deionized water and placed into individual three L glass jars. The jars contained two L of ASTM soft water [33] that was gently aerated by a central aeration unit (Sweet Water Air Pump SL24 Aquatic Eco-Systems, Inc., Apopka, FL, USA). Standard methods [34] were used for all measurements of water-quality parameters. All test water was measured for pH with a Beckman Model  $\Phi$  240 (Beckman Instruments, Fullerton, CA, USA) calibrated meter. Alkalinity was determined by titration of 0.02 N H<sub>2</sub>SO<sub>4</sub> to pH 4.5 and hardness by titration with 0.01 M ethylenediaminetetra-acetic acid (EDTA).

### ***Experimental Procedures***

Alkalinity, hardness and pH were all tested before the test initiation and for quality assurance of every water batch throughout the study. The six remaining mussels were weighed and measured, gently pried open, had ~ 1 mL of hemolymph extracted from the anterior adductor muscle, had 3 gill samples dissected per mussel (~ 15 mg each), and were then bagged and stored frozen (-20°C) for Pb analysis. The hemolymph was divided equally into two cryotubes for analysis of Pb (-20°C), and ion (Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>, Ca<sup>2+</sup>) concentrations (-80°C). The gill tissue was stored frozen at -80°C in SEI buffer solution. These six mussels constituted the baseline concentrations.

The 58 test mussels were acclimated to test conditions for five days with a complete renewal of fresh ASTM soft water on the third day of acclimation. Prior to the start of the test, the mussel in each jar was fed 20 mL of a suspension containing 2 mL of Instant Algae<sup>®</sup> Shellfish Diet and 1 mL *Nannochloropsis* concentrate (Reed Mariculture, Campbell, CA,

USA) in one L of DI water. During the test, mussels were fed three times a week. On day 0 of the test, three mussels were selected as day 0 controls and were removed from their jars. They were gently pried open, and a 25 gauge syringe was used to withdraw 1 mL of hemolymph from the anterior adductor muscle of each, which was then divided into two 1.2 mL cryotubes for ion ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ ) and Pb analysis. These mussels were then dissected and three samples of gill tissue (~ 15 mg each) were removed per mussel for  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity assessment, and each placed in 1 mL centrifuge tubes, on ice, with 100  $\mu\text{L}$  of SEI buffer solution. Ion hemolymph samples along with gill samples were then frozen at  $-80^\circ\text{C}$ . Mussels were bagged and frozen along with Pb hemolymph samples at  $-20^\circ\text{C}$ . The remaining jars were renewed with fresh ASTM soft water and labeled with initial target concentration and replicate: 9 mussels per concentration (control, 1.95, 7.8, 31.25, 125 and 500  $\mu\text{g}$  Pb/L). On day 7, 14 and 28, three mussels from each concentration were sampled for hemolymph and gill tissue, and then bagged as described above.

Water and toxicant renewals were conducted three times per week. Before each renewal the mussel in each jar was fed and allowed to siphon for two hours. Water chemistry was measured with a calibrated multi-probe (YSI Model 556 MPS, Yellow Springs Instruments, Yellow Springs, OH, USA) at 48 and 72 hour time points for controls, 31.25 and 500  $\mu\text{g}$ /L Pb spiked jars for quality assurance. Water samples were taken, five mL from three jars per concentration, at 0, 48 and 72 hour time points for Pb concentration verification, and stored preserved (75  $\mu\text{L}$  of concentrated trace metal grade nitric acid) for analysis.

Physiochemical characteristics of water in the test jars averaged 20.2°C (range 18.9 – 21.0) for temperature, 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<sub>3</sub> (range 28 – 32), and hardness 42 mg/L (range 40 – 44).

#### ***Na<sup>+</sup>,K<sup>+</sup>-ATPase Activity Assay***

The Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was determined using the method of McCormick [35], modified for use with mussel tissue (Appendix 1). Ouabain is sometimes used to inhibit Na<sup>+</sup>,K<sup>+</sup>-ATPase activity by binding to the alpha-subunit of the enzyme after it has released 3Na<sup>+</sup> and before it binds 2K<sup>+</sup>, thus stopping the cycle [24]. However ouabain proved insufficient in inhibiting Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in *Elliptio complanata* gill tissue. For the most part, there were no differences between the non-ouabain and ouabain inhibited activities of total ATPase. It is known that there are various isoforms of the alpha subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase that are species specific and have varying degrees of sensitivity to ouabain inhibition [36, 37]. It has also been shown that certain regions of the alpha subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase, when mutated, will reduce ouabain sensitivity [38]. Therefore, I used a K<sup>+</sup>-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<sup>+</sup>,K<sup>+</sup>-ATPase activity was not present in measurable amounts in the mussel *Anodonta cataracta*, with both salt depletion and ouabain showing negative results [28]. This method has also been used to detect Na<sup>+</sup>,K<sup>+</sup>-ATPase activity in the gills of goldfish which are insensitive to ouabain, requiring an extremely high concentration (10mM) for inhibition [39]. Chasiotis and Kelly [39] found that inhibition with a K<sup>+</sup>-free salt solution was just as effective as using the high concentration of ouabain, and less expensive. Because

the other ATPase which would be effected 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 [40], the majority of inhibition seen 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 I found high activity, about 50% of total ATPase in *Elliptio complanata* gill tissue, I propose that *Elliptio complanata* contain  $Na^+,K^+$ -ATPase with an alpha-isoform highly resistant to inhibition by ouabain. For our experiment then, we used the assay with  $K^+$ -free salt solution in place of ouabain to determine the mussels'  $Na^+,K^+$ -ATPase activity.

In detail (for 50 samples, amounts are doubled if more is required), a refrigerated centrifuge (Allegra™ 25R Centrifuge, Beckman Coulter, Fullerton, CA, USA) was set to 4°C, and aliquots of phosphoenolpyruvate (PEP) and adenosine diphosphate (ADP) were removed from the -80°C freezer and placed on the counter to thaw for about 30 minutes. 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 imidazole buffer. Lactate dehydrogenase (LDH) (12.2 µL) and pyruvate kinase (PK) (23.2 µL) were added in the same tube and centrifuged at 12,000 x g for eight minutes at 4°C. The supernatant was removed, re-suspended with imidazole 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 imidazole 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. For the B solution which measures ATPase activity minus  $\text{Na}^+, \text{K}^+$ -ATPase activity, 15 mL of AM was mixed with 5 mL of the  $\text{K}^+$ -free salt solution prepared previously. 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  $\mu\text{L}$  of 4 mM ADP and 200, 175, 150 and 100  $\mu\text{L}$  imidazole buffer, respectively, were added for 0, 5, 10 and 20 nMoles ADP/10  $\mu\text{L}$  concentrations. A Fusion<sup>TM</sup> Universal Microplate Analyzer (A153600 Meriden, CT) was allowed to warm for 30 minutes prior to analysis. Mussel gill samples were thawed immediately prior to running the assay. Sample tubes were homogenized with 25  $\mu\text{L}$  0.3% SEID (3X concentrate; 0.0751 g in 25 mL SEI (sucrose 250 mM, disodium ethylenediaminetetraacetic acid dihydrate ( $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ ) 10 mM, imidazole 50 mM)) for 20 – 30 seconds using 1.5 mL centrifuge tube pellet pestles. Activity decreases after homogenization, so all samples were read within 0.5 hours of processing. The homogenates were centrifuged at 5,000 x g for one minute at 4°C, and 10  $\mu\text{L}$  is pipetted into each of four wells per sample. The ADP standard curve was run once at the beginning of each batch of samples, 10  $\mu\text{L}$  per 3 wells per concentration was pipetted into the plate, and 200  $\mu\text{L}$  of ice cold A w/salt was added to each well. The ADP standard curve rapidly decreases in the first 2 – 3 minutes, but then stabilizes, and is read in mOD/nmole ADP. Then 200  $\mu\text{L}$  of ice cold B w/salt ( $-\text{K}^+$ ) was added to two of the four wells for each sample, and 200  $\mu\text{L}$  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 minutes at 1 minute intervals. ATPase activity was measured as

mOD/10  $\mu$ l /minute. The Bradford Protein Assay [41] of Kit (IBI/Shelton Scientific; VWR #14221-496) was used to determine protein concentration, and the final  $\text{Na}^+,\text{K}^+$ -ATPase activity as  $\mu$ moles ADP/mg protein/hour.

### ***Analytical Procedures***

Mussels were prepared and analyzed at Research Triangle Institute (Research Triangle Park, NC) for Pb concentrations. Mussel tissues were 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 mass spectrometry (Thermo Element 2 Magnetic Sector ICP-MS). The average percent recovery of Pb from samples of mussel tissue was 109.3%, and ranged from 99.6 – 119%. Recovery of Pb in samples (n = 17) of test water averaged 100.7% and ranged from 95 – 103%. Hemolymph Pb recovery averaged 96% and ranged from 84 – 101%.

### ***Statistical Analysis***

Results for  $\text{Na}^+,\text{K}^+$ -ATPase activity were analyzed following a generalized linear model with concentration and time considered as fixed-effect factors, and mussels and subsamples within each mussel considered as random factors. Analyses were performed with Proc MIXED procedure in SAS v9.1.3 (SAS Institute, Cary, NC). Data were tested by residual plot and log transformed (natural log: base e) prior to analysis when necessary to achieve homogeneity of variances. Estimated least squares and their 95% confidence intervals were back transformed for presentation purposes.

Results for the Pb and ion data were analyzed following a generalized linear model with concentration and time considered as fixed-effect factors and mussel samples considered as random factors. Analyses were performed with Proc MIXED procedure in SAS v9.1.3. Data were tested by residual plot and log transformed (natural log: base e) prior to analysis when necessary to achieve homogeneity of variances. Statistical significance level was determined at  $\alpha = 0.05$  for all tests, unless otherwise stated.

