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Platinum and Lead Markers as Indicators of Transportation Impact

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16. Abstract

The intent of this study was to provide information necessary to characterize the present concentrations of an emerging transportation related contaminant, the platinum group metals (PGM), and to provide background information necessary to explore the potential uses of these metals as a specific marker of transportation related contamination that results from the discharge of road runoff into streams. Thirty-seven road crossings were evaluated in the Atlantic Slope of central North Carolina. Mussel tissue and sediment samples were collected from upstream and downstream of each road crossing. Samples were analyzed for the platinum group metal platinum (Pt), as well two other common transportation pollutants, lead and cadmium, and a common atmospheric pollutant, mercury. The enrichment of pollutants (downstream concentration – upstream concentration) was compared to the number of vehicles crossing the stream. Local stream environmental variables and landscape scale variables were used to model the concentration of Pt in mussel tissue. Two 28-day laboratory toxicity tests, one with Pt and one with Pb, were conducted to assess the potential threat that current concentrations of these metals pose to native mussel fauna.

The correlation of enrichment of the metals at bridge sites was weak for all metals studied. We hypothesize that complex hydrologic alteration caused by highway crossing structures was responsible for increased variation in downstream samples. Multiple samples taken from a single stream over an 8 km stream reach indicated that Pt concentrations in mussel tissue and sediment was enriched downstream of a road crossing, but the enrichment was not uniform and enrichment does not peak for several km downstream from the source. Results from the 28-day tests indicated that PGM may not pose an immediate threat to adult mussels and that mussels are a good sentinel species for studying PGM.

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Executive Summary

The intent of this study was to provide information necessary to characterize the present concentrations of an emerging transportation related contaminant, the platinum group metals (PGM), and to provide background information necessary to explore the potential uses of these metals as a specific marker of transportation related contamination that results from the discharge of road runoff into streams. Thirty-seven road crossings were evaluated in the Atlantic Slope of central North Carolina. Mussel tissue and sediment samples were collected from upstream and downstream of each road crossing. Samples were analyzed for the platinum group metal platinum (Pt), as well two other common transportation pollutants, lead and cadmium, and a common atmospheric pollutant, mercury. The enrichment of pollutants (downstream concentration – upstream concentration) was compared to the number of vehicles crossing the stream. Local stream environmental variables and landscape scale variables were used to model the concentration of Pt in mussel tissue. Two 28-day laboratory toxicity tests, one with Pt and one with Pb, were conducted to assess the potential threat that current concentrations of these metals pose to native mussel fauna.

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INTRODUCTION

Lead (Pb) 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 discharge, as well as the ban of Pb from gasoline (Gatz et al. 1989; Schmitt 2004), there is still considerable amounts residing 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 (Norton et al. 1990; Pirkle et al. 1998; Mielke 1999; Schmitt et al. 2002; Jackson et al. 2004; Schmitt 2004; Gillikin et al. 2005; Vermillion et al. 2005; Angelo et al. 2007; Schmitt et al. 2007). 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 (Mielke 1999). 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 (Norton et al. 1990). 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 (Klaminder et al. 2006). Many studies have shown greater levels of Pb in roadside sediments (Latimer et al. 1990; Mielke 1999; Sutherland and Tolosa 2000; Sutherland 2003 a; Weiss et al. 2006) from historic transportation, with most of the Pb found in the small grain fraction (<63 μm), which is more likely to be re-suspended or eroded into rivers and streams adjacent to these roads (Sutherland and Tolosa 2000; Weiss et al. 2006).

The introduction of catalytic converters into automobiles in the United States in the mid 1970s and in Europe in the 1980s coincided with new laws intended to reduce air pollution including the phase-out of leaded gasoline, which is not compatible with catalytic converters, leading to the ban in 1996. However, the use of catalytic converters for automobile exhaust purification has led to the emission and environmental contamination by the platinum group metals (PGM) platinum (Pt), palladium (Pd), and rhodium (Rh) in place of Pb. The PGM are the active components in catalytic converters that help to reduce emissions of hydrocarbons, carbon monoxide, and nitrogen oxides. Consequently, concentrations of PGM are increasing in dust and soils along highways and are being transported into aquatic habitats through surface runoff, where they are accumulating in the sediments of streams and in the tissues of aquatic organisms (Sures et al. 2001; Zimmermann et al. 2002, 2005). Due to this phenomenon, PGM are an emerging class of contaminants with potential human and environmental health implications, due to suspected mutagenic and carcinogenic activity (Ravindra et al. 2004). Also, because the influx of PGM to the environment can be almost entirely attributed to their use in automobile catalytic converters, they have potential to serve as excellent indicators of highway and transportation impacts to aquatic and terrestrial ecosystems.

Platinum group metals are a family of similar transition metals belonging to Group VIII of the periodic table. PGM include the elements ruthenium (Ru), rhodium (Rh), palladium (Pd), osmium (Os), iridium (Ir) and platinum (Pt). They are rare in the earth's crust, and ores containing PGM are only found in a small number of places. The most productive mines are found in Russia and South Africa, with smaller deposits being

exploited in Montana (USA), Canada, Zimbabwe, and Australia (Mineral Information Institute 2000).

PGM have outstanding catalytic properties, high resistance to wear and tarnish, resistance to chemical attack, excellent high temperature characteristics, and stable electrical properties. These physical and chemical properties make them especially useful in the industrial production of chemicals by catalysis and in the electronics industry as conductive components. Other uses include alloys of PGM used in dental prosthetics and jewelry (USGS 2007). Platinum is also used in various pharmaceuticals, particularly in anti-cancer drugs. However, automobile catalytic converters represent the largest use of PGM (Ravindra et al. 2004).

In response to the 1970 Clean Air Act, catalytic converters containing PGM began to be added to the exhaust system of automobiles to catalyze the combustion of fuels and reduce the concentrations of nitric oxides, carbon monoxide and residual hydrocarbons present in automobile exhaust. Beginning in the mid-1970s catalytic converters were added to all new automobiles sold in the United States and Japan. Catalytic converters were made mandatory for all new European Union vehicles in 1993 (Sutherland 2003 b). Since their introduction, the concentration of PGM in the roadside environment has increased from background levels that were among the lowest of any element in the earth's crust (0.4 ng/g Pt and Pd, 0.06 ng/g Rh, Whedepol 1995) to concentrations of Pt in urban tunnel dust as high as 730 ng/g (Helmers and Mergel 1998). The global emission of transportation derived Pt to the environment has been estimated between 0.8 and 6.0 metric tons per year, assuming 500 million catalyst equipped vehicles with an average mileage of 15,000 km/yr (Rauch et al. 2005).

Auto catalysts represent a large fraction of the world wide demand for PGM. In 2000, Pt, Pd and Rh used in auto catalysts accounted for 31%, 61% and 99%, respectively, of worldwide demand (Ravindra et al. 2004). Due to the high cost of these metals—$39,324, $7,342, and $39,712 U.S. per kg of Pt, Pd and Rh, respectively, as of March 23, 2009 (Johnson Matthey)—they tend to be highly conserved and recycled in their other uses (Tuit et al. 2000). Another source of Pt to the environment is its use in anti-cancer drugs, primarily cisplatin and carboplatin. These drugs are a component of hospital effluent and are estimated to account for 3.3-12.3% of Pt in municipal sewage, based on a study of five European hospitals (Kummerer et al. 1999). The majority of PGM found in the environment is directly attributable to automobile emission (Tuit et al. 2000).

The emission of Pt from automobile catalytic converters can be mainly attributed to thermal sintering, evaporation and mechanical erosion (Palacios 2000). Emission rates found in the literature have a wide range. Rates of speed, engine size, catalyst type, and the age of the catalyst appear to play a major role in the emission rates (Ravindra et al. 2003). There is high variation in emission rates reported in the literature from studies that used various methods of direct determination by exhaust collection under laboratory conditions. These reported rates range 2-1,900 ng/km. Emission rates estimated from lab tests are often conducted under the best conditions, with well maintained engines operating at a small number of operating speeds. Methods of indirect determination, those that assessed PGM in the roadside environment or in organisms, generally reported a greater mean emission rate (Palacios 2000). Emission rates of vehicles under real world driving conditions are likely to emit larger amounts of PGM. A synthesis of results

from several approaches including bench testing and data collected from roadside soils and grasses indicate a Pt emission rate between 500 and 800 ng/km (Helmers 1997).

Emission of PGM from automobile exhaust is primarily in particulate form in the (0) oxidation state or as oxides (Moldovan et al. 2002). The size of the emitted particles fall primarily into the (>10 μm) class, representing 62-67% of particulate emission. Particles in the (3.1-10 μm) and (<3.1 μm) represent ~21% and ~13%, respectively (Ravindra et al. 2004). Direct methods of emissions testing have estimated the soluble fraction of platinum at 1% (Artlet et al. 2000) and 10% (Konig et al. 1992). Moldovan et al. (2000) confirmed that the soluble fraction of Pt in both diesel and gasoline exhaust in most cases represented less than 10% of the total emission; however, the soluble fraction of Pd and Rh were significantly greater, ranging between 20% and 40%.

The emission of PGM from automobiles has caused significant increases in the concentrations found in road-deposited sediment and roadside soils (Cicchella et al 2003, Sutherland 2003 b, Lesniewska et al. 2004, Zereini et al. 2007). PGM is also being transported to aquatic systems by runoff and waste water discharges and can be found in sediment (Laschka et al. 1996, Tuit et al. 2000, Rauch et al. 2005, Turner et al. 2006). PGM is detectible in urban air as particulate matter of widely varying size (Gomez et al. 2002) and can be transported long distances by the wind. Ice cores taken from the Greenland ice sheet indicate global anthropogenic deposition of these metals, but this likely represents significant contributions from Russian smelters (Rauch et al. 2005).

Bioavailability and Toxicity

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* (Metcalfe-Smith et al. 1996), there are many environmental factors affecting Pb bioavailability and uptake. Both the pH of the water and the amount of calcium sulfate ($CaSO_4$) and dissolved organic carbon (DOC) present have affected the availability of Pb to fathead minnow (Grosell et al. 2006), with variations from pH 7.4 increasing, and addition of $CaSO_4$ or DOC decreasing toxicity. Two forms of Pb (lead acetate trihydrate and lead carbonate) were both found highly adsorbed onto organic matter in sediment (Darling and Thomas 2005). 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 (Graham et al. 2006). Humic water increases the bioavailability of most metals compared with tap water (Zimmermann et al. 2002) 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 (Stewart 1999).

While several studies have found little or no correlation between Pb accumulation in freshwater mussels and the amount present in the environment (Ravera et al. 2003; Anderson et al. 2004), 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 (Gale et al. 2002, 2004). In a

study with *Elliptio complanata*, it was shown that Pb accumulation was reduced by the presence of iron oxyhydroxides and organic mater, and that once sediment levels were "normalized" for organic carbon content (Tessier et al. 1984), 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 (Lingard et al. 1992), can still be used as a good sentinel of environmental concentrations of Pb. Because mussel growth is reduced in the presence of heavy metals (Naimo 1995) 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 (Dermott and Lum 1986).

When bivalves are exposed to lead, lysosomes are the key intracellular structures where lead storage takes place in the tissues (Amiard et al. 1995; Marigómez et al. 2002), and these lysosomes are then removed by cellular apex extrusion. This process explains the observed increase in lysosomal numbers and size (Giamberini and Pihan 1997). Storage has also been seen in granular concretions (Adams and Shorey 1998; Vesk and Byrne 1999; Byrne and Vesk 2000; Marigómez et al. 2002). 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 (Wiesner et al. 2001). 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 (Marigómez et al. 2002), 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 (Marigómez et al. 2002).

When mussels are exposed to heavy metals, uptake occurs mainly in the gill and mantle (Salánki and V.-Balogh 1989; Naimo 1995; Gundacker 2000; Yap et al. 2004) and observed effects have included changes in growth (Naimo 1995), filtration (Salánki and V.-Balogh 1989; Salánki and Hiripi 1990; Bleeker et al. 1992; Kraak et al. 1994; Naimo 1995), enzyme activity (Naimo 1995) and behavior (Salánki and Hiripi 1990; Naimo 1995). An increase in lysosomal numbers and size (Giamberini and Pihan 1997) and DNA strand-breakage (Black et al. 1996) 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.