## Results

Test water was found to have an average hardness of 42 mg/L throughout the study ranging from 40 to 44, with an average alkalinity of 30 mg/L ranging from 28 to 32. Concentrations of Pb in the test water averaged 100.6% of target concentrations after renewals over the 28 day study (Table 1), ranging from 91.5% for the highest concentration to 108.2% for the lowest. Thus the treatments had average measured concentrations of 2, 8, 31, 121 and 458  $\mu\text{g/L}$  immediately following renewals. By 48 and 72 hours, the concentration of Pb in the test water was significantly reduced to 7% and 6% of the target concentration respectively over the study. For the first half of the study, concentrations averaged 5.1% of target at 48 hours and 4.1% of target at 72 hours. This proportion increased slightly for the second half of the study where concentrations averaged 8.7% of target at 48 hours and 7.3% of target at 72 hours. The average daily exposure concentrations over the 28 day study was calculated as the weekly average, with three T0, three T48 and one T72, to be 1, 4, 14, 57 and 245  $\mu\text{g Pb/L}$ , and were the values used as the treatment groups.

Average Pb concentrations in mussel tissue for each treatment group over the 28 day study ranged from 0.33 to 897.85  $\mu\text{g/g}$  (Table 2), and was strongly correlated ( $R^2 = 0.952 - 0.999$ ;  $P < 0.001$ ) with exposure concentration (Figure 1), showing a linear increase up to day 14. Using the differences of Least Squares Means (LSM), an increase was observed with treatment group and time on day 7 and day 14 (Figure 2). By day 14, all treatment groups were significantly different from each other. There was an increase in tissue Pb concentration from day 14 to day 28, but there was no statistically significant change in Pb concentration after day 14.

Average hemolymph Pb concentrations for each treatment group over the 28 day study ranged from below detection in the controls, to 821.83  $\mu\text{g/L}$  in the 245  $\mu\text{g/L}$  treatment concentration (Table 3). When analyzed by LSM, hemolymph was found to be more variable than the tissue Pb concentration, and averages for concentrations of Pb in hemolymph from the 1 to 57  $\mu\text{g/L}$  treatments all increased over the 28 day study, though not significantly (Figure 3). The hemolymph in mussels from the 245  $\mu\text{g/L}$  Pb treatment however, while increasing in concentration by day 7, decreased thereafter with a final day 28 concentration significantly less than on day 7. This hemolymph Pb concentration on day 28 was also statistically similar to levels for the 57  $\mu\text{g/L}$  Pb treatment group (Figure 3).

Ion analysis of the hemolymph revealed that while calcium ( $\text{Ca}^{2+}$ ) levels were reduced below baseline values for the controls on day 0 along with controls and lower treatment groups from day 7 on (Figure 4),  $\text{Ca}^{2+}$  levels increased significantly in the hemolymph from mussels at the highest treatment (245  $\mu\text{g/L}$ ). Chloride ( $\text{Cl}^-$ ) levels were reduced significantly with time in all treatment groups, including the controls, compared to

the baseline (Figure 5) mussel samples. Hemolymph  $\text{Cl}^-$  was also reduced in the controls with each time point, with day 28 controls significantly less than day 0 controls. On day 28,  $\text{Cl}^-$  was positively correlated with increasing Pb exposure, with the 245 treatment group significantly greater than the control at the  $\alpha = 0.10$  level. Sodium ( $\text{Na}^+$ ) levels were similar to baseline levels on day 0, however were significantly reduced in all controls for day 7, 14 and 28 (Figure 6). By day 28, the 245  $\mu\text{g/L}$  treatment significantly reduced  $\text{Na}^+$  below the controls at that time point, while the 1  $\mu\text{g/L}$  treatment on day 7 and the 57  $\mu\text{g/L}$  treatment on day 14 were significantly greater than their respective time point controls. Potassium ( $\text{K}^+$ ) levels were reduced significantly with time to below the baseline level at the  $\alpha = 0.05$  level for all treatments (Figure 7), except the 1  $\mu\text{g/L}$  treatment group on day 7 which was significantly lower at the  $\alpha = 0.10$  level.

The average  $\text{Na}^+, \text{K}^+$ -ATPase activity for each treatment group at each time point ranged from 0.95 to 3.46  $\mu\text{Moles ADP/mg protein/hour}$  (Table 4). Activity of the baseline mussels and the day 0 control mussels were analyzed by LSM and shown not to be significantly different ( $P = 0.76$ ). Activity of each treatment group over the 28 day study (Figure 8) showed no significant change in activity of the controls with time compared to the baseline and day 0 control measurements. Activity decreased with increasing Pb exposure on day 7 and 28. On day 7, the 245  $\mu\text{g/L}$  treatment group was significantly less than the day 7 control group as well as the baseline and day 0 controls, and on day 28, the 57  $\mu\text{g/L}$  treatment group was significantly less than the day 28 control ( $P = 0.033$ ), baseline ( $P = 0.033$ ) and day 0 control ( $P = 0.046$ ). In addition, the 245  $\mu\text{g/L}$  treatment group on day 28 was significantly ( $P < 0.05$ ) less than all other treatment groups at that time point as well as

significantly less ( $P < 0.0001$ ) than the day 28 control, day 0 control and baseline. The 245  $\mu\text{g/L}$  treatment group significantly reduced  $\text{Na}^+, \text{K}^+$ -ATPase activity by 70% from the control on day 28 (Table 5). On day 14 there was reduced activity in the lower Pb concentrations and a significant increase (though only at the  $\alpha = 0.1$  level) in activity over the control for the 245  $\mu\text{g/L}$  treatment group. When the average  $\text{Na}^+, \text{K}^+$ -ATPase activity was compared to exposure concentration, a strong correlation ( $R^2 = 0.82$ ;  $P = 0.013$ ) was observed (Figure 9). This correlation holds ( $R^2 = 0.82$ ;  $P = 0.013$ ) when the average measured tissue Pb concentration was used (Figure 10). Correlations between individual mussels'  $\text{Na}^+, \text{K}^+$ -ATPase activity (Figure 11) increased over time for tissue Pb, and decreased for hemolymph Pb. The correlation of activity and tissue concentration was observed on day 7 as well (Figure 12) with an  $R^2 = 0.69$  and  $P = 0.04$ .

## Discussion

Mussels accumulated quantities of Pb by day 7 and day 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*, which found no further accumulation after two weeks [6]. The fact that concentrations on day 14 were similar to concentrations of the next higher treatment group on day 7 shows that the rate of Pb accumulation was dependent on exposure concentration, as shown in exposures with the zebra mussel (*Dreissena polymorpha*) [42]. 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

*Elliptio complanata*, as other freshwater mussels have been suggested [1-4], is a good sentinel of recent Pb contamination.

Lead concentrations in hemolymph were more variable than those in tissue; however, significant increases ( $\alpha = 0.05$ ) were observed with increasing exposure concentrations for most treatment groups, except for the 245  $\mu\text{g/L}$  exposure, which showed a significant reduction in Pb by the end of the study. Because Pb levels in the test water continued to be depleted through the study, levels in hemolymph did not significantly increase with time, and levels in tissue did not significantly increase after two weeks, I conclude the mussels were actively eliminating the Pb from their bodies. Storage of Pb in the shell [43-45] was likely taking place (although un-measured in this study), but the majority of the elimination was most likely in lysosomes or granulocytes associated with pseudo-feces [6, 46]. The fact that the greatest exposure concentration resulted in a rapid increase in hemolymph Pb, followed by subsequent reductions, suggests a lag time between initial exposure and increased lysosomal production and size [47] allowing the mussels to better manage the transport and elimination of Pb.

Calcium concentrations were reduced from the baseline and remained low throughout the experiment in controls and the treatment groups from 1 to 57  $\mu\text{g Pb/L}$ . At the greatest exposure of 245  $\mu\text{g Pb/L}$ , however,  $\text{Ca}^{2+}$  was significantly increased above the controls with an  $\alpha = 0.05$ , and significantly above the baseline with an  $\alpha = 0.1$  on day 7 and 14 and 0.05 by day 28. The overall reduction of  $\text{Ca}^{2+}$  is most likely attributed to the mussels adjusting from the stream water with a hardness of 25 mg/L to the ASTM soft test water with an average hardness of 42 mg/L. The baseline hemolymph average of 195 mg  $\text{Ca}^{2+}/\text{L}$  for this

experiment was similar to the median of 175 mg Ca<sup>2+</sup>/L from other observations with *Elliptio complanata* [12] and well within the 95% confidence interval (CI) of 131 to 237 mg Ca<sup>2+</sup>/L as derived by Gustafson, et al. [48]. Acclimation of the mussels to ASTM soft water brought the Ca<sup>2+</sup> averages down to the low end of this CI, with a few (day 7: control and 1, day 14: 14, day 28: control, 1, 4 and 57) below the CI. And while no observable changes were seen after day 0 at exposure concentrations of 57 µg Pb/L or less, 245 µg Pb/L resulted in an average Ca<sup>2+</sup> concentration on day 28 significantly greater ( $P < 0.05$ ) than all other treatments, and the upper CI [48]. This increase was observed despite the overall decreases from changes in environment. Thus, I conclude that high concentrations of Pb significantly increased hemolymph Ca<sup>2+</sup> concentrations. The high concentration of Pb required to obtain this effect, however, makes Ca<sup>2+</sup> levels alone unsuitable as a biomarker of Pb exposure.

Chloride concentrations were reduced significantly from the baseline by day 0, and in all treatment groups throughout the exposure. The baseline average was nearly half that from Gustafson et al. (638 mg/L) [12]. The controls lost Cl<sup>-</sup> with each time point with the day 28 control significantly less than the day 0 control, indicating continuous loss as a result of the transition to soft ASTM water. And while on day 28 there seems to be a trend with increased Cl<sup>-</sup> and Pb exposure, differences were only significantly higher from the control at the greatest exposure, and only at the 0.10  $\alpha$  level.

Sodium concentrations were not changed from the baseline by day 0, however were significantly reduced in controls on days 7, 14 and 28. The baseline average was very similar to the average (368 mg/L) found by Gustafson et al. [12]. By day 28, the high exposure caused a significant decrease in Na<sup>+</sup> hemolymph levels from the control, suggesting adverse

effects by Pb, but again this effect was seen at concentrations too high for practical use as a biomarker.

Potassium concentrations were reduced significantly from the baseline in all treatment groups over the exposure. The baseline average was about 1.8 times higher than Gustafson's (21 mg/L) [12], but was reduced to similar concentrations throughout the rest of the exposure. This again suggests acclimation by the mussels to the ASTM soft water. No trends were observed with  $K^+$  levels and Pb exposure.

From the ion results, I conclude that effects were observed at too high a concentration of Pb exposure (if seen at all) for any useful application as biomarkers to environmentally relevant concentrations. However the reduced  $Na^+$  in hemolymph at the greatest concentration by day 28 was expected because  $Na^+,K^+$ -ATPase activity was shown to be reduced to 30% of the controls by that time point. Moreover, because  $3Na^+$  ions are exchanged for  $2K^+$  ions, we would expect to see changes in ion concentration from  $Na^+$  before it is observed in  $K^+$ . Increased  $Ca^{2+}$  in hemolymph at the greatest Pb exposure could be a result of the mussels attempting to protect their tissues from Pb uptake and toxicity. The presence of  $Ca^{2+}$  would reduce Pb uptake and toxicity by competing for the same uptake sites [49]. Therefore, if mussels were to re-mobilize  $Ca^{2+}$  from their shell [50-52], they could incorporate Pb while generating a  $Ca^{2+}$  buffer, thereby reducing Pb uptake and toxicity to the tissues.

By day 28 of the experiment,  $Na^+,K^+$ -ATPase activity was significantly reduced in gill tissue with increasing concentrations of Pb in the exposure water. As early as day 7 though, the highest exposure was significantly ( $P < 0.01$ ) lower than the baseline and day 0

control as well as significantly ( $P = 0.045$ ) below the day 7 control. On day 28 the greatest exposure reduced activity significantly from that of all other treatments ( $P < 0.03$ ) at that time point, as well as the baseline and controls ( $P < 0.0001$ ). About 82% of the reduction in  $\text{Na}^+, \text{K}^+$ -ATPase activity was explained by the concentration of Pb in the tissue. The mussels at the lowest exposure concentration of 1  $\mu\text{g/L}$  of Pb had an average  $\text{Na}^+, \text{K}^+$ -ATPase activity 18% lower compared to the controls (Table 5), and because  $\text{Na}^+, \text{K}^+$ -ATPase is responsible for maintaining  $\text{Na}^+, \text{K}^+$  transmembrane gradients, it has been suggested that inhibition may lead to gill tissue damage [53]. In addition, disruption of  $\text{Na}^+$  levels in the cell would affect  $\text{Na}^+/\text{H}^+$  exchange altering pH levels, and thereby affecting shell formation and dissolution [40] along with  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  levels. And as Pb will compete for  $\text{Ca}^{2+}$  uptake sites [17], its presence will directly affect ion concentrations and shell formation and dissolution as well.

The use of  $\text{Na}^+, \text{K}^+$ -ATPase as a biomarker of Pb exposure has potential for assessing the health of mussels, however the variation in activity among individual *Elliptio complanata* indicates that additional research is needed. Also, because gill dissection is not a non-lethal sampling technique, it is not a tool suitable for threatened and endangered species. I conclude that current measured environmental concentrations of Pb from this study in North Carolina may be sufficient to cause reductions of  $\text{Na}^+, \text{K}^+$ -ATPase activity in *Elliptio complanata*. And if more mussels are used in generating the range for a particular site's activity level, it is possible that variability could be reduced enough for successful implementation in the field. More research is needed to determine if  $\text{Na}^+, \text{K}^+$ -ATPase activity can be used as a realistic biomarker for Pb exposure.

Overall, ion concentrations in hemolymph of the freshwater mussel *Elliptio complanata* do not appear to be useful as biomarkers for Pb exposure. Activity of the enzyme Na<sup>+</sup>,K<sup>+</sup>-ATPase has potential as a biomarker for Pb exposure. Also, because eastern *Elliptio* were found to reach an equilibrium with exposure levels and tissue concentration relatively quickly (about two weeks), this suggests useful application of these mussels for biomonitoring assays, given they are collected from a relatively clean reference site. Such methods could be used to indicate the bioavailable amount of Pb at a contaminated site.

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## Tables

**Table 1. Measured water Pb concentrations immediately following renewals (T0), 48 hours after renewal (T48), and 72 hours after renewal (T72), including percentages of the target concentration. The average daily exposure concentration over the 28 days is given as the weekly mean of three T0, three T48 and one T72.**

Target Pb Concentration (ug/L)	Mean Measured Concentration (ug/L)						Average Daily Exposure
	T0 (*n=10)	% of Target	T48 (*n=5)	% of Target	T72 (*n=3)	% of Target	
<b>Control (0)</b>	0.0	n/a	0.0	n/a	0.0	n/a	0.0
<b>2</b>	2.1	105.5	0.1	4.4	0.1	5.2	1.0
<b>8</b>	8.3	104.2	0.3	3.4	0.3	3.6	3.7
<b>31</b>	31.1	100.3	1.0	3.2	1.3	4.2	13.9
<b>125</b>	121.0	96.8	9.3	7.5	6.0	4.8	56.7
<b>500</b>	457.5	91.5	88.9	17.8	73.1	14.6	244.6

\* Each 'n' represents 3 pooled samples.

**Table 2. The results of tissue Pb analysis as the average of the three mussels for each treatment group at every timepoint.**

Treatment Group (ug/g)	Day 7		Day 14		Day 28	
	Average	SD	Average	SD	Average	SD
Control (0)	0.34	0.20	0.37	0.03	0.36	0.04
1	0.47	0.28	1.32	0.44	2.27	0.91
4	1.93	0.41	3.39	1.53	6.94	3.28
14	6.23	4.67	18.61	3.96	40.95	19.53
57	42.34	18.19	89.66	4.05	181.33	59.28
245	93.46	25.98	728.31	343.38	897.85	144.55
	<b>Average</b>	<b>SD</b>				
Baseline	0.30	0.18				
d0 Control	0.24	0.08				

**Table 3. The results for hemolymph Pb concentrations as the average of the three mussels for each treatment group and timepoint, and the standard deviation.**

Treatment Group (ug/L)	Day 7		Day 14		Day 28	
	Average	SD	Average	SD	Average	SD
Control (0)	0.13	0.00	0.17	0.07	0.24	0.28
1	0.33	0.26	0.95	0.85	1.31	0.79
4	0.79	0.06	1.28	0.81	5.38	3.91
14	6.33	3.78	17.38	12.31	19.54	21.40
57	77.73	36.54	171.63	60.04	224.30	112.61
245	821.83	214.78	434.13	374.73	56.16	14.49
	<b>Average</b>	<b>SD</b>				
Baseline	0.71	0.80				
0 Control	0.52	0.68				

**Table 4. The average  $\text{Na}^+, \text{K}^+$ -ATPase activity for each treatment group at every timepoint, as well as the Baseline and day 0 Control averages. Activity is expressed as  $\mu\text{moles of ADP/mg protein/hour}$ .**

Treatment Group (ug/g)	Day of Exposure			Baseline
	7	14	28	
Control (0)	2.54	2.16	3.13	3.00
1	3.25	1.73	2.56	<b>0 Control</b> 3.13
4	2.55	1.39	2.50	
14	2.25	1.97	2.31	
57	2.02	2.97	1.77	
245	1.44	3.46	0.95	

**Table 5. The  $\text{Na}^+, \text{K}^+$ -ATPase activity for each treatment group as a percentage of the Control for day 28 of the study.**

Inhibition of $\text{Na}^+, \text{K}^+$ -ATPase Activity: Day 28		
Exposure (ug/L)	Activity(% of C)	% Reduced
1	81.7	18.3
4	79.9	20.1
14	73.7	26.3
57	56.5	43.5
245	30.4	69.6