The bioavailability of PGM is primarily determined by the solubility of the metals under environmentally relevant conditions (Sutherland 2003 b). Zerenini et al. (1997) tested the solubility of catalyst material in direct contact with soil of varying pH, salt, and sulfur concentrations. They found that metallic PGM particles are largely immobile over short periods of time (Zereini et al. 1997). However, it has been found that organic acid solutions can mobilize metallic PGM over an experimental period of one year. These conditions are comparable to roadside drainage ditches, where decaying vegetation and automobile emitted catalyst are deposited (Bowles and Gize 2005). Additionally, siderophores (organic ligands produced by bacteria, fungi and plants) that increase the

bioavailability of iron in the soil, are also effective at increasing the solubility of PGM (Dahlheimer et al. 2007).

Bioaccumulation has been demonstrated in a variety of different taxa. Plants grown on soil treated with soluble forms of PGM may accumulate the metals, especially Pd (Ek et al. 2004). A variety of species of roadside plants have been shown to accumulate PGM as well as other traffic related metals (Schafer et al. 1998, Djingova et al. 2003, Lesniewska et al. 2004). The sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, has been shown to accumulate PGM. This bacterium is similar to bacteria naturally found in decaying detritus in aquatic systems (Yong et al. 2002). Aquatic species, European eels, *Anguilla anguilla*, and zebra mussel, *Dreissena polymorpha*, have been shown to bioaccumulate PGM when exposed to road dust under laboratory conditions (Sures et al. 2001, Zimmermann et al. 2002).

Wolterbeek and Verburg (2001) ranked 80 metals by toxicity based on the average relative toxicity from 30 literature data sets using various species and toxic endpoints. In their analysis, PGM species Pt(II), Pt(IV), Rh(I), Rh(III), and Pd(II) ranked 10^{th}, 11^{th}, 20^{th}, 25^{th} and 28^{th}, respectively. In this ranking, the toxicity of Pt was similar to lead, Pb(IV).

PGM causes phytotoxicity and chlorosis in plants at high concentrations, and Pt complex compounds are mutagenic in bacteria (Gebel et al. 1997). PGM are known to cause allergic reactions in humans, causing asthma, conjunctivitis, dermatitis, rhinitis, and uticaria. Pt is used in cancer drugs due to its cytotoxic properties (Caroli et al. 2001). Zebra mussels exposed to PGM from road dust produce elevated levels of heat shock

protein (Singer et al. 2005) and metallothionein (Frank et al. 2008), providing evidence for cellular effects in bivalves.

Freshwater Mussels as Bioindicators

Freshwater mussels (family Unionidae) may be among the groups of aquatic organisms adversely affected by persistent, low-level exposure to PGM in surface waters. Unionid mussels are filter and deposit feeding, long-lived (30-100 yr) organisms that live burrowed in sediments of streams and rivers. They are one of the most rapidly declining faunal groups in North America. About 67% of the nearly 300 freshwater mussel species found in North America are considered vulnerable to extinction or already extinct (Bogan 1993; Williams et al. 1993). The decline of mussel populations in North America has occurred steadily since the mid 1800s and has been attributed to pollution, construction of dams and impoundments, sedimentation, navigation, and habitat degradation (Fuller 1974, Bogan 1993, Neves 1997, Brim Box and Mossa 1999, Vaughn and Taylor 1999). The surface waters of North Carolina have historically supported 56 species of unionid mussels (Bogan 2002). Today, approximately 78% of these species are listed as endangered, threatened, or of special concern by the U.S. Fish and Wildlife Service and the State of North Carolina (LeGrand et al. 2008) or are already extinct. Many of the same human-mediated and environmental stressors such as highways and associated landscape development (e.g., Wheeler et al. 2005) responsible for the declines of freshwater mussels throughout North America have also contributed to the declines in North Carolina. Principally, the stressors associated with human development and urbanization in almost all of the State's 17 river basins has hastened these declines over the past 20 to 50 years.

Freshwater mussels employ a unique reproductive strategy that involves a parasitic larva (glochidia) that must attach itself to a host fish in order to undergo a metamorphosis to its final stage as a primarily sessile juvenile. The complexity of this reproductive strategy makes freshwater mussels highly vulnerable to extirpation due to environmental alterations (Bogan 1993, Neves et al. 1997). Mussels have a unique life history that includes low trophic level, long life, and sensitive early life stages (Cope et al. 2008). These traits make them a potentially sensitive indicator species for anthropogenic PGM.

They also meet many of the prerequisites for an ideal biomonitor of stable trace metals (Phillips and Rainbow 1993) 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 (Salánki and V.-Balogh 1989; Bleeker et al. 1992; Amiard et al. 1995; Ingersoll 2005), allowing for laboratory studies, and are strong accumulators of Pb (Tessier et al. 1984; Salánki and V.-Balogh 1989; Anderson et al. 2004) with an established correlation between metal accumulation and the ambient bioavailable concentration (Tessier et al. 1984).

Biomarkers

Biomarkers in the ecological discipline have been described as "any biological response to an environmental chemical at the below-individual level, measured inside an organism or in its products (urine, feces, hairs, feathers, etc.) indicating a departure from the normal status, that cannot be detected from the intact organism" (van Gestel and van Brummelen 1996). Biomarkers are restricted to biochemical, physiological, histological and morphological measurements of health. For example, the salt water mussel *Perna*

virdis has been shown to be sensitive to silver and chromium contamination and display reduced oxygen uptake, filtration rates, and inhibition of Na^{+} K^{+}-ATPase, Ca^{2+} ATPase and Mg^{2+}-ATPase at sublethal exposure concentrations (Vijayavel et al. 2007), indicating ion transporters as a potential biomarker of metal contamination in bivalves.

Study Objectives

The fate of PGM in aquatic systems is poorly understood. The objective of this present study was to characterize the present tissue concentration of PGM in a common freshwater mussel and assess its correlation with vehicle traffic and other landscape scale anthropogenic activities, as well as compare those measurements to those found for Pb as a historical traffic indicator to assess current environmental concentrations. Accompanying laboratory studies of uptake of PGM and Pb from aqueous medium were intended to elucidate the levels of environmental contamination that are necessary to attain tissue concentration at those observed in the field study and to evaluate potential biomarkers of metal exposure.

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FIELD STUDIES

Chapter 1: Assessing platinum concentrations in freshwater mussel tissue and sediment at road crossings from streams in central North Carolina

Abstract

The aim of this study was to assess the level of platinum group metals (PGM) accumulation in the freshwater mussel *Elliptio complanata* and to assess local and landscape scale environmental variables that could be used to explain or predict exposure. A total of 37 sites were sampled throughout North Carolina and were chosen based on availability of *E. complanata*, geographic distribution, land use patterns and traffic density. At each site, samples were collected from an upstream and downstream location within 50-150 m of a highway crossing. A sample consisted of three adult *E. complanata* and sediment. Mussel tissue and sediment samples were analyzed for concentrations of rhodium (Rh), palladium (Pd), and platinum (Pt). Additional related stream, mussel, and local variables investigated included total organic carbon content, co-occurring metal contamination cadmium (Cd) and mercury (Hg), pH, conductivity, temperature, and estimated vehicle traffic density at the stream crossing. Landscape variables included human population density, land use, and density of transportation infrastructure. During development of the analytical techniques for PGM, we discovered that measurement of Pd and Rh had unresolvable interference from yttrium and strontium oxide, respectively, and were unable to be accurately quantified. Therefore, Pt was the only PGM measured and its concentrations ranged 0.09-1.98 ng/g in mussel tissue and

0.06-1.86 ng/g in sediment. A biota-sediment accumulation factor for Pt, calculated as the mean [tissue dry weight]/[soil dry weight], was 3.2, compared to 87.0 and 87.8 for Hg and Cd, respectively. Pt contamination of mussels and sediment at the stream crossing sites was not significantly correlated with the amount of vehicle traffic crossing the structure. Multiple regression modeling indicated a significant relation between Pt concentration in mussel tissue and the human population of the watershed.

Introduction

Native freshwater mussels (family Unionidae) may be among the groups of aquatic organisms adversely affected by persistent, low-level exposure to PGM in surface waters. Unionid mussels are filter and deposit feeding, long-lived (30-100 yr) organisms that live burrowed in sediments of streams and rivers. They are one of the most rapidly declining faunal groups in North America. About 67% of the nearly 300 freshwater mussel species found in North America are considered vulnerable to extinction or already extinct (1, 2). The decline of mussel populations in North America has occurred steadily since the mid 1800s and has been attributed to pollution, construction of dams and impoundments, sedimentation, navigation, and habitat degradation (3-6). The surface waters of North Carolina have historically supported 56 species of unionid mussels (7). Today, approximately 78% of these species are listed as endangered, threatened, or of special concern by the U.S. Fish and Wildlife Service and the State of North Carolina (8) or are already extinct. Many human-mediated and environmental stressors, such as highways and associated landscape development are responsible for the declines of

freshwater mussels throughout North America (9) and have also contributed to the declines in North Carolina. Mussels have a unique life history that includes low trophic level, long life, and sensitive early life stages (10). These traits make them well-suited to serve as a sentinel or bioindicator species for anthropogenic PGM.

Presently, no information on the bioaccumulation of PGM in freshwater mussels is published in the primary literature. Laboratory investigations of PGM uptake in the distantly related zebra mussel (*Dressinia polymorpha*) indicate that PGM derived from automobile catalytic convertors is bioavailable to filter feeding bivalves and uptake is enhanced by the presence of humic substances and organic acids in the exposure water (11). The zebra mussels used in that study had a background level of PGM that was above detection limits, indicating that anthropogenic sources of PGM were being accumulated by this species in its natural environment. Freshwater mussels are known to bioaccumulate other anthropogenically derived metal pollutants, such as cadmium (Cd), copper (Cu) and zinc (Zn); however, the relationship between tissue burden and local environmental contamination is sometimes weak, indicating that complex environmental dynamics are important (12). The rarity of many unionid mussel species and the demonstrated toxicity of PGM in other species (13), make the potential of bioaccumulation of PGM in this faunal group a cause for concern.

The freshwater mussel species *Elliptio complanata* is widely distributed and abundant in North Carolina, relative to other mussel species. *E. complanata* is suited to the role of a bioindicator species due to its long life, sessile nature, and body size adequate to be used for analysis of trace metals and other contaminants (14). It has been

demonstrated to be robust to nonlethal sampling techniques and a suitable bioindicator of chronic water quality conditions (15, 16).

Unpublished data collected previously by our laboratory suggested that *E. complanta* was accumulating Pt and Pd, and that there was a correlation with vehicle traffic density at the crossing site (Figure 1) (17). The present study was designed to define current levels of accumulated PGM in *E. complanata*, test its correlation with vehicle traffic at the site, and to investigate other local and watershed scale variables that might contribute to PGM concentrations in mussel tissue.

Materials and Methods

Mussel and Sediment Collection

Mussels and sediment were collected from 37 stream sites in central North Carolina (Figure 2). Sites were selected based on the presence of the target species at the site, vehicle traffic density, and geographic distribution. A sample was taken from an upstream and downstream location within an area of stream 50-150 m from the road crossing. A sample consisted of three adult *E. complanata* and a surfical (top 5 cm) single grab sample of sediment. Mussels were selected to represent roughly the average size of adult mussel from the site and were taken at random. Mussels were placed in a labeled plastic bag and stored in a cooler on ice, until they could be frozen in a standard freezer ($\geq$-20 ºC). Sediment samples were collected from within areas inhabited by *E. complanata* using a stainless steel scoop and placed in an acid washed jar. Other water quality variables measured at the site were water temperature, pH, conductivity, and

dissolved oxygen (YSI Model 556 MPS, Yellow Springs Instruments, Yellow Springs, Ohio, USA). Additional large scale habitat variables were estimated using geographic information systems (GIS) software (Environmental Systems Research Institute, ArcInfo 9.2, 2008). Landscape variables assessed included land cover, watershed size, human population, and vehicle traffic densities.

Single mussels for PGM analysis did not provide adequate tissue mass to reach detection limits for the PGMs. Therefore, homogenates from three mussels were combined to form a single composite sample for analysis of PGM; one composite sample from the upstream reach and one from the downstream reach at each site. This method produced detectable results, but an assessment of individual variation was lost for mussels from a single location. To estimate potential variation in PGM in mussels within a site, a second experimental design was employed within New Hope Creek, Orange County, North Carolina. Here, six locations were sampled at various distances upstream and downstream of the Interstate 40 (I-40) and NC Hwy 86 crossing of New Hope Creek. Vehicle crossings per day (vc/d) were estimated to be 58,000 vc/d for I-40 at its crossing of New Hope Creek, and that for NC Hwy 86 6,800 vc/d. At each sampling location, mussels were taken at random and stratified into four samples of three individuals each. A surficial sediment sample was also taken at each of these locations using the same method previously described.