## Figures

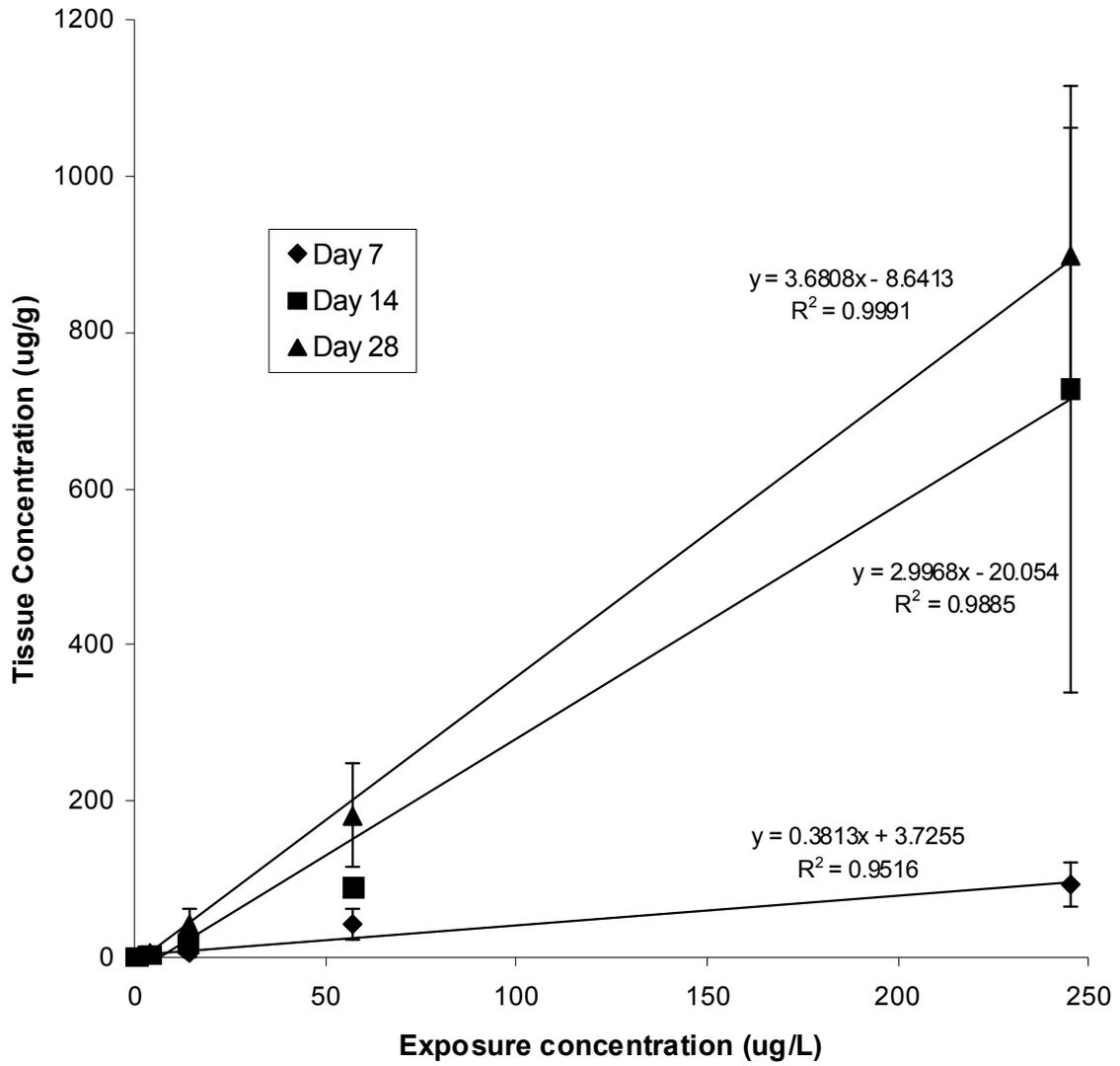


Figure 1. Correlations between mussel tissue Pb concentration and exposure concentration on days 7, 14 and 28. Error bars represent 95% confidence intervals.

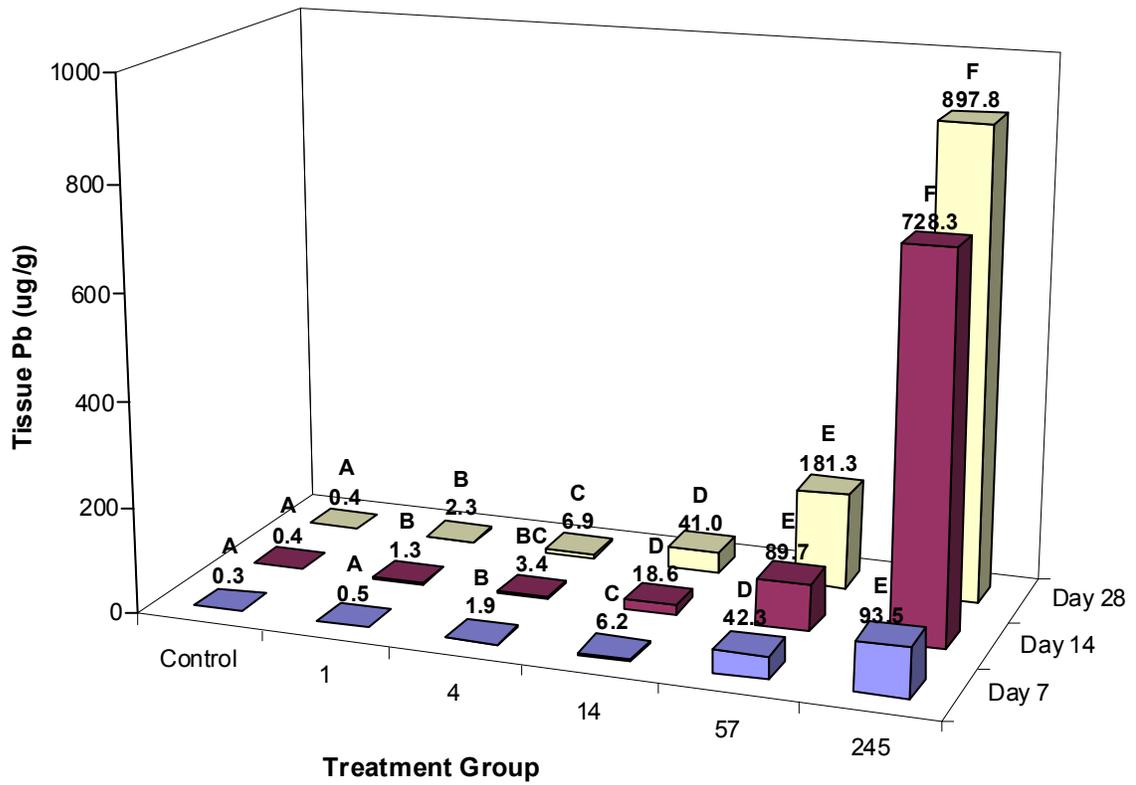


Figure 2. The average tissue Pb concentration for each treatment group, over the 28 day study. Values with the same letters are not significantly different.

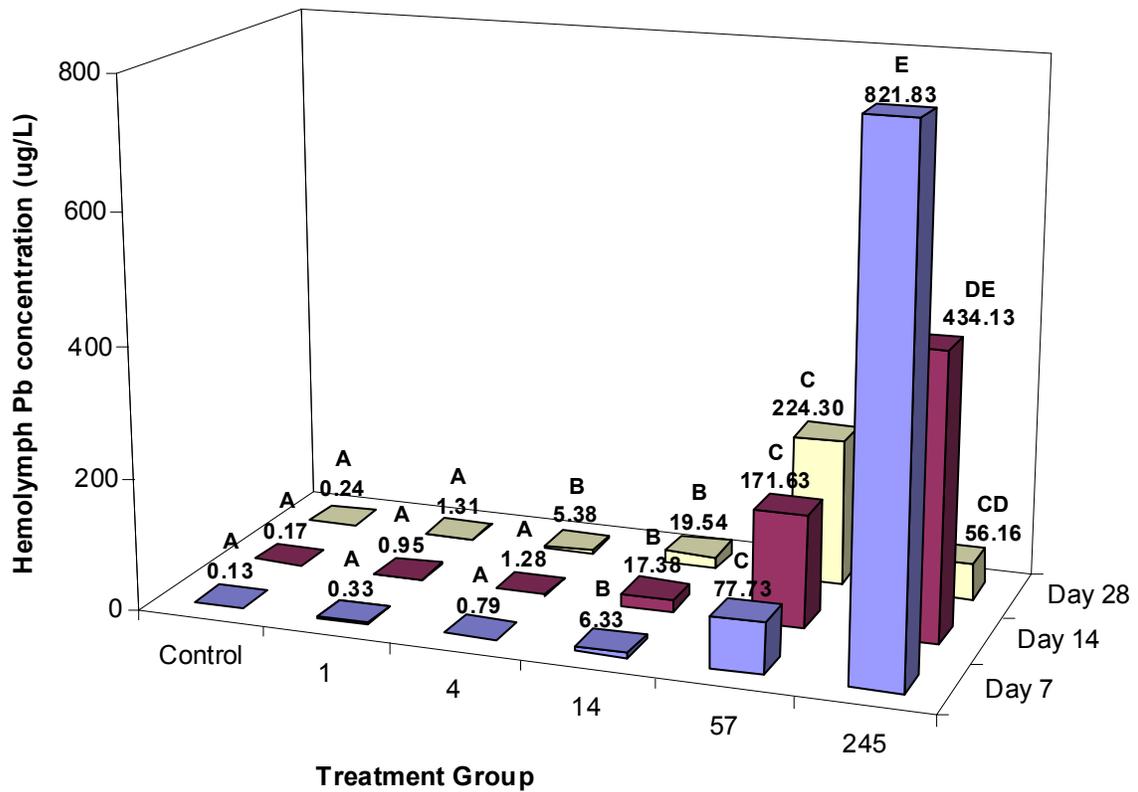


Figure 3. The average hemolymph Pb concentration, for each treatment group, over the 28 day study. Values with the same letters are not significantly different.

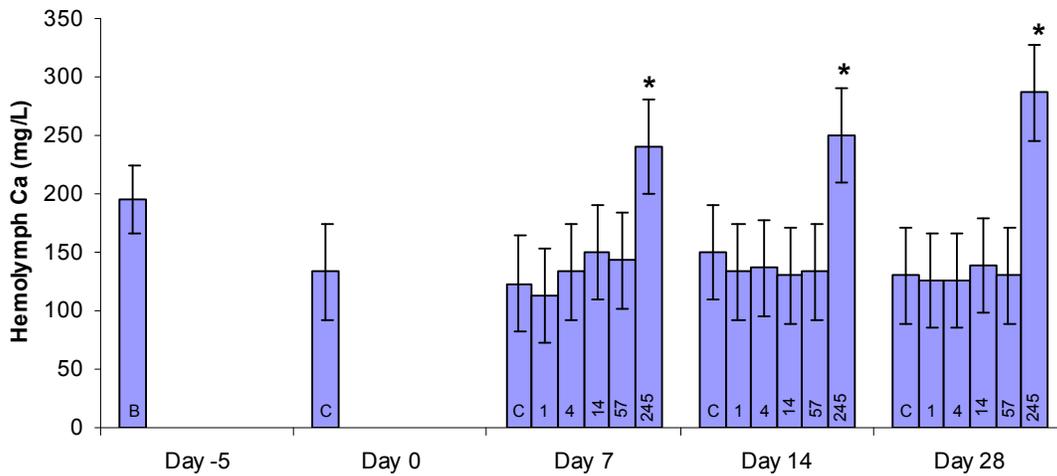
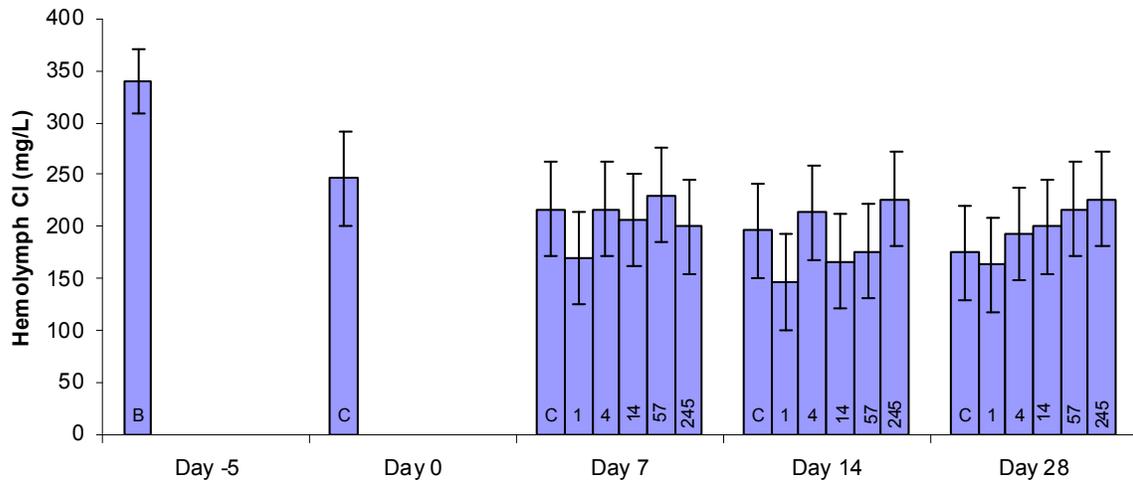
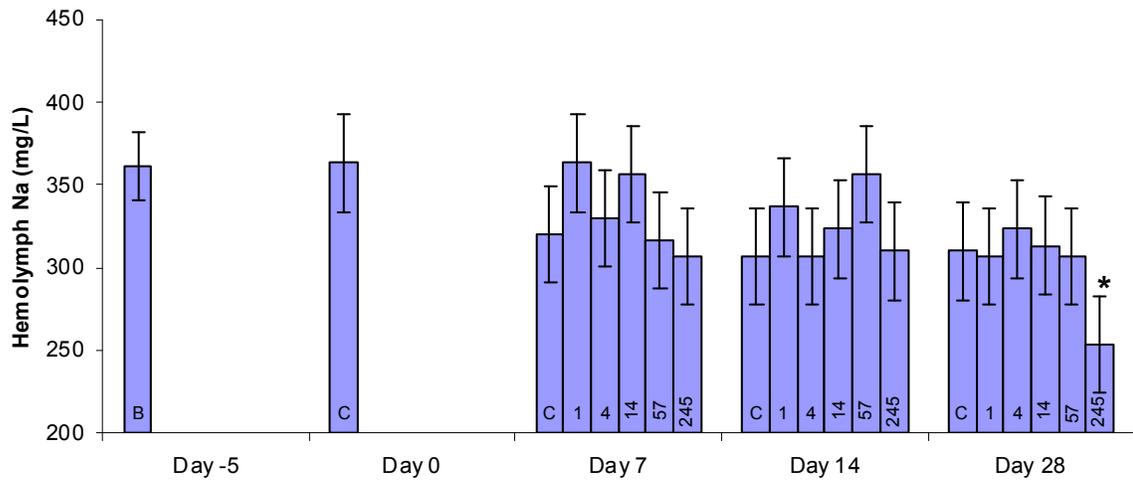


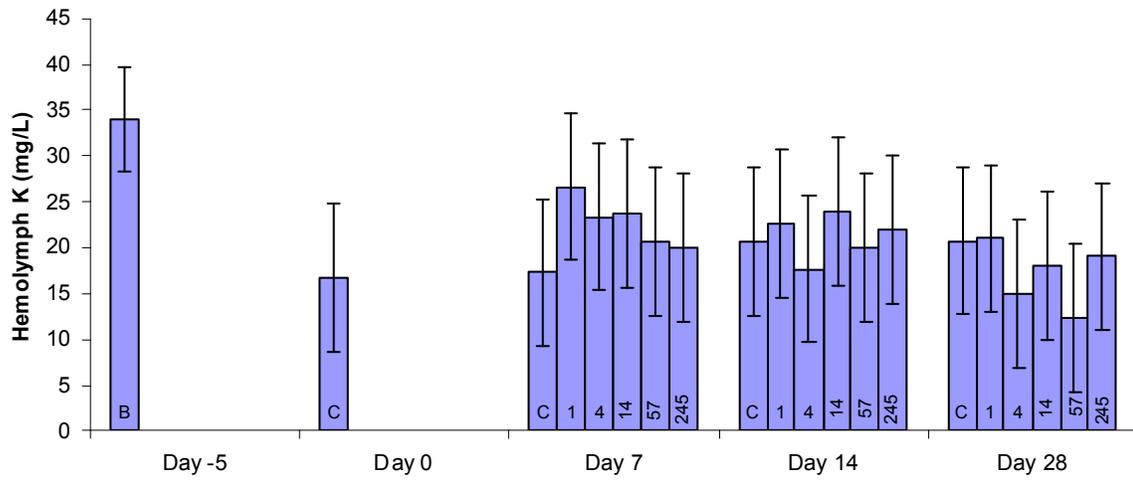
Figure 4. Hemolymph Calcium levels as mg/L during the second 28 day exposure for each treatment group and time point. All Controls were significantly lower than the Baseline. The asterisk '\*' signifies values significantly greater than the Baseline. Error bars represent 95% confidence intervals.



**Figure 5. Hemolymph Chloride levels as mg/L during the second 28 day exposure for each treatment group and time point. All treatment groups were significantly less than the Baseline. Controls decreased with each time point, with the day 28 Control significantly lower than the day 0 Control. Error bars represent 95% confidence intervals.**



**Figure 6. Hemolymph Sodium levels as mg/L during the second 28 day exposure for each treatment group and time point. The asterisk '\*' signifies values significantly lower than that time point's Control. Controls on day 7,14 and 28 are significantly lower than the Baseline and day 0 Control. Error bars represent 95% confidence intervals.**



**Figure 7. Hemolymph Potassium levels as mg/L during the second 28 day exposure for each treatment group and time point. All treatment groups were significantly lower than the Baseline with an alpha = 0.05 level, except day 7: 2 which is significant at the alpha = 0.1 level. Error bars represent 95% confidence intervals.**

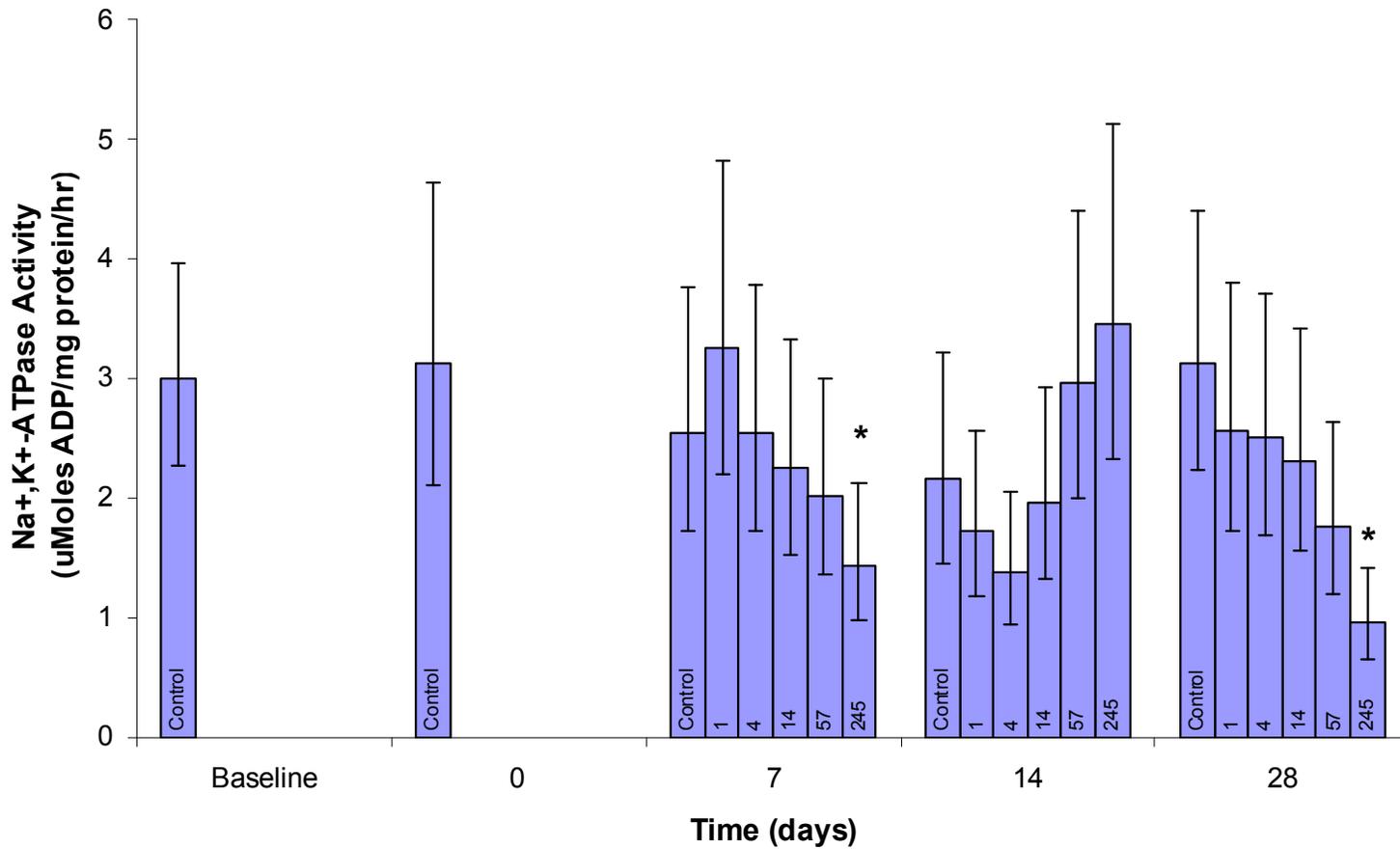


Figure 8. The average Na+,K+-ATPase activity for each treatment group over the 28 day study. Bars represent 95% confidence intervals. The " \* " indicates values which are significantly different from the Control of that time point, as well as the baseline and day 0 values.