Sample Analysis

Samples were analyzed at Research Triangle Institute (Durham, North Carolina, USA) for concentrations of Hg, Cd, Pt, Pd, Rh, with the PGM being the primary targets

of interest. Measured concentrations of the target elements were expressed in ng/g dry weight. At the time of processing, mussel samples were removed from the freezer, partially thawed, dissected from their shells and placed into tared, acid-cleaned 50 mL digestion tubes. The samples were then weighed to the nearest 0.01 g wet weight. The samples were then frozen for a minimum of 24 h at ≥ -20 ºC. After freezing, the samples were placed in a freeze dryer for a minimum of 36 h. Samples were lyopholized then were reweighed to determine the dry weight of the sample. All samples were coarsely ground in the plastic digestion tubes with plastic spatulas.

The samples were treated with 2.0 mL of concentrated nitric acid (Ultrex®) and 2.0 mL of concentrated hydrochloric acid (Ultrex®) and allowed to stand at room temperature for approximately 1 h. The samples were then placed in a graphite digestion block with a digital temperature control module. The samples were heated on an automated program for 1 h at 50 ºC and 1 h at 80 ºC. The samples were removed, allowed to cool, and then 0.5 mL each of concentrated nitric and hydrochloric acids and 3 mL of deionized water were added to each sample. The samples were returned to the block digestion unit and heated for 6 h at 101 ºC. Once the digestion program was completed, the samples were allowed to cool and brought to a final volume of 40 mL using deionized water. The samples were tightly capped, shaken, and a 4 mL aliquot was taken for analysis.

A nominal 4 g aliquot of sediment was transferred from the sample container to an acid washed 50 mL digestion tube. The samples were treated with 2.5 mL of concentrated nitric acid (Ultrex®) and 2.5 mL of concentrated hydrochloric acid

(Ultrex®). All samples were then placed in a graphite digestion block with a digital temperature control module. The samples were heated on an automated program for 1 h at 50 ºC, 1 h at 80 ºC, and then 6 h at 101 ºC. Once the digestion program was completed, the samples were allowed to cool and brought to a final volume of 40 mL using deionized water. The samples were tightly capped, shaken, and a 4 mL aliquot was taken for analysis.

All mussel and sediment extracts were analyzed with a Thermo X-Series II Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Prior to analysis, the instrument was optimized for signal to noise and a system suitability check consisting of 10 replicates of a multi-element standard was run. The Percent Relative Standard Deviation (%RSD) of the 10 replicates was required to be <2% for the instrument to be considered ready for analysis. The ICP-MS was calibrated at the beginning of each analytical run using a series of dilutions prepared from a NIST-traceable stock solution matched to the acid matrix of the samples. The minimum acceptable correlation coefficient for the standard curve was 0.995. A calibration check was performed immediately after the calibration, at an interval of no more than every 10 samples, and at the end of the analysis so that all samples analyzed in a batch were bracketed by calibration checks. The calibration check was prepared from a separately prepared NIST-traceable stock solution than the calibration standards and at a concentration that was not used in the calibration curve.

Model Selection

An information-theoretic approach was used to develop models that best described the relationship between instream and landscape attributes (18). Instream habitat variables collected at field sites and landscape-level parameters estimated using GIS were tested for redundancy using a correlation matrix in SAS Corporation JMP7 software. Variables that were significantly correlated (Pearson product-moment correlation <0.05) were considered redundant and the number of variables included in model selection were reduced to nine as follows: the percent of the total watershed area in the forested land use category (%forested), human population of the watershed, the average number of vehicle crossings per day at the site (vc/d), water pH, water specific conductivity (μS/cm), % total organic carbon (%TOC) content of both the upstream and downstream sediment samples, the mean whole body mass (g) of the mussels collected at a site, and concentration of tissue cadmium (ng/g) in mussels at the site. The human population of the watershed and vc/d were log(base e) transformed. All-subsets regression was used to model relationships for the response variables upstream mussel tissue Pt concentration and downstream mussel tissue Pt concentration. Models were then ranked by corrected Akaike's Information Criterion (AICc) and associated statistics to identify the most parsimonious models (i.e. those that explain the most variance with fewest parameters).

Results

During development of the analytical techniques for measuring PGM in mussel tissue, we discovered that yttrium oxide interfered with the detection of Pd and that strontium oxide interfered with Rh in the mass spectrometer. At low concentrations of Pd and Rh in the samples, the interferences were unable to be resolved and thus, results for Pd and Rh were not quantified. Therefore, Pt is the only PGM reported, and its results were calculated based on the concentration of 195Pt isotope in the sample. Among all samples, Pt concentrations ranged from 0.06 to 1.98 ng/g (mean 0.58 ng/g) dry weight in mussel tissue and from 0.06 to 1.86 ng/g (mean 0.28 ng/g) dry weight in sediment (Table 1). The biota-sediment accumulation factor for Pt, calculated as the mean [tissue]/[soil], was 3.2, compared to 87.0 and 87.8 for Hg and Cd, respectively (Table 2).

For the elements Pt and Cd, mean concentrations from all sites with an upstream and downstream sampling location were greater in the downstream samples for both tissue and sediment. Mean downstream Pt concentration was elevated 9% in tissue and 4% in sediment compared to mean upstream values. Tissue Hg concentrations were greater in the upstream samples, and sediment Hg concentrations were greater in the downstream samples. Variation among sites was high and none of the site comparisons were statistically different at the $P<0.05$ level (Tables 3-6). Pt concentration in mussel tissue was not correlated with Pt concentration in sediment among sites (Figure 3.)

The relative change in Pt concentrations between upstream and downstream locations among sites was inconsistent. At some sites the concentrations in tissue or sediment was greater in the upstream samples (17 of 37, 46% in tissue; 14 of 37, 38% in

sediment; Table 6). The difference in Pt concentration was not correlated with vehicle traffic, estimated as vehicle crossings per day (vc/d), for sediment or mussel tissue concentrations (Figure 4.).

The mussel and sediment samples collected from New Hope Creek demonstrated that there is considerable variation within and among locations within a single stream. The six sites sampled within New Hope Creek had mean mussel tissue Pt concentration of 0.12 ng/g and ranged from 0.07 to 0.23 ng/g. At these sites, standard deviation (SD) ranged 0.008-0.165 ng/g, demonstrating variation among sites within the same stream. The coefficient of variation (CV) within a sampling location ranged 12%-70% (n=4 at each location), with the greatest variation occurring immediately downstream of the Interstate 40 crossing of New Hope Creek.

The variables that most parsimoniously explained variance in the Pt concentration in mussel tissue collected from upstream of the highway crossing were human population, %TOC from the upstream samples, tissue Cd concentration and mussel mass (R^2=0.72, P<0.0001, Table 7). The variables that most parsimoniously explained variance in the Pt concentration in mussel tissue collected from downstream of the highway crossing were human population, %TOC from the downstream samples, tissue Cd concentration, mussel mass, and % forested (R^2= 0.71, P<0.0001, Table 7). Relationships were positive with all explanatory variables except mussel mass, which was negative.

Discussion

The concentration of Pt in mussel tissue and sediment of central North Carolina streams was low relative to the other metal pollutants (Cd and Hg) studied. Concentrations of Pt in mussel tissue measured in this study were less than 2 ng/g dry weight and were as low as 0.07 ng/g dry weight. Sediment Pt concentrations were similarly low, ranging 0.06-1.86 ng/g dry weight. Compared to a natural background concentration of 0.4 ng/g in the earth's crust (19), this level of enrichment does not seem particularly high.

The biota-sediment accumulation factor for Pt calculated in this study (mean 3.2) was comparatively less than that for the other metals investigated, 87.0 and 87.8 for Hg and Cd, respectively. Analysis of the sediment Pt concentration involved a total digestion of the sample and may not be representative of the relatively small bioavialable fraction of Pt particles emitted from automobiles. For example, experiments with ground catalyst material in circumneutral water (pH 6-8) demonstrated that a relatively constant percentage (0.01-0.25%) of the Pt present in the catalyst matrix was dissolved after 3 months (20). The concentrations of dissolved Pt in water were not measured in the present study, however; an investigation of Pt from the Mölndalsån River in Sweden found that sediment concentrations ranged from 8.3 ng/g to 11.2 ng/g, whereas surface water Pt concentrations were <0.1 ng/L and pore water concentrations were 0.5 ng/L (21). A bioaccumulation factor calculated from Pt in water, based on a similar ratio to sediment concentrations, would be several orders of magnitude greater than that calculated from sediment.

In our study, there was little correlation between the concentration of Pt in mussel tissue and the surrounding sediment. This suggests that the route of Pt exposure for mussels is something more complex than simple physical contact with sediment bound Pt. It has been demonstrated that *Dreissena sp.*, zebra mussels, can take up particulate metals as well as dissolved metals (22). Zebra mussels can directly use dissolved organic carbon (DOC) as a food source, and complexation of the metals Cd, silver (Ag) and Hg with high molecular weight DOC significantly increased uptake by the mussels; for Cd the increase was 32-fold (23). A study investigating Cd uptake in two unionid mussel species, *Unio pictorum* and *Anodonta sp.*, in the Danube River found significant positive correlation with sediment concentrations in *Unio* and no correlation in *Anodonta*, despite the two species co-occurring and being collected from the same habitat, indicating differing routes of uptake or metabolism (12).

The amount of vehicle traffic crossing a stream in our study was not correlated with the change in tissue or sediment concentrations between upstream and downstream samples for any of the contaminants investigated. When averaged over all sites, the mean Pt concentration in tissue and sediment was slightly elevated in the downstream samples compared to the upstream group, 9% for tissue and 4% for sediments; however, due to high variation among sites these changes were not statistically significant at the $P<0.05$ level. In 46% of samples, the difference in tissue concentration between downstream samples and upstream samples was negative, indicating greater concentrations of Pt in the upstream sample. Although Pt emission into the environment from automobiles is a non-point source form of emission, its transfer from the roadside environment into the

linear stream environment was hypothesized to behave like a point source discharge, where upstream samples would be unaffected by the influence of the roadside discharge and downstream samples would be enriched. Because emission of Pt into the roadside environment is known to be correlated with vehicle traffic density (24) and runoff from highways has been shown to be enriched with Pt (25), a correlation between traffic and enrichment in the aquatic environment immediately downstream of highway crossings seemed plausible.

One possible reason for the lack of correlation is high variation of Pt concentrations within locations. A necessary tradeoff in the analytical procedure for this study required that soft tissue digestates from three mussels be combined to obtain results that were above the detection limit of the technique, which precluded estimation of the variation between individuals. Indeed, multiple samples collected from a single stream, New Hope Creek, revealed that variation among composite samples collected from a single location was high and that variation differed among locations within the stream over a 7-km distance. Among these samples, the CV ranged from 12-70%, with the greatest CV immediately downstream of a major interstate highway crossing. If this trend was common in the streams that were sampled, significantly greater variation in samples collected from downstream of a highway crossing could produce the observed results.

The form of Pt emitted into the environment from automobiles is in a metallic form with relatively low solubility (26). The majority of the Pt transferred from the road-side environment to the stream would presumably remain in this form. Pt particles would

then settle and become part of the sediment where they can be transformed by geochemical and biological processes into a more bioavailable form. In this case, the deposition of Pt particles would be dispersed for some distance from the source, and enrichment of mussel tissue concentrations might be expected to increase with distance from the source. Longitudinal trends in results from New Hope Creek confirmed this hypothesis, displaying decreased concentrations in mussel tissue and sediment in the first kilometer downstream of the interstate highway crossing compared to the samples from the first kilometer upstream, but samples collected from bridge crossings approximately 2.5 and 4.5 km downstream of the Interstate were highly enriched with no obvious source of significant additional input. The concentrations from a site approximately 6.7 km downstream were slightly reduced from those at 2.5 and 4.5 km, indicating that the maximum enrichment of Pt from a road source might not occur for some distance downstream (~ 3.5 km) from the primary source (Figure 5).