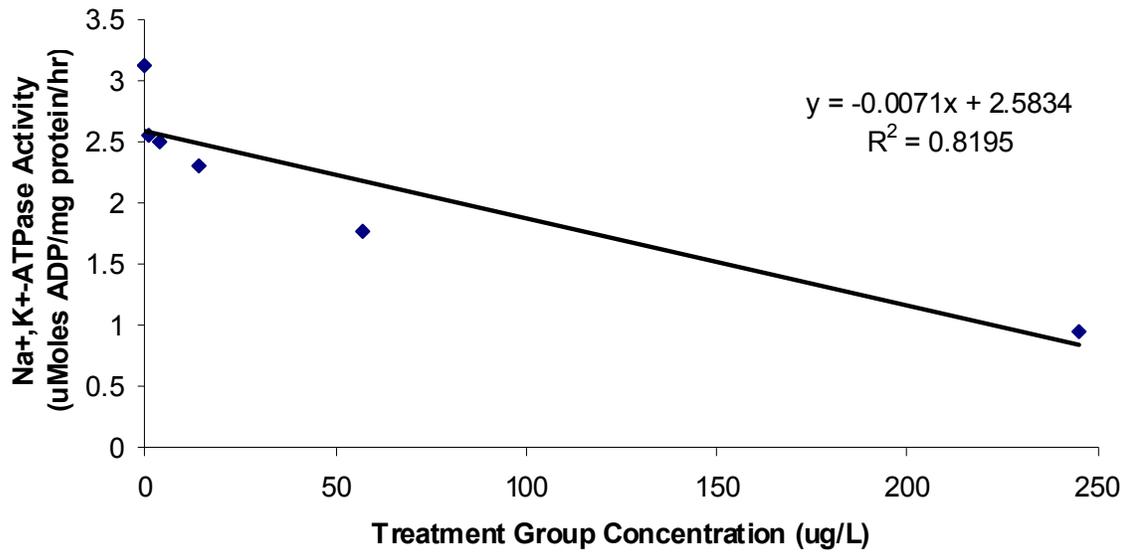


Figure 9. Na+,K+-ATPase activity correlations with treatment groups as it relates on day 28 of the study.

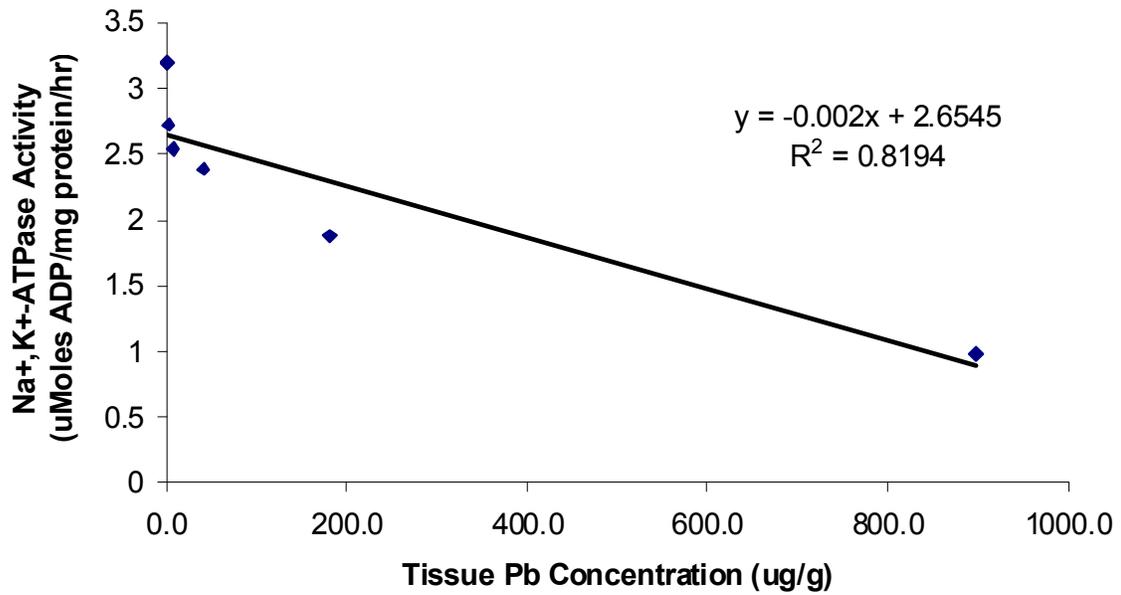


Figure 10. The correlation of average Na+,K+-ATPase activity and average tissue Pb concentration, per treatment group, on day 28.

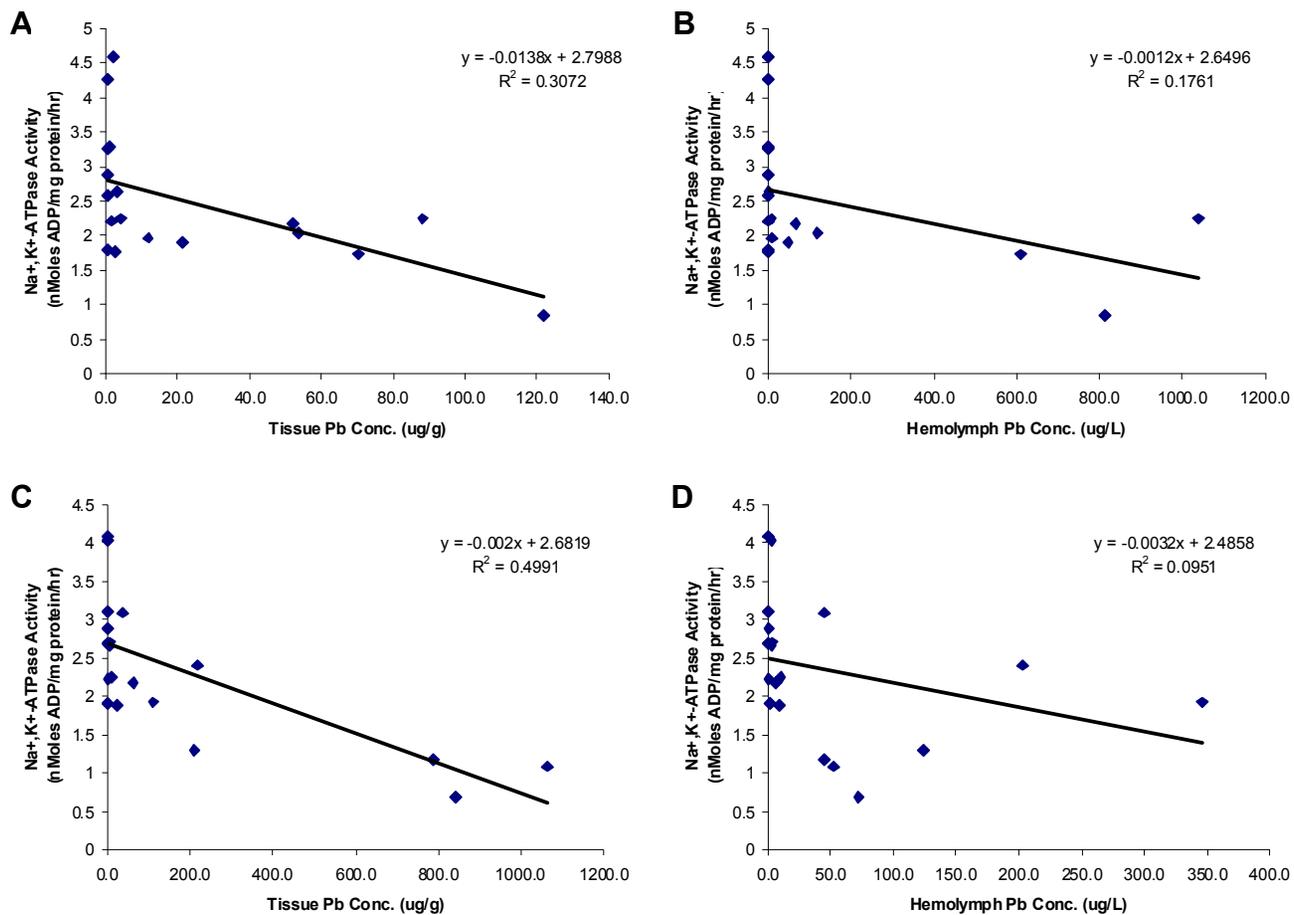


Figure 11. Na<sup>+</sup>,K<sup>+</sup>-ATPase activity correlations: A) with tissue Pb concentration on day 7, B) with hemolymph Pb concentration on day 7, C) with tissue Pb concentration on day 28, D) with hemolymph Pb concentration on day 28.

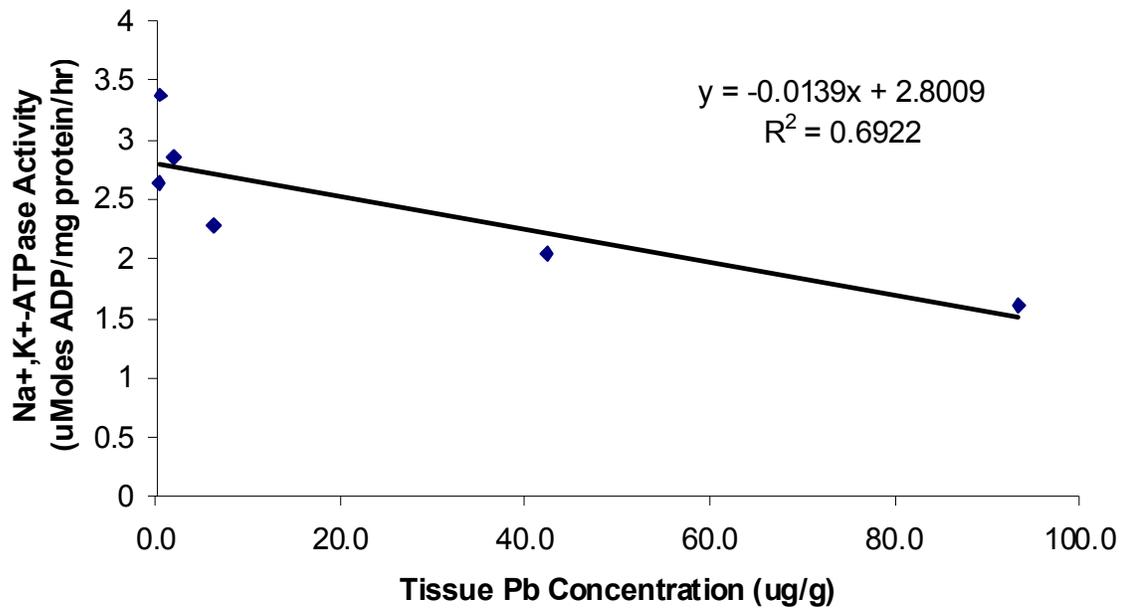


Figure 12. The correlation of average Na+,K+-ATPase activity and average tissue Pb concentration, per treatment group, for day 7.

**APPENDIX 1:**  
**Na<sup>+</sup>, K<sup>+</sup>-ATPase Assay – Microplate Assay**  
**I. Buffer preparation and reaction mixtures.**

**Salt Solution**

Imidazole (50 mM)	1.702g
NaCl (189 mM)	5.52g
MgCl <sub>2</sub> * 6H <sub>2</sub> O (10.5 mM)	1.02g
KCl (42 mM)	3.14g

Add 450 ml DiH<sub>2</sub>O, adjust to pH 7.5 with HCl, qs to 500 ml. Store for **3 months** at 4°C.

**1X SEI Buffer**

Sucrose (250 mM)	42.79g
Na <sub>2</sub> EDTA (10 mM) 2H <sub>2</sub> O	1.86g
Imidazole (50 mM)	1.70 g

Add 450 ml DiH<sub>2</sub>O, adjust to pH 7.3 with HCl, qs to 500 ml. Store for **1 month** at 4°C.

**0.3% SEID (3X concentrate)**

0.1g Na deoxycholic acid in 33.3 ml SEI, (0.0751g in 25 ml). Store for **1 week** at 25°C.

**Assay Mixture (AM) Reagents**

<u>Sigma #, \$</u>	<u>Buffer, Abbreviation, Molecular Weight</u>	<u>Stock Conc.</u>	<u>Final Conc.</u>	<u>Conc. in AM</u>
I-2399 \$29.40	<b>Imidazole Buffer (IB; MW = 68.08)</b> 1.702 g Imidazole in 475 ml DiH <sub>2</sub> O, adjust to pH 7.5 with HCl, qs to 500 ml, store <b>3 months</b> at 4°C	50 mM	50 mM	50 mM
P-7252 \$90.90	<b>Phosphoenolpyruvate (PEP; MW = 465.6)</b> 0.978 g in 100 ml IB, divide into 10 or 20 ml aliquots, store for <b>6 months</b> at -80°C	21 mM	2 mM	2.8 mM
N-6005 \$73.90	<b>NADH- reduced (MW = 709.4)</b> add to assay mixture, make <b>fresh</b> with each batch of AM	--	0.16 mM	0.22 mM

A-3377 \$53.30	<b>Adenosin Triphosphate (ATP; MW = 551.1)</b> add to assay mixture, make <b>fresh</b> with each batch of AM	--	0.5 mM	0.7 mM
L-2500 \$66.30	<b>Lactic Dehydrogenase (LDH)</b> Spin for 8 minutes at 12,000g at 4°C; a distinct pellet should result, remove supernatant, suspend pellet in several mls IB; add to assay mixture, make <b>fresh</b> with each batch of AM	--	3.3 U/ml	4.6 U/ml
P-1506 \$62.30	<b>Pyruvate Kinase (PK)</b> Spin for 8 minutes at 12,000g at 4°C; a distinct pellet should result, remove supernatant, suspend pellet in several mls IB; add to assay mixture, make <b>fresh</b> with each batch of AM (centrifuge LDH and PK together)	--	3.6 U/ml	5.1 U/ml
O-3125 \$103.50	<b>Ouabain</b> 0.382 g in 50 ml IB, mix in hot water bath to dissolve Store for <b>3 months</b> at 25°C	10.5 mM	0.5 mM	0.7 mM

The final concentration calculations include the salt solution and homogenate volumes (210ul)

## II. Assay Standards

### ADP Standards (MW = 427.2)

4mM Stock Solution:

0.0427g in 25 ml Na Acetate (57 mM)

(0.4627g Na Acetate in 100 ml DiH<sub>2</sub>O, pH 6.8)

Store in 500 µl aliquots at -80°C. On day of assay, thaw aliquot and dilute:

<u>nmoles/10 ul</u>	<u>IB</u>	<u>4 mM ADP Stock</u>
0	200 µl	0 µl
5	175 µl	25 µl
10	150 µl	50 µl
20	100 µl	100 µl

Each standard is added to the plate in quadruplicate of 10 µl, after which, 200 µl of AM/Salt Sol'n is added. Standard curve slope should be in the range of 17-19 mOD/nmole.

### NADH Standards (MW = 709.4) Must be made the day of the assay

4 mM NADH stock solution:

Add 28 mg to 10 ml Imidazole buffer

<u>nmoles/10 ul</u>	<u>IB</u>	<u>4 mM NADH Stock</u>
0	200 $\mu$ l	0 $\mu$ l
10	150 $\mu$ l	50 $\mu$ l
20	100 $\mu$ l	100 $\mu$ l
40	0 $\mu$ l	200 $\mu$ l

Each standard is added to the plate in quadruplicate of 10  $\mu$ l, after which, 200  $\mu$ l of IB is added. Standard curve should be in the range of 18-20 mOD/nmole.

### III. Assay Mixture Recipe

**Make fresh with every assay to ensure quality.**

<u>Solution</u>	<u>4 microplates (96 assays)</u>	<u>8 microplates (192)</u>
PK	46.3 $\mu$ l *	92.6 $\mu$ l *
LDH	24.3 $\mu$ l *	48.6 $\mu$ l *
NADH	10.9 mg	21.8 mg
PEP	9.33 ml	18.66 ml
ATP	27 mg	54 mg
IB	qs to 70 ml	qs to 140 ml
	Divide into 35 ml halves Add 2.5 ml IB to one (A) Add 2.5 ml Ouabain in IB to the other (B)	Divide into 70 ml halves Add 5 ml IB to one (A) Add 5 ml Ouabain in IB to the other (B)

**NOTE:** If using a 200  $\mu$ l repipetter (this is advised to hasten the process) the salt solution: assay mixture ratio, which may be used, is 50 parts: 150 parts.

#### Example for 1 plate:

- Add 2.5 ml Salt solution to 7.5 ml of assay mixture A
- Add 2.5 ml Salt solution to 7.5 ml of assay mixture B
- Pipet 200  $\mu$ l per well

\* Swirl Sigma vial; remove indicated volume; centrifuge for 8 minutes at 12,000 g at 4°C; remove supernatant; resuspend in IB; add to assay mixture. **These values change with each vial bought. Recalculate amount every time enzymes are bought.**

## IV. Sample Preparation and Assay Notes (Read at 340 nm)

### 1. Run standard curves for ADP and NADH

- a) **ADP standard curve** is run to ensure that reagents for that batch of assay mixture are prepared correctly and in good condition. Additionally, this is the slope that is used to calculate ATPase activity.
- b) The ADP curve is usually 13-14 mOD/nmole ADP.
- c) When running the ADP standard curve there should be rapid equilibrium of ADP (within 3 – 4 minutes) and the optical density of the 0 Standard should be between 0.4 and 1.2 OD units.
- d) If either of these 2 observations are not made then one or more reagents have gone bad or is not present in high enough concentrations.
- e) **NADH standard curve** is run to establish that reagents and plate provide for optimal absorbance. This only needs to be run once per NADH vial purchased to check for optimum absorbance conditions.
- f) A curve of 18 - 20 mOD/nmole is desirable.

### 2. Sample preparation and assay protocol

- a) Gill tissue is stored in 100  $\mu$ l SEI buffer at  $-20^{\circ}\text{C}$  for up to 2 months and  $-80^{\circ}\text{C}$  for 6 months
- b) Thaw samples immediately prior to assay and add 50  $\mu$ l SEID (3X concentrate).
- c) Homogenate in tube (20 – 30 seconds), ensuring all tissue is homogenized.
- d) Centrifuge at 5,000g for 1 minute at  $4^{\circ}\text{C}$  to remove insoluble material.
- e) Pipet 10  $\mu$ l of sample into 4 wells (2-salt, 2-K free salt). Uses a total of 40 $\mu$ l homogenate.
- f) With AM/Salt solutions still in ice bath, add 200  $\mu$ l of either solution A or B to respective wells.
- g) Read plate at 340 nm for 10 minutes with 60-second intervals at room temperature.