An additional source of variation in Pt enrichment downstream of a highway crossing might come from attributes of the crossing structure itself. Highway bridges and culverts vary widely in their construction. Crossing structures invariably cause constriction of a stream's channel and in doing so, alter the natural hydrology and sediment dynamics. Structures that significantly constrict the flow of the stream can cause drastic changes in stream morphology and during high flow events will destabilize the substrate downstream of the constriction, potentially reducing the deposition of fine particulates and pollutants or periodically scouring them out. The degree of hydraulic influence of the structure would depend on the design of the structure and the

morphology of the stream, but would diminish some distance downstream from the structure to a point that would allow for deposition of sediment, Pt, and other substances to resume.

Pt in mussel tissue collected from upstream of the crossing structure was intended to represent a site specific background concentration for the stream that reflected human activity in the watershed upstream from the location. The variables included in the candidate model set describing both upstream and downstream concentrations (Table 7) support a watershed scale influence on the Pt concentrations at the site and that anthropogenic activity within the watershed is the overall source of Pt contamination. Candidate models for both upstream and downstream Pt displayed significant correlation with the total organic carbon content of the sediment from upstream of the crossing structure and neither was correlated with the total organic carbon content of the sediment from downstream.

This shared correlation with organic carbon content between locations could be the result of Pt transformation taking place in the sediment throughout the watershed, with the sediment TOC of the upstream sediment sample being representative of the average sediment TOC of the watershed where the substrate is less disturbed by hydraulic alterations of the road crossing. The solubility of fine Pt particles is known to increase in organic matrices and is further increased by complexation with humic acid (27, 28). Mussels are known to feed on suspended particulate organic matter and detritus, apparently preferring the living algal and bacterial fraction (29). A process of dissolution of fine Pt particles in the organic fraction of the sediment during its breakdown and

eventual assimilation into bacteria and algae would lead to bioavailable Pt being incorporated into food sources being utilized by the mussels. The production of food utilized by the mussels takes place throughout the watershed and if the primary route of Pt contamination in mussels is dietary, then significant enrichment of Pt in mussel tissue would not be expected for some distance downstream from the source. This pattern of enrichment was confirmed by the distribution of enrichment from the intensive samples collected longitudinally within New Hope Creek and could partially explain the lack of correlation with vehicle traffic density at the sites.

Our results support the hypothesis that mussels uptake Pt from both waterborne and sediment routes. Waterborne Pt may be either dissolved or particulate. During the process of filtration, mussels come into contact with waterborne Pt and could absorb dissolved Pt and may ingest particulate Pt. Pt not ingested or absorbed may pass through the mussel and exit via the excurrent siphon or be incorporated with pseudofeces and biodeposited. Mussels in hydraulic refugia may increase the organic content of the sediment through biodeposition. Mussels burrowed in sediment with high organic content may be able to deposit feed and could uptake Pt via this route. Additionally, close contact with contaminated sediments may facilitate the transformation of Pt to more bioavailable forms that can be directly absorbed from the sediment. Mussels that are present in an area that does not permit the deposition of organic material, such as those downstream of a highway crossing with a scoured substrate, may not be able to deposit feed and only uptake Pt from the waterborne route and consequentially accumulate less Pt than mussels in nearby areas where biodeposition and deposit feeding occur.

Differences in the amount of biodeposition and deposit feeding could account for the measured variation between upstream and downstream locations within a single stream.

The results of this study demonstrate that *E. complanata* is accumulating Pt and that the pattern of accumulation in a stream may be affected by hydraulic factors. The spatial lag in Pt accumulation measured in New Hope Creek shows that Pt can be carried several km from its source ($\geq$4.5 km in New Hope Creek). Because Pt is only one of many pollutants associated with vehicular transportation, other pollutants may also be transported a considerable distance in aquatic systems. Because of the potential for long distance transport of pollutants from road sources, it is important for transportation planners to consider the effects of transportation infrastructure over large sections of stream.

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Tables

Table 1-1. Platinum (Pt), cadmium (Cd), and mercury (Hg) concentrations in mussel tissue and sediment collected from all samples.

		Metal Concentrations		
Source	n	Mean (ng/g)	Standard Deviation (ng/g)	Range (ng/g)
Pt tissue	80	0.58	0.41	0.07 - 1.98
Pt sediment	80	0.28	0.28	0.06 - 1.86
Cd tissue	74	1,002.45	678.92	33.26 – 3,065.36
Cd sediment	74	16.53	13.4	0.5 - 96.92
Hg tissue	74	470.72	240.27	28.55 – 1,147.83
Hg sediment	74	8.72	7	47.14 - 645.61

Table 1-2. Biota-sediment accumulation factors calculated as [tissue (ng/g dry weight)]/ [sediment (ng/g dry weight)] for platinum (Pt), cadmium (Cd), and mercury (Hg).

		Biota-sediment accumulation factor [tissue]/[sediment]			
Element	n	Mean	Standard Deviation	Median	Range
Pt	80	3.2	3.1	2.0	0.2 - 11.0
Cd	74	87.8	93.0	60.7	10.8 -582.8
Hg	74	87.0	153.4	61.5	13.4 -1313.1

Table 1-3. Platinum concentrations in mussels and sediment from upstream and downstream of road crossings.

	Platinum concentration in mussels and sediment (ng/g dry wt)			
Stream	Upstream Tissue (n=3)	Downstream Tissue (n=3)	Upstream Sediment (n=1)	Downstream Sediment (n=1)
Crabtree Creek	0.116	0.226	0.179	0.180
Bear Creek	0.136	0.094	0.155	0.193
Six Mile Creek	0.165	0.158	0.224	0.250
Harlands Creek	0.171	0.215	0.203	0.191
Long Creek	0.184	0.200	0.137	0.199
Tar River	0.201	0.353	0.176	0.257
Tar River	0.242	0.302	0.426	0.208
Buckhorn Creek	0.262	1.208	0.118	0.117
Little River	0.282	0.383	0.127	0.114
Densons Creek	0.292	0.414	0.308	0.247
Eno River	0.293	0.750	0.189	0.516
Terrels Creek	0.353	0.459	0.150	0.247
Stony Creek	0.397	0.438	1.390	1.861
Swift Creek	0.401	0.294	0.149	0.260
Sevenmile Creek	0.451	0.386	0.421	0.271
New Hope Creek	0.498	0.399	0.808	0.436
Little Fishing Creek	0.500	0.715	0.239	0.358
Troublesome Creek	0.508	0.500	0.117	0.152
New Hope Creek	0.513	0.321	0.751	0.891
Shocco Creek	0.523	0.516	0.119	0.268
Stony Creek	0.538	0.305	0.302	0.245
Little River	0.597	0.821	0.176	0.164
Tar River	0.603	0.666	0.143	0.233
Troublesome Creek	0.654	0.502	0.292	0.305
Contentnea Creek	0.669	0.611	0.061	0.187
Black Creek	0.689	0.669	0.075	0.069
Sevenmile Creek	0.693	0.580	0.546	0.197
Six Mile Creek	0.773	0.303	0.072	0.084
Contentnea Creek	0.829	0.805	0.114	0.074
Little Fishing Creek	0.962	1.163	0.235	0.309
Flat River	0.966	0.714	0.480	0.171
Contentnea Creek	0.998	1.173	0.154	0.207
Cane Creek	1.056	1.419	0.400	0.263
Little River	1.103	1.341	0.128	0.146
Troublesome Creek	1.126	0.988	0.177	0.219
Crains Creek	1.540	1.984	0.296	0.346
Moccasin Creek	1.595	1.505	0.146	0.137
Mean	0.591	0.645	0.275	0.286
Standard Deviation	0.374	0.436	0.257	0.302

Table 1-4. Cadmium concentrations in mussels and sediment from upstream and downstream of road crossings.

	Cadmium concentration in mussels and sediment (ng/g dry wt)			
Stream	Upstream Tissue (n=3)	Downstream Tissue (n=3)	Upstream Sediment (n=1)	Downstream Sediment (n=1)
Swift Creek	39	33	0.5	1.7
Bear Creek	65	36	1.6	1.6
Crabtree Creek	131	669	8.4	11.4
Tar River	377	427	21.4	39.4
Shocco Creek	382	476	17.4	7.7
Little River	391	716	11.3	30.3
Buckhorn Creek	404	1,128	9.6	6.0
Sevenmile Creek	434	387	25.8	26.7
Six Mile Creek	489	490	4.8	7.8
Harlands Creek	541	722	17.3	27.8
Eno River	575	801	11.6	16.5
Little Fishing Creek	595	1,010	22.2	16.2
Sevenmile Creek	694	843	19.2	21.8
Long Creek	696	574	10.9	11.5
Contentnea Creek	766	809	9.2	10.8
New Hope Creek	784	909	19.1	30.6
Little River	842	687	13.9	30.6
Little River	853	756	9.1	7.4
Densons Creek	874	1,032	14.4	23.0
Stony Creek	889	986	17.4	25.4
Contentnea Creek	917	1,192	11.9	17.6
Cane Creek	971	1,126	15.7	15.8
Little Fishing Creek	980	692	17.6	19.6
Black Creek	1,008	1,083	3.8	7.7
Stony Creek	1,016	657	10.1	15.0
Terrels Creek	1,034	1,025	11.2	11.5
Six Mile Creek	1,079	590	4.2	5.1
Flat River	1,182	1,646	28.6	16.5
Troublesome Creek	1,190	1,394	4.8	7.8
Tar River	1,213	454	10.2	28.8
Tar River	1,296	2,233	15.3	10.3
New Hope Creek	1,492	787	29.5	20.2
Contentnea Creek	1,663	2,500	49.3	96.9
Moccasin Creek	2,391	1,988	6.1	22.6
Crains Creek	2,451	3,055	35.5	13.9
Troublesome Creek	2,745	2,152	12.2	10.3
Troublesome Creek	3,065	1,600	5.3	13.3
Mean	987	1,018	14.5	18.6
Standard Deviation	703	663	9.9	16.1

Table 1-5. Mercury concentrations in mussels and sediment from upstream and downstream of road crossings.

	Mercury concentration in mussels and sediment (ng/g dry wt)			
Stream	Upstream Tissue (n=3)	Downstream Tissue (n=3)	Upstream Sediment (n=1)	Downstream Sediment (n=1)
Terrels Creek	33	29	1.2	1.1
Troublesome Creek	42	114	2.8	4.1
Harlands Creek	48	37	1.7	1.5
Densons Creek	197	206	8.1	5.8
Contentnea Creek	235	270	4.9	10.1
New Hope Creek	238	382	9.7	5.8
Six Mile Creek	272	239	8.6	17.9
Eno River	287	405	7.0	0.3
Shocco Creek	311	275	3.4	6.1
Little River	322	389	17.9	23.2
Tar River	366	349	17.3	12.4
Swift Creek	371	359	3.9	3.2
Stony Creek	373	338	5.9	8.8
Black Creek	388	328	6.4	4.8
Little Fishing Creek	397	409	2.3	2.4
Tar River	398	470	14.5	17.1
Troublesome Creek	442	326	4.8	7.8
Little River	447	329	3.6	5.5
Stony Creek	451	431	8.9	18.2
Sevenmile Creek	455	546	15.9	6.8
Contentnea Creek	500	375	3.9	4.9
Sevenmile Creek	500	555	10.8	8.1
Flat River	513	632	4.6	4.7
Crains Creek	542	570	6.1	7.0
Troublesome Creek	545	397	8.9	4.9
Cane Creek	617	652	9.1	5.8
Buckhorn Creek	630	552	6.7	6.6
Long Creek	673	899	6.7	5.3
Bear Creek	706	716	24.7	47.1
Little River	709	638	9.8	10.7
Six Mile Creek	709	550	11.4	22.1
Little Fishing Creek	749	679	4.3	12.2
Crabtree Creek	789	755	7.0	9.0
New Hope Creek	814	162	2.5	3.6
Moccasin Creek	832	568	6.1	12.8
Contentnea Creek	987	898	8.4	11.5
Tar River	1148	968	15.5	10.9
Mean	487	454	8.0	9.5
Standard Deviation	254	228	5.2	8.5

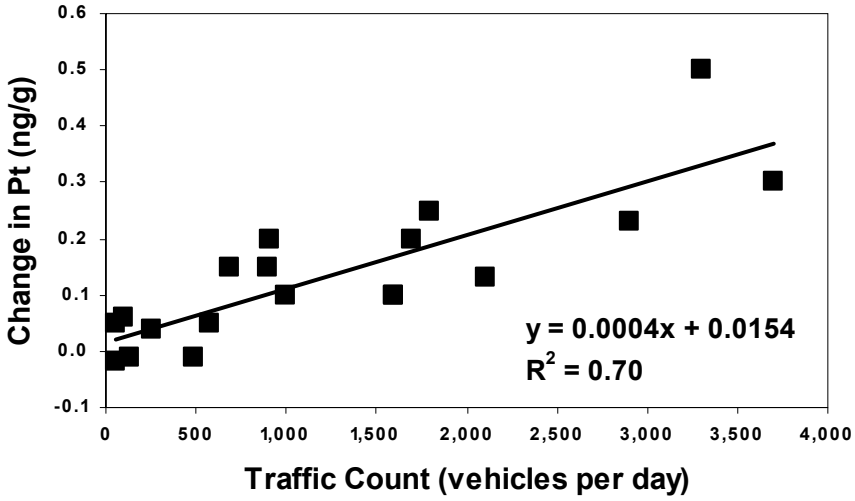
Table 1-6. Change in mean platinum (Pt) concentrations of mussel and sediment between upstream and downstream locations.

		Change in Pt concentration [downstream]-[upstream] ng/g	
Stream	Traffic (vc/d)	Δ[Sediment]	Δ[Tissue]
Long Creek	30	0.06	0.02
Contentnea Creek	390	0.05	0.18
Tar River	430	0.08	0.15
Cane Creek	470	-0.14	0.36
Troublesome Creek	540	0.03	-0.01
Densons Creek	610	-0.06	0.12
Little Fishing Creek	610	0.07	0.20
Little Fishing Creek	610	0.12	0.21
Tar River	680	-0.22	0.06
Bear Creek	690	0.04	-0.04
Troublesome Creek	910	0.04	-0.14
Moccasin Creek	935	-0.01	-0.09
Flat River	1,000	-0.31	-0.25
New Hope Creek	1,000	0.14	-0.19
Eno River	1,200	0.33	0.46
Buckhorn Creek	1,400	0.00	0.95
Contentnea Creek	1,600	0.13	-0.06
Sevenmile Creek	1,800	-0.15	-0.06
Shocco Creek	1,800	0.15	-0.01
Little River	2,000	0.02	0.24
New Hope Creek	2,000	-0.37	-0.10
Terrels Creek	2,200	0.10	0.11
Black Creek	2,300	-0.01	-0.02
Little River	2,400	-0.01	0.22
Stony Creek	2,800	-0.06	-0.23
Swift Creek	3,300	0.11	-0.11
Little River	5,700	-0.01	0.10
Contentnea Creek	5,850	-0.04	-0.02
Troublesome Creek	9,600	0.01	-0.15
Crains Creek	9,650	0.05	0.44
Harlands Creek	12,000	-0.01	0.04
Six Mile Creek	21,500	0.01	-0.47
Six Mile Creek	21,500	0.03	-0.01
Tar River	28,000	0.09	0.06
Stony Creek	43,000	0.47	0.04
Sevenmile Creek	87,000	-0.35	-0.11
Crabtree Creek	147,000	0.00	0.11
Mean		0.01	0.05
Standard Deviation		0.16	0.24

Table 1-7. Five most parsimonious models explaining variance in Pt concentration among upstream and downstream mussel tissue samples. K is the number of model parameters; ΔAICc is the difference between successive model Akaike's Information Criterion values corrected for bias; and w_i is the Akaike's weight, or probability that the model is the most informative model.

Model	K	ΔAICc	w_i
Upstream Mussel Tissue Platinum (ng/g)			
0.1055(ln(population))+0.2811(upstream % TOC)+0.0003(tissue Cd (ng/g))-0.0069(mass)-0.4075	6	0	0.305
0.1092(ln(population))+0.2831(upstream % TOC)+0.0003(tissue Cd (ng/g))-0.0068(mass)+0.0022(%forested)-0.5982	7	2.45	0.089
0.0963(ln(population))+0.2732(upstream % TOC)+0.0003(tissue Cd (ng/g))-0.0067(mass)-0.0732(pH)+0.2030	7	2.95	0.070
0.1085(ln(population))+0.2937(upstream % TOC)+0.0003(tissue Cd (ng/g))-0.0070(mass)-0.0337 (downstream % TOC)-0.3937	7	3.06	0.066
0.1080(ln(population))+0.2849(upstream % TOC)+0.0003(tissue Cd (ng/g))-0.0066(mass)-0.03903(specific conductivity)-0.4027	7	3.25	0.060
Downstream Mussel Tissue Platinum (ng/g)			
0.1258(ln(population))+0.3750(upstream % TOC)+0.0003(tissue Cd (ng/g))-0.0093(mass)+0.0057(%forested)-0.7480	7	0	0.274
0.1185(ln(population))+0.3748(upstream % TOC)+0.0003(tissue Cd (ng/g))-0.0084(mass)-0.3657	6	1.25	0.147
0.1374(ln(population))+0.3923(upstream % TOC)+0.0003(tissue Cd (ng/g))-0.0085(mass)+ 0.0061(%forested) -1.3844(specific conductivity)-0.7672	8	2.36	0.084
0.1146(ln(population))+0.3647(upstream % TOC)+0.0003(tissue Cd (ng/g))-0.0093(mass)+ 0.0062(%forested) -0.0972(pH)+0.0281	8	3.14	0.057
0.1318(ln(population))+0.3730(upstream % TOC)+0.0003(tissue Cd (ng/g))-0.0090(mass)+0.0060(%forested) -(ln(traffic(vc/d))-0.6862	8	3.26	0.054

Figures



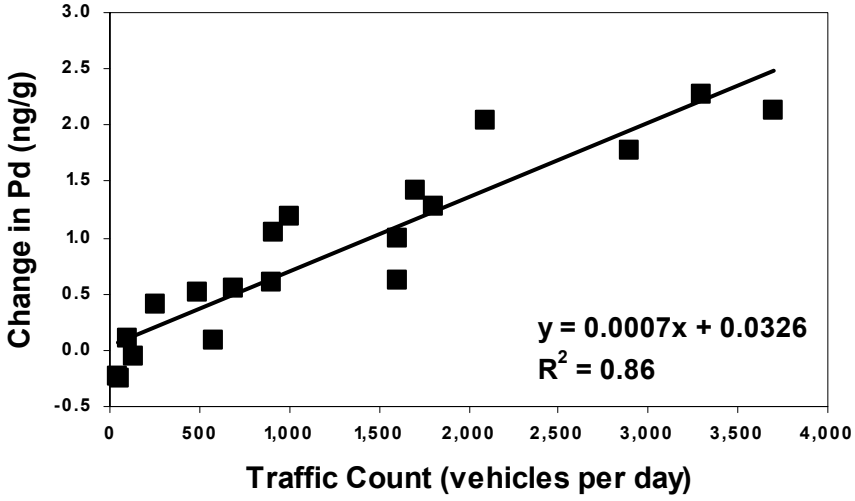


Figure 1-1. Preliminary data on correlation between traffic count and change in Pt and Pd in *Elliptio complanata* tissue between upstream and downstream samples at bridge crossings. Change in elemental concentration was calculated as the downstream concentration minus the upstream concentration (18).

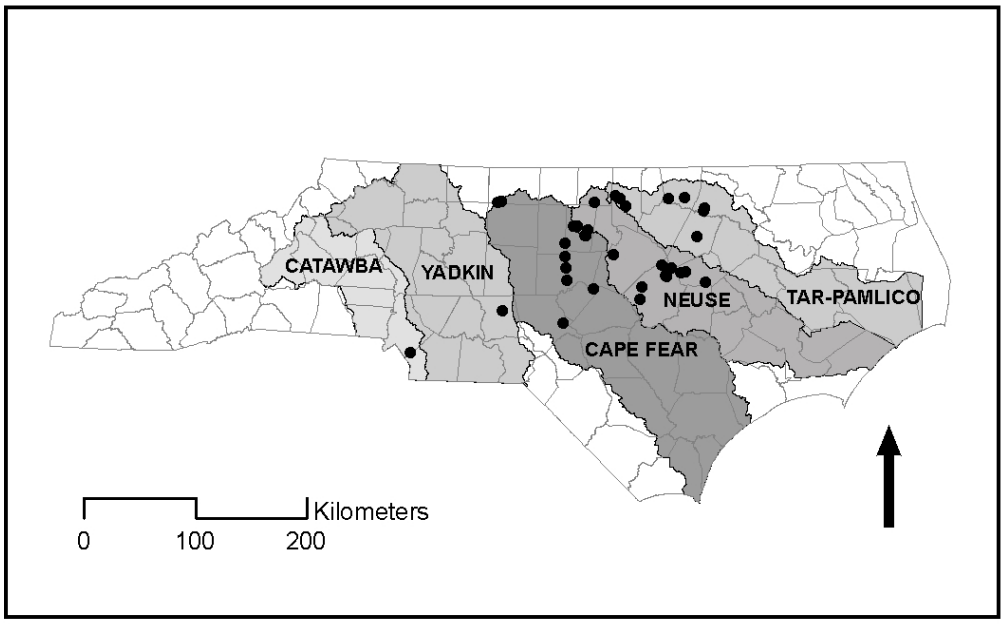


Figure 1-2. Site locations and river drainage basins where upstream and downstream samples of mussels and sediment were collected.

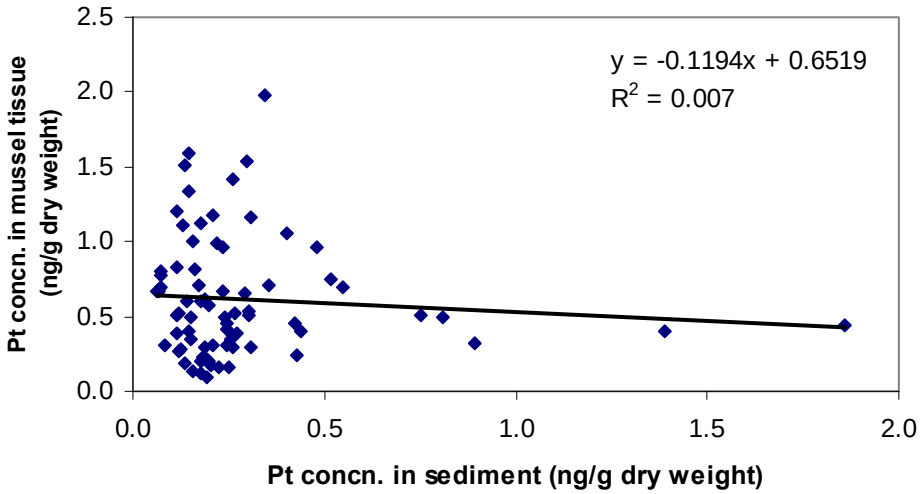
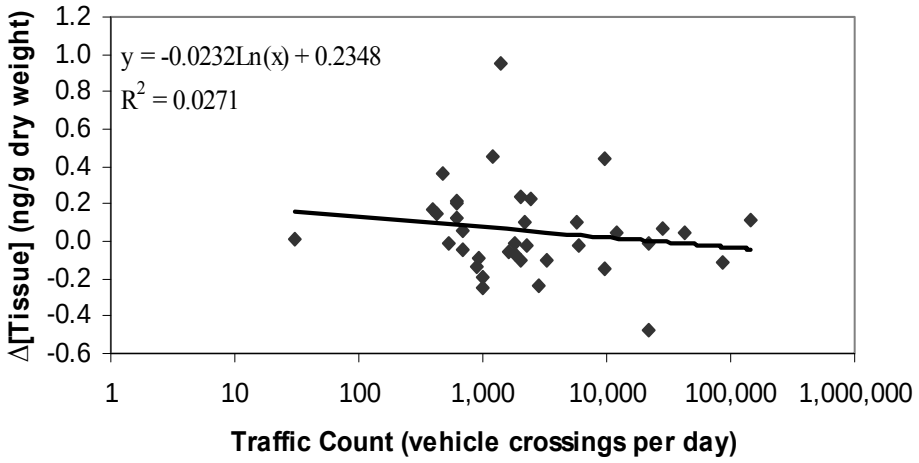


Figure 1-3. Correlation of tissue Pt concentration and sediment Pt concentration from all sampling locations.



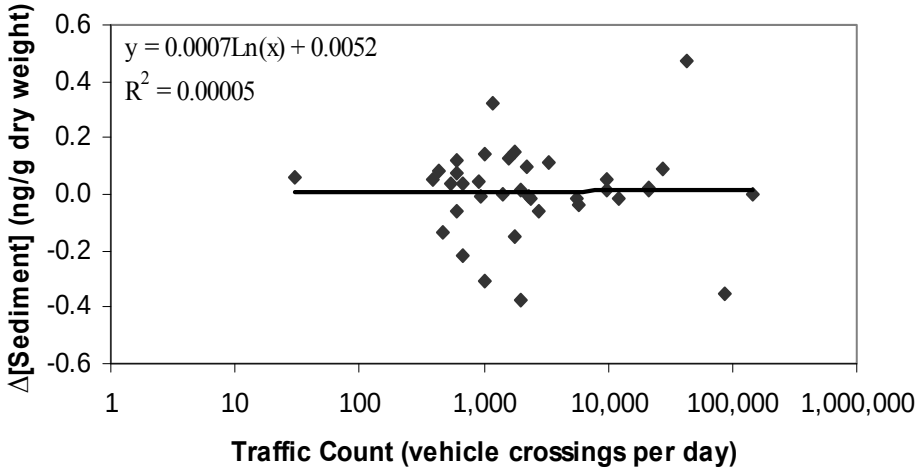


Figure 1-4. Correlation between traffic count at a highway crossing and the change in mussel tissue Pt and sediment Pt concentrations between upstream and downstream samples at the highway crossing.

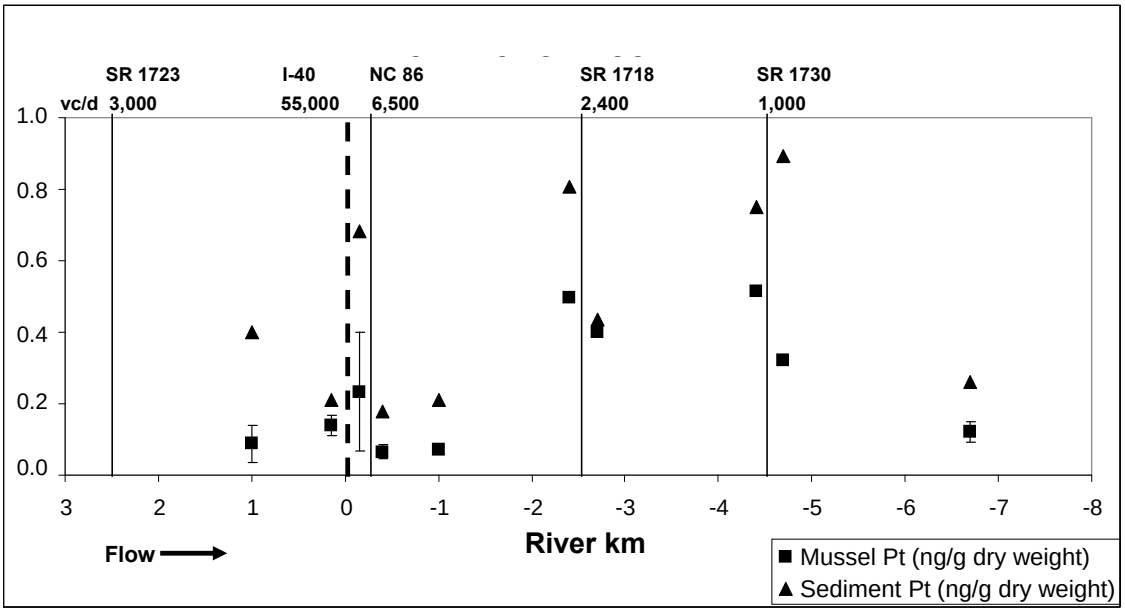


Figure 1-5. Longitudinal pattern of mussel tissue and sediment Pt concentrations in New Hope Creek. Vertical lines represent road crossings of the stream. Road number and traffic count in vehicle crossings per day (vc/d) are displayed across the top. The flow of the stream is from left to right with river kilometers measured from Interstate 40 crossing displayed as a broken vertical line. Where multiple samples were collected error bars display ± one standard deviation.

Chapter 2: 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 μg/g (dry weight), and sediment Pb concentrations ranged from 1.53 – 10.28 μg/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. Our results indicate a trend of decreasing Pb concentrations in sediment with time, and we 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 μm), 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, we 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_2, H_2O, N_2, and SO_2 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 µg/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 µg/g (16%) more Pb than samples taken from upstream and 2 µg/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, we 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, we 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. Our 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 2-1. 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).

	Mean lead concentrations in Mussels (µg/g dry wt)			
vc/d	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 2-2. The average lead concentration in sediment for each site, with the difference between downstream and upstream.

	Mean lead concentration in Sediment (µg/g dry wt)	
vc/d	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

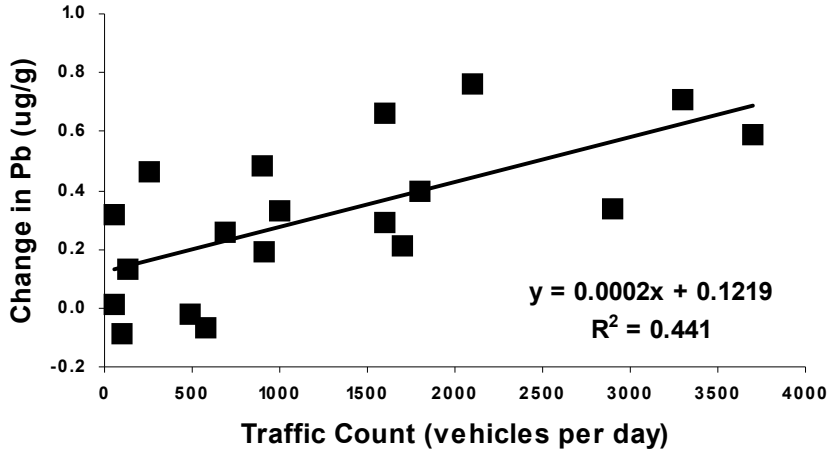

Figure 2-1. Preliminary data on correlation between traffic count and change in Pb among the freshwater mussel Elliptio complanata. (Data from Levine et al. 2005)

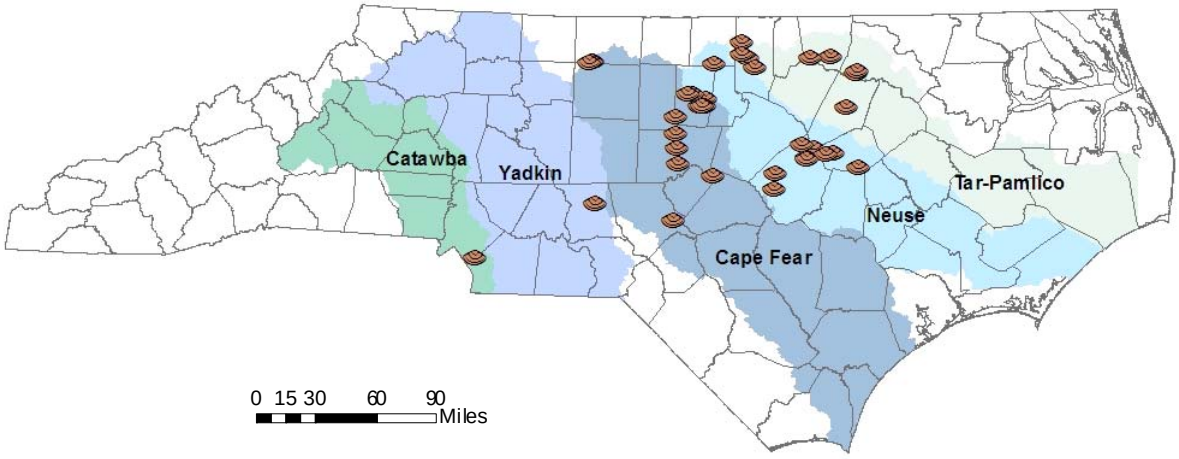

Figure 2-2. Site locations where mussel and sediment samples were taken within North Carolina.

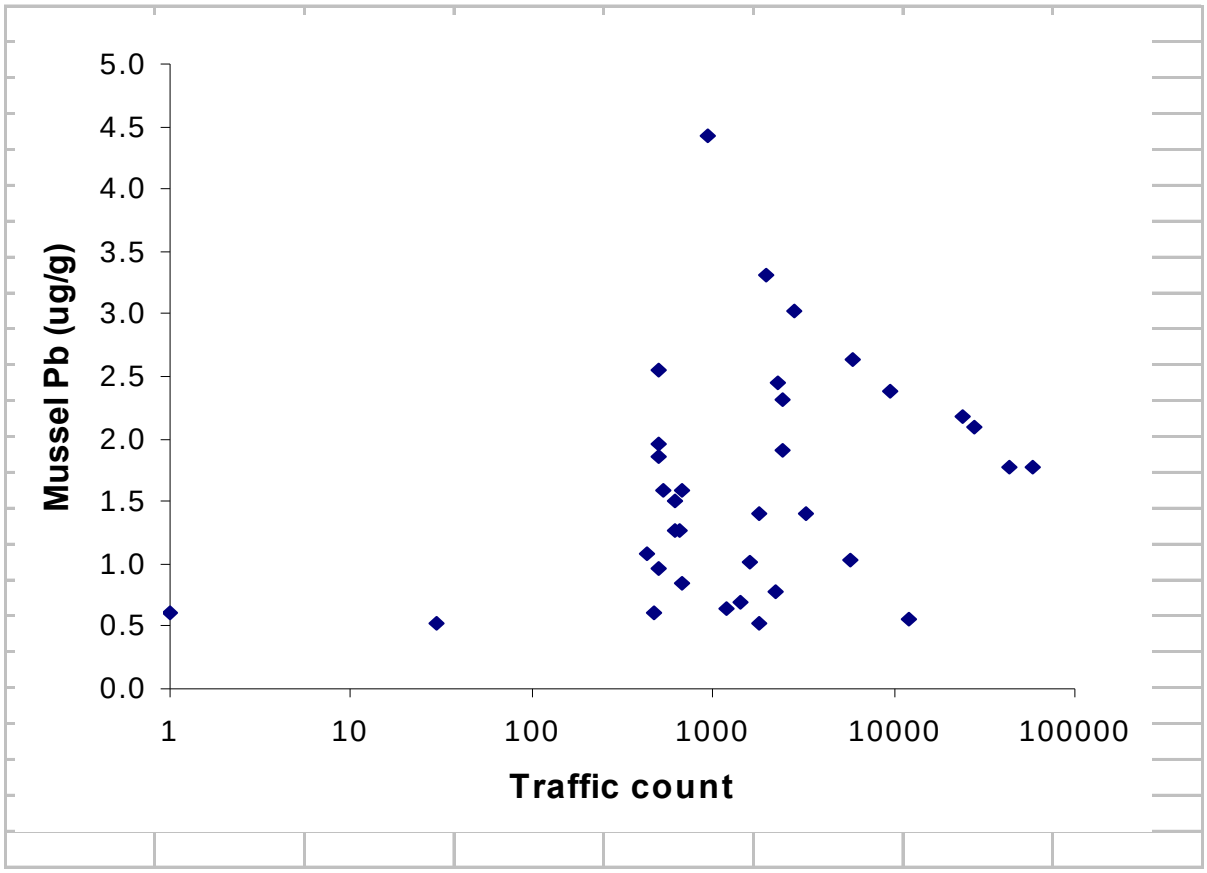

Figure 2-3. The average Pb concentration of mussels for each site with the corresponding traffic count.

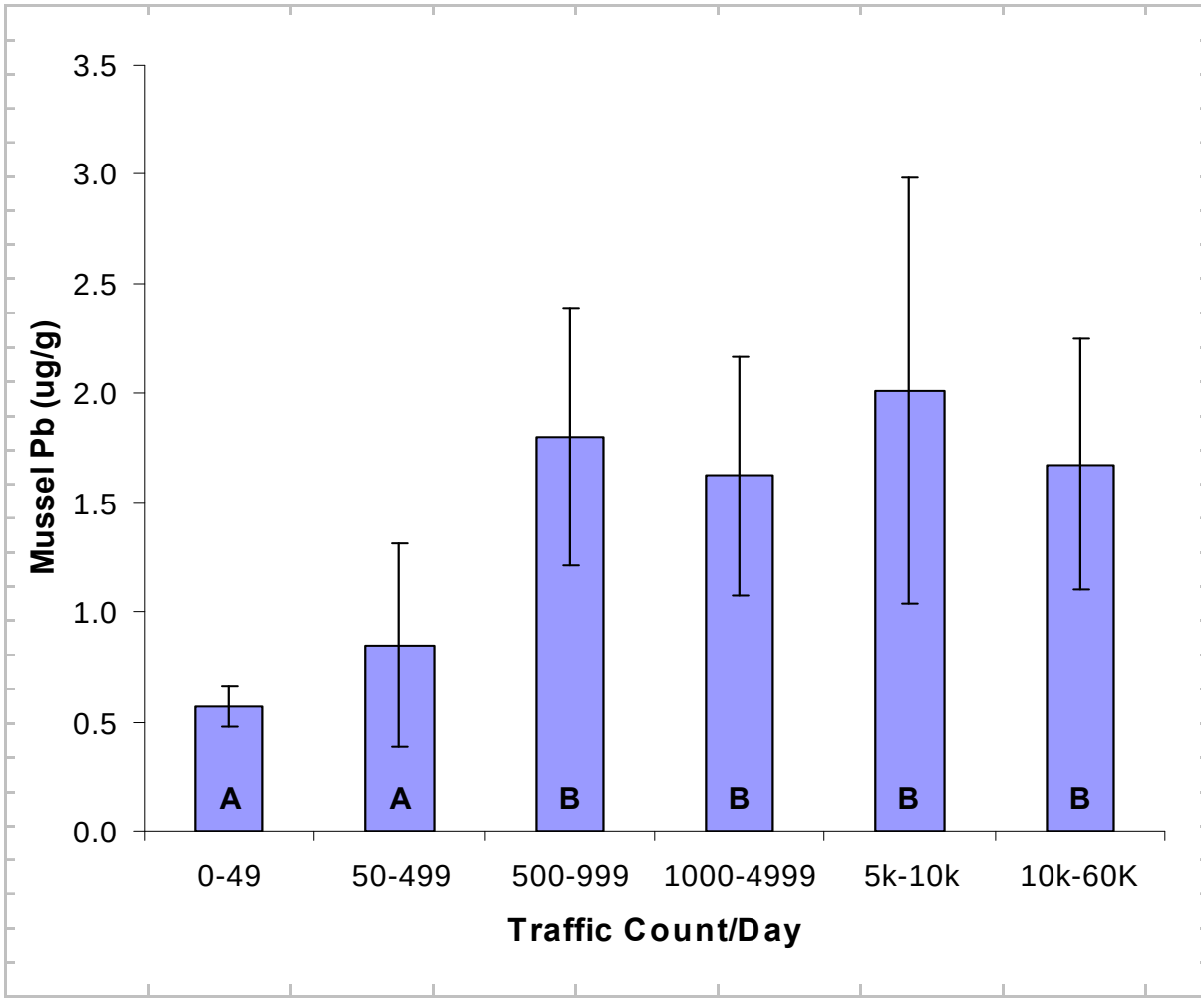

Figure 2-4. The average mussel Pb concentration per traffic group, with error bars representing 95% confidence intervals. Means with different letters are significantly different.

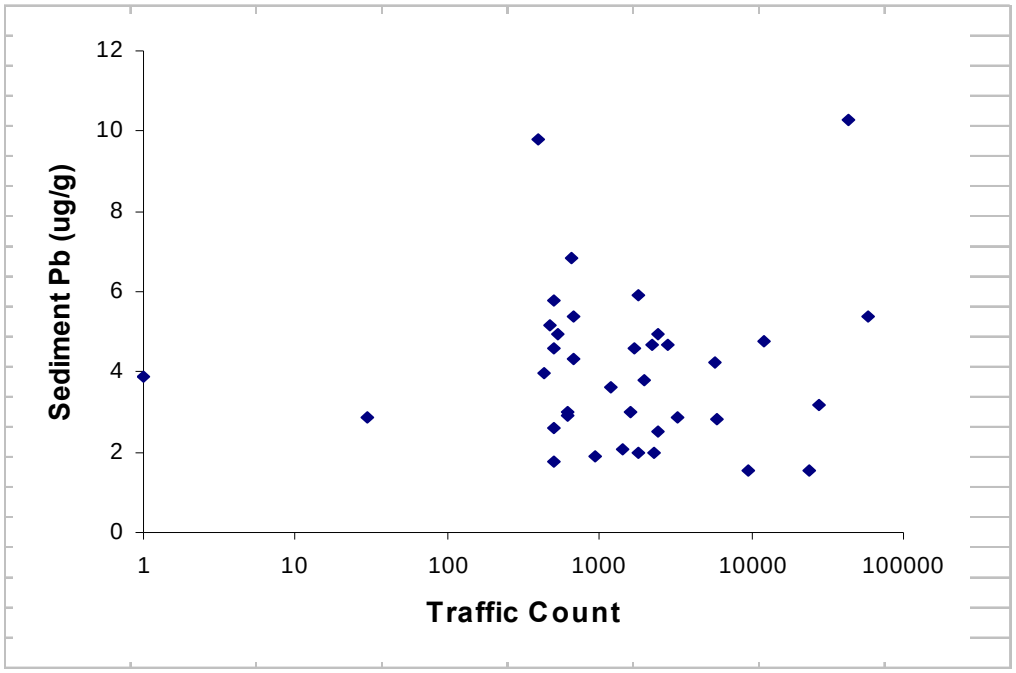

Figure 2-5. Scatter plot of sediment Pb concentrations with traffic count.

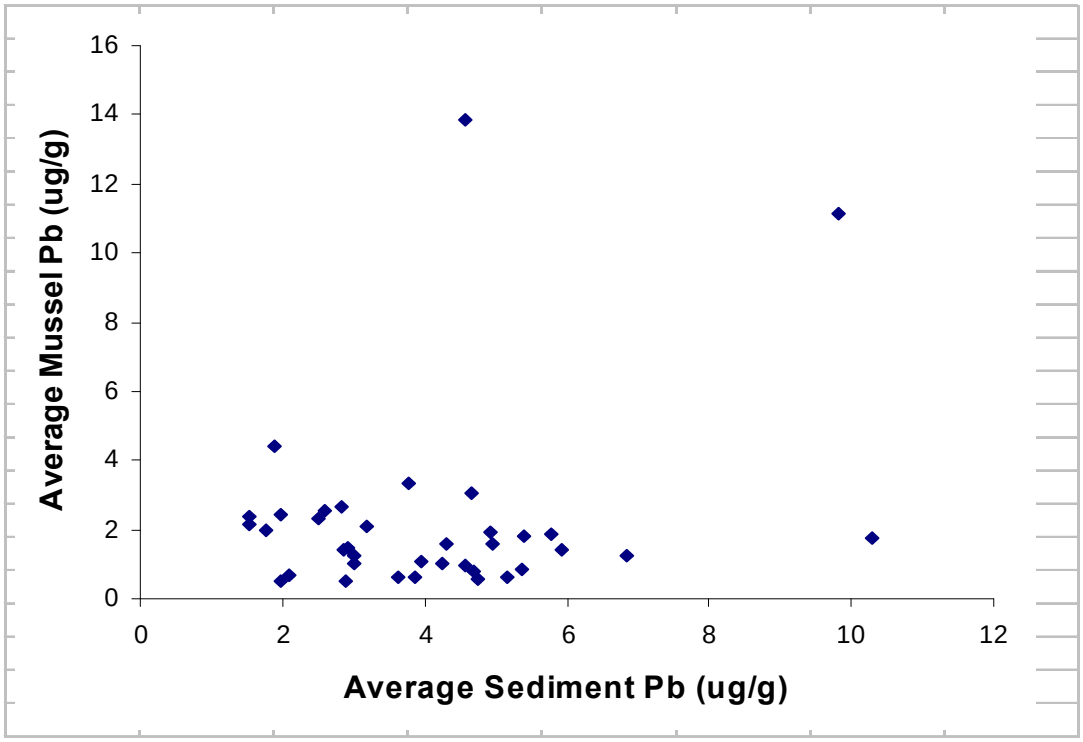

Figure 2-6. Average mussel Pb concentration at each site with average sediment Pb concentration.

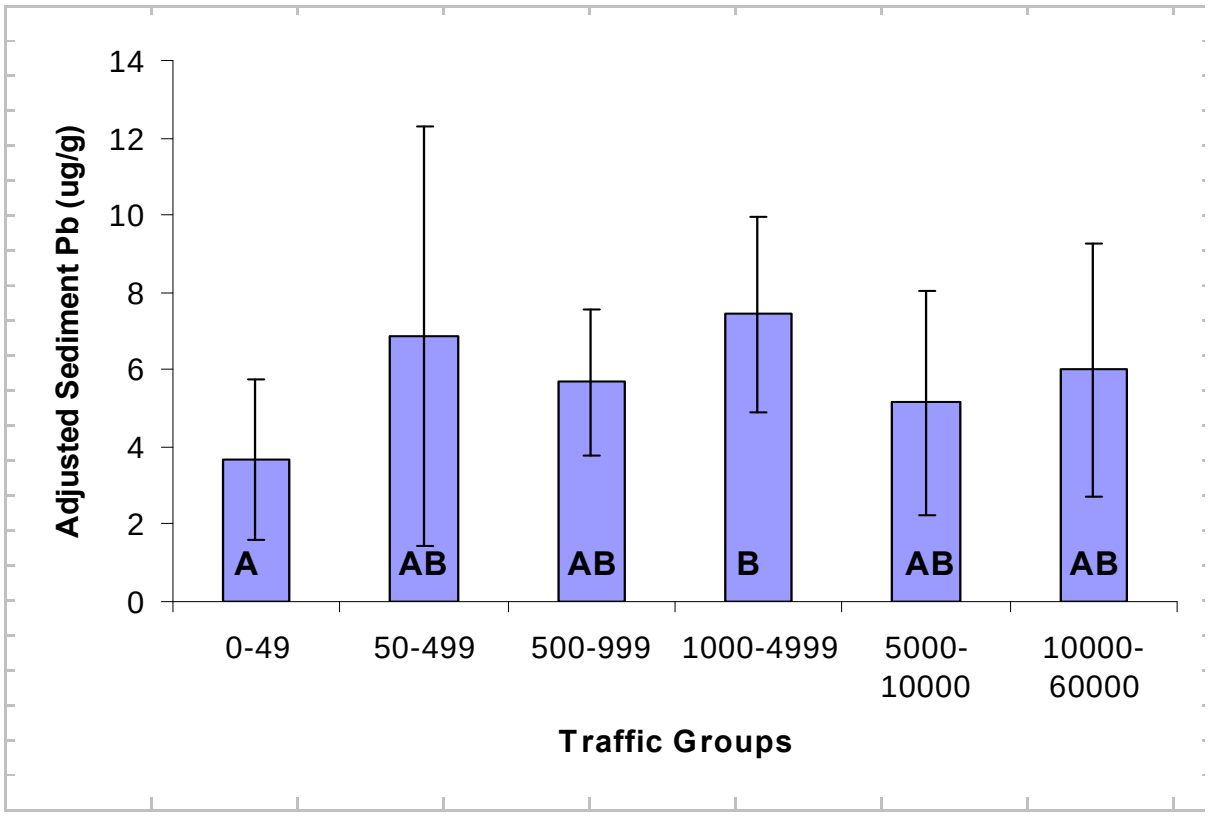

Figure 2-7. The average sediment Pb levels for each traffic group adjusted for the total carbon (TC) content. Error bars represent 95% confidence intervals.

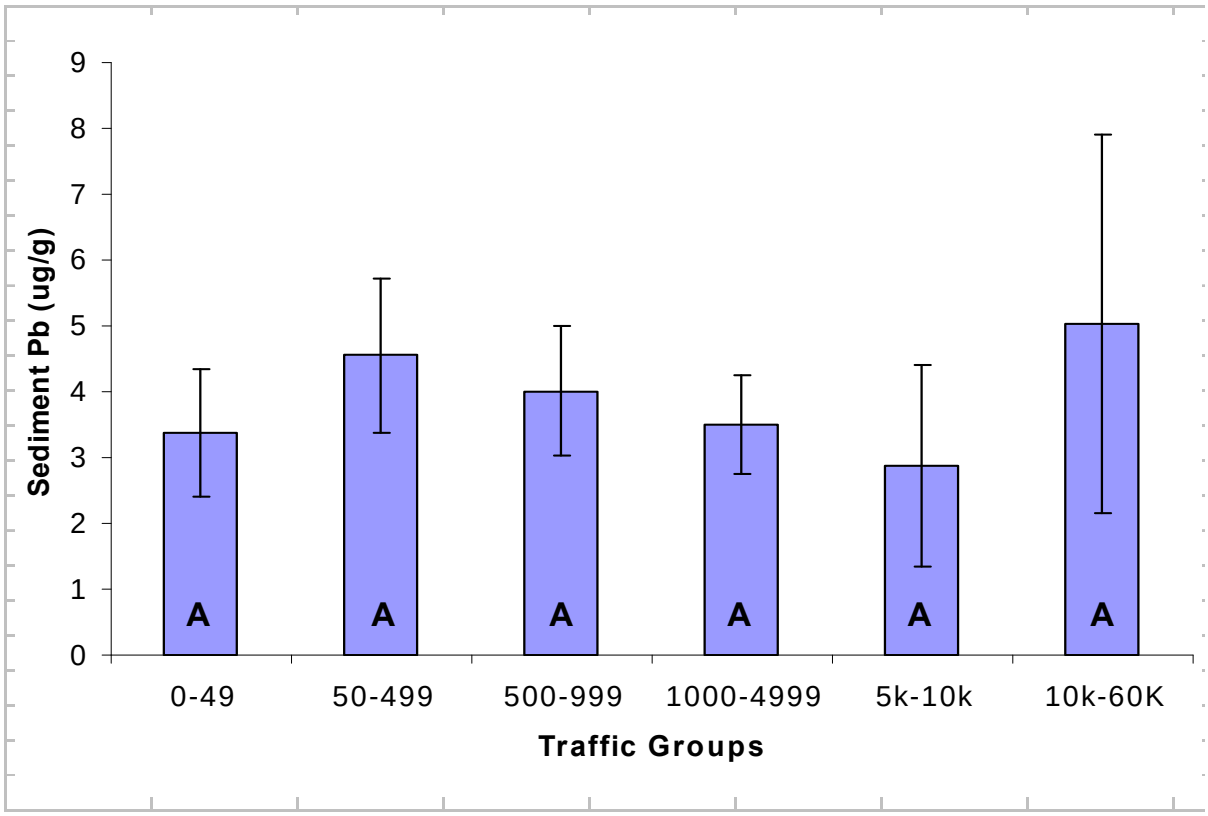

Figure 2-8. Average concentration of Pb in sediment for each traffic group. Error bars represent 95% confidence intervals.

LABORATORY STUDIES

Chapter 3: Evaluation of Na^+, K^+-ATPase activity and hemolymph ion concentrations as biomarkers of Pt and Pd exposure in freshwater mussels

Abstract

A 28-d laboratory test was conducted with waterborne Pt and Pd to determine toxicity, bioaccumulation, and to assess several potential biomarkers of exposure to Pt and Pd. Test mussels were exposed to five concentrations of an equal mixture of Pt and Pd salts, ranging from 0.05 to 500 µg/L of each metal, in a static renewal test. The 500 µg/L concentration resulted in high mortality (4 of 9 dead by day 12) of test mussels; all individuals in each of the other test concentrations survived to the end of the test. There were nine replicate mussels per treatment concentration, allowing three mussels from each treatment to be sampled on days 7, 14 and 28. Tissue and hemolymph were assessed for concentrations of Pt, Pd, sodium (Na^+), calcium (Ca^{2+}), potassium (K^+), and chloride (Cl^-) concentrations. Na^+,K^+-ATPase activity was assessed in gill tissue as a potential biomarker of exposure. Tissue concentrations of Pt ranged from 0.10 ng/g dry weight in controls to 34,486 ng/g dry weight in the 500 µg/L treatment, and Pd ranged from 0.06 ng/g dry weight in controls to 34,404 ng/g dry weight in the 500 µg/L treatment. Concentrations of Pt in hemolymph ranged from 0.08 ng/mL in controls to 50.1 ng/mL in the 500 µg/L treatment, and Pd ranged from 0.06 ng/mL in controls to 12.2 ng/mL in the 500 µg/L treatment. On day 28, Na^+,K^+-ATPase activity displayed a logarithmic trend ($y=0.489\ln(x)+2.054$; $R^2=0.73$) of increasing activity with increasing PGM exposure concentration; however, activity was only significantly increased

($P<0.05$) at 5.0 µg/L and 50 µg/L concentrations. High variation and weak correlation of Na^+,K^+-ATPase activity with Pt and Pd exposure concentration indicate that it may not be a suitable biomarker of PGM exposure. Hemolymph Ca^{2+} levels were increased on day 7 at the 50 µg/L concentration. Hemolymph Na^+ levels were decreased on day 28 at the 5.0 µg/L and 50 µg/L concentrations. Cl^- and K^+ levels were decreased at the 50 µg/L concentration. Hemolymph ion measurement does not appear to be sensitive enough to serve as a biomarker of PGM exposure at environmentally relevant exposure concentrations.

Introduction

In the United States in the mid 1970s and in Europe in the 1980s, laws were enacted to reduce air pollution that necessitated the introduction of catalytic converters for automobiles. The use of catalytic converters for automobile exhaust purification has led to the emission and environmental contamination by the platinum group metals (PGM) platinum (Pt), palladium (Pd), and rhodium (Rh). Recently, there were an estimated 500 million vehicles equipped with catalytic converters world-wide (Rauch 2005). Consequently, concentrations of PGM, formerly among the lowest of any element in the earth's crust (0.4 ng/g Pt and Pd, 0.06 ng/g Rh, Whedepol 1995), are increasing in dust and soils along highways and are being transported into aquatic habitats through surface runoff, where they are accumulating in stream sediments and tissues of organisms (Sures et al. 2001; Sutherland 2003; Zimmermann et al. 2002, 2005).

Bioaccumulation of PGM has been demonstrated in a variety of species. Plants grown on soil treated with soluble forms of PGM accumulate the metals, especially Pd (Ek et al. 2004). A variety of roadside plant species have been shown to accumulate PGM, as well as other traffic related metals (Schafer et al. 1998; Djingova et al. 2003; Lesniewska et al. 2004). The sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, has been shown to accumulate PGM, and this bacterium is similar to bacteria naturally found in decaying detritus in aquatic systems (Yong et al. 2002). European eels (*Anguilla anguilla*) have been shown to bioaccumulate PGM when exposed to road dust solutions under laboratory conditions (Sures et al. 2001, Zimmermann et al. 2002).

Laboratory investigations of PGM uptake in the zebra mussel (*Dreissena sp.*) indicate that PGM derived from automobile catalytic convertors is bioavailable to filter feeding bivalves (Zimmermann et al. 2002). The zebra mussels used in their study had a background level of PGM that was above the detectable limit, indicating that anthropogenic sources of PGM are being accumulated by this species in its natural environment (Zimmermann et al. 2002).

Presently, no information on the bioaccumulation of PGM in unionid mussels is available. Freshwater mussels are known to bioaccumulate other anthropogenically derived metal pollutants, such as cadmium (Cd), copper (Cu), and zinc (Zn); however, the relationship between tissue burden of these metals and local environmental contamination varies among species (Gundacker 2000). *Elliptio complanata* sampled from the St. Lawrence River, Canada, accumulated 2.5 times more nickel (Ni), an element similar to the PGM, and displayed less variation than a co-occurring and

intermixed species, *Lampsilis radiata* (Metclafe-Smith 1995), indicating that *E. complanata* might be a good sentinel of PGM contamination.

Freshwater mussels are of particular interest in assessing effects of aquatic pollutants due to their sensitive early life stages (glochidia, juveniles) and their imperiled status world-wide (Cope et al. 2008). About 67% of the nearly 300 freshwater mussel species found in North America are considered vulnerable to extinction or are already extinct (Bogan 1993). The decline of many freshwater mussel species and the demonstrated toxicity of PGM in other species, including bivalves (Wolterbeek and Verburg 2001, Frank et al. 2008), make the potential of bioaccumulation of PGM in this faunal group cause for concern.

The purpose of this study was to investigate the toxicity and bioaccumulation of Pt and Pd in *E. complanata* from laboratory waterborne exposures. Mussels were assessed for mortality and sublethal changes in hemolymph sodium (Na^+), calcium (Ca^+), potassium (K^+) and chloride (Cl^-) ion concentrations. Additionally, the membrane bound ion transporter enzyme Na^+,K^+-ATPase was assessed as a potential sublethal biomarker of PGM exposure.

Materials and Methods

Collection, Transport, and Holding of Mussels

Laboratory toxicity testing followed the ASTM guidelines for conducting laboratory toxicity tests with freshwater mussels (ASTM 2006), as modified for use with adults. Sixty-three adult *E. complanata* were collected from a relatively uncontaminated,

rural, forested section of the Eno River near Hillsborough, North Carolina. Mussels were collected by hand, placed in ice chests, and covered with damp nylon mesh bags to prevent desiccation and reduce stress during transport (Cope et al. 2003). Mussels were transported directly to the laboratory (30 min transportation time), where they were scrubbed with a soft-bristle brush and rinsed with deionized water. Fifty-nine mussels were assigned at random to individual test aquaria containing two L of ASTM soft water (ASTM 1993). Aquaria were labeled with one of six exposure concentrations (0-500 µg/L) and day to be sampled. Four remaining mussels were placed in cold storage (-80 ºC) to serve as a baseline measurement for PGM contamination at the collection site. Test water was continuously aerated by a central aeration unit (Sweet Water Air Pump SL24 Aquatic Eco-Systems Inc., Apopka, Florida, USA). Each batch of ASTM soft water was measured for pH with a Beckman Model 240 (Beckman Instruments, Fullerton, California, USA) calibrated meter. Alkalinity was determined by titration of 0.02 M H_2SO_4 to pH 4.5. Hardness was determined by titration with 0.01 M ethylenediaminetetra-acetic acid (EDTA). Water in the test aquaria was periodically tested for temperature, pH, conductivity, and dissolved oxygen at 0, 48, and 72 hour time points with a calibrated multi-probe (YSI Model 556 MPS, Yellow Springs Instruments, Yellow Springs, Ohio, USA). Physiochemical characteristics of water in the test jars averaged 18.9 °C (range 18.6 – 19.2 °C) for temperature, dissolved oxygen 9.1 mg/L (range 8.77- 9.37 mg/L), pH 7.5 (range 7.3-7.6), alkalinity 30 mg/L as $CaCO_3$ (range 29 – 31 mg/L as $CaCO_3$), and hardness 42 mg/L (range 42– 44 mg/L). Composite water samples were taken, 5 mL from 3 jars per concentration, at 0, 48 and 72 hour time points

for Pt and Pd concentration verification. Water was stored in 25 ml vials with 75 µL of concentrated trace metal grade nitric acid (Ultrex®) for analysis.

Experimental Procedures

Test mussels were allowed to acclimate to test conditions 5 d prior to test initiation, with a 100% water renewal of ASTM water on the third day prior to the test. Mussels were fed for the first time on the morning on the first day of the 28 d exposure and then every 48 h or 72 h prior to test water renewal and introduction of the Pt/Pd test solution. Each jar received 25 mL of a food suspension containing 2 mL of Instant Algae® Shellfish Diet and 1 mL *Nannochloropsis* concentrate (Reed Mariculture, Campbell, California, USA) in 1 L of deionized water. The test solution was a mixture of dissolved Pt and Pd, both at 1,000 ppm in 5% HCl (High-Purity Standards catalog # 100038-2 (Pd) and 100040-2 (Pt)). Test solution was added to jars by pipette to yield the appropriate test concentration.

Mussels were sampled and processed on days 0, 7, 14, and 21. During processing, mussels were weighed (to the nearest 0.1 g) and measured (to the nearest mm), gently pried open, and had 1 mL of hemolymph extracted from the anterior adductor muscle with a 25 gauge syringe (Gustafson et al. 2000). The hemolymph was divided equally into two 1.2 mL cryotubes for analysis of Pt, Pd concentration and ion concentration (Na^+, K^+, Cl^-, Ca^{2+}); samples were stored at $\geq$-20°C and -80°C, respectively. From each individual, mussel gill tissue