## V. Bradford Protein Assay

### Methods

- 1) Prepare standards for curve in centrifuge tubes.
- 2) Pipet 90  $\mu\text{l}$  of Salt solution into centrifuge tubes for samples.
- 3) Pipet 10  $\mu\text{l}$  of each homogenate sample into appropriate centrifuge tubes.
- 4) Add 1 ml of reagent to each tube.
- 5) Vortex samples briefly.
- 6) Incubate for 2 minutes at 25°C, and transfer to 1 mL microcuvetes.
- 7) This is an endpoint assay read at a wavelength of 595 nm

### Preparation of standards:

<u>ug/10ul</u>	<u>BSA standard</u>	<u>Salt Sol.</u>
0	--	100 $\mu\text{l}$
5	5 $\mu\text{l}$	95 $\mu\text{l}$
10	10 $\mu\text{l}$	90 $\mu\text{l}$
15	15 $\mu\text{l}$	85 $\mu\text{l}$
20	20 $\mu\text{l}$	80 $\mu\text{l}$

## VI. ATPase Activity Calculation

- The ATPase standard curve should be read in mOD/nmole ADP
- The ATPase activity measurements should be read in mOD/10 $\mu$ l /minute

### Sample calculation:

Standard curve = 20 mOD/nmole ADP

ATPase (without Ouabain) = 30.0 mOD/10 $\mu$ l /minute

ATPase (with Ouabain) = 13.5 mOD/10 $\mu$ l /minute

Na<sup>+</sup>,K<sup>+</sup>-ATPase = 30.0 – 13.5 = 16.5 mOD/10 $\mu$ l /minute

Protein reading = 8.5  $\mu$ g/10 $\mu$ l

$$\frac{16.5 \text{ mOD}/10\mu\text{l} / \text{minute}}{20 \text{ mOD.nmole ADP}} = 0.825 \text{ nmoles ADP}/10\mu\text{l} / \text{minute}$$

$$\frac{0.825 \text{ nmoles Adp}/10\mu\text{l}/\text{minute}}{8.5 \mu\text{g}/10\mu\text{l} \text{ protein}} = 0.097 \mu\text{moles ADP}/\text{mg protein}/\text{minute}$$

$$(0.097)(60\text{min}) = 5.82 \mu\text{moles ADP}/\text{mg protein}/\text{hour}$$

## VII. References

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## CONCLUSIONS

The successful completion of this study has resulted in a better understanding of the current state of Pb contamination in freshwater ecosystems, the contribution from traffic both in the past and present and physiological effects of Pb exposure on the freshwater mussel *Elliptio complanata*.

Both of the laboratory studies have shown that the freshwater mussel *Elliptio complanata* accumulates dissolved Pb very rapidly from the surrounding water, and deposits it in the tissues as well as hemolymph. Concentrations of Pb in tissue and hemolymph, however, only increased linearly up through day 14. Because Pb was rapidly depleted from the water throughout the exposures, yet not increasing in the tissues or hemolymph after day 14, I conclude that the mussels were binding, metabolizing and excreting the Pb. Storage of Pb in the shell was no doubt taking place (although un-measured in this study), but the majority of elimination was most likely in lysosomes and granulocytes as has been shown for other mussel species [1, 2], being transported in pseudo-feces, where it would collect on the bottom of the jar, out of the water column and therefore not detected in the water samples.

Multiple biomarkers for Pb exposure and effect were evaluated in both laboratory studies. The classic biomarker for Pb in vertebrates and some invertebrates,  $\delta$ -aminolevulinic acid dehydratase (ALAD), was shown not to be present in detectable levels in hemolymph or tissues (gill, mantle, foot and visceral) of *Elliptio complanata*. The non-repeatedly sampled mussels from the first laboratory study suggested that  $\text{Ca}^{2+}$  might be adversely affected by Pb exposure, and the findings from the second laboratory study

confirm this. Hemolymph  $\text{Ca}^{2+}$  was found to be significantly increased at the greatest exposure, likely due to re-mobilization from the shell in order to buffer against uptake and toxicity, which is reduced in the presence of  $\text{Ca}^{2+}$  [3]. Sodium concentrations were also reduced by the end of the study in the greatest exposure, as expected from disruption of  $\text{Na}^+, \text{K}^+$ -ATPase activity. Mussel tissues in both laboratory exposures were found to accumulate Pb with very strong correlations ( $R^2 = 0.984$  and  $0.999$ , respectively) to exposure concentration. These correlations were observed at all time points, and concentrations of Pb in tissues increased up to day 14. This indicates that the mussels may have reached equilibrium with the bioavailable Pb. The sodium pump, or  $\text{Na}^+, \text{K}^+$ -ATPase activity in mussel gill tissue was shown to be negatively correlated with mussel tissue Pb concentration ( $R^2 = 0.82$ ) at the end of the 28 day exposure. The results for  $\text{Na}^+, \text{K}^+$ -ATPase activity suggest its potential use as a biomarker of Pb exposure in mussels as has been shown for fish [4]. However, other inhibitors and confounding factors of  $\text{Na}^+, \text{K}^+$ -ATPase need to be taken into consideration before its considered as a definitive biomarker of Pb exposure. For example, if sulfhydryl donors and L-cysteine are present, they can reduce Pb inhibition of  $\text{Na}^+, \text{K}^+$ -ATPase by as much as an order of magnitude [5, 6]. Other inhibitors shown to reduce  $\text{Na}^+, \text{K}^+$ -ATPase activity include the pesticide fenitrothion [7] as well as the metals mercury and copper [8, 9], but not cadmium [10]. Because Pb accumulation in mussel tissue on day 28 was not significantly greater than on day 14, I conclude that the freshwater mussels may have reached equilibrium with the bioavailable Pb concentration. However, because Pb contamination of freshwater mussel tissue has been shown to have no observed

loss over time [11, 12], concentrations in tissues are an indication of the greatest contamination level of that site over the lifespan of the mussel.

These findings from the laboratory studies are in agreement with the results from the field study. While mussels from sites with a traffic count over 500 vehicles per day had significantly ( $P \leq 0.0425$ ) greater levels of Pb in their tissues than mussels from lower traffic sites, there was no correlation found between the higher traffic counts, with current sediment levels, or between traffic count and current sediment levels. Because vehicles today release minimal or no levels of Pb, as it is no longer added to gasoline, the historic correlation observed between mussel tissue and traffic count [13] or Pb in sediment [14] is not apparent. Erosion and remobilization of Pb from roadside sediment has been shown to be a current problem in Hawaii [15]. After 28 days, the lowest Pb exposure concentrations of 0.9  $\mu\text{g/L}$  in the first laboratory study, and 1.0  $\mu\text{g/L}$  in the second laboratory study, both resulted in mussel tissue Pb concentrations (1.5 and 2.3  $\mu\text{g/g}$  respectively) similar to those measured in mussels collected from streams adjacent to high traffic ( $> 500$  vc/d) areas. This tissue Pb concentration was also enough to cause an observed 18% reduction in the average  $\text{Na}^+, \text{K}^+$ -ATPase activity (Table 1) as measured in the second laboratory study.

In conclusion, Pb was found to accumulate rapidly in mussel tissue in the first two weeks of exposure, and then slower increases for the next two weeks were observed. Accumulation of Pb was also observed in the hemolymph, however it did not significantly ( $P > 0.05$ ) increase with exposure time and actually decreased significantly at the highest 245  $\mu\text{g/L}$  treatment group, leading us to conclude that equilibrium was being reached and the mussels were eliminating the Pb in pseudo-feces, likely contained in lysosomes and

granulocytes. Tissue Pb concentration was strongly correlated with exposure concentration at every time point tested (day 7, 14 and 28). The enzyme activity of Na<sup>+</sup>,K<sup>+</sup>-ATPase was shown to be inhibited, with a strong negative correlation to Pb tissue concentration ( $R^2 = 0.82$ ;  $P = 0.013$ ), with observed decreases in hemolymph Na<sup>+</sup> when inhibited to 30% of controls (or 1  $\mu$ Mole ADP per mg protein per hour), and has potential as a biomarker. Calcium was also increased in hemolymph with the greatest exposure, likely in order to buffer tissue cells against uptake and toxicity. Stream dwelling freshwater mussels are still affected by transportation related impacts from historic Pb deposition, but the effects observed are much less than the period of active use of leaded gasoline. The historic correlation of mussel tissue Pb concentration and traffic count will likely diminish further with time.

Further research should focus on the Platinum Group Elements (PGEs) Pt, Pd and Rh, which are now being released by automobiles in place of Pb through the use of catalytic converters [16]. Little is known of the contamination or effects of these elements on aquatic ecosystems, but it has been shown in one recent study that concentrations of palladium in sediments along roads in Germany increased by 15 times from 1994 to 2004 [17], so these elements should be monitored closely. Lead is still a problem in the environment, with the effects of its contribution from leaded gasoline still evident, but it is unclear if concentrations are currently at levels which alone would cause reductions in the freshwater mussel *Elliptio complanata*'s population. And sub-lethal effects, such as Na<sup>+</sup>,K<sup>+</sup>-ATPase activity, appear to not be adversely affected at environmentally relevant Pb concentrations. However with Pb (along with Cd and Zn) shown to cause reductions in mussel communities and species

diversity [18], it is critical to continue monitoring aquatic ecosystems and avoid further contamination of the environment.

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## Table

**Table 1. The Na<sup>+</sup>,K<sup>+</sup>-ATPase activity for each treatment group as a percentage of the Control for day 28 of the study.**

<b>Inhibition of Na<sup>+</sup>,K<sup>+</sup>-ATPase Activity: Day 28</b>		
<b>Exposure (ug/L)</b>	<b>Activity(% of C)</b>	<b>% Reduced</b>
<b>1</b>	81.7	18.3
<b>4</b>	79.9	20.1
<b>14</b>	73.7	26.3
<b>57</b>	56.5	43.5
<b>245</b>	30.4	69.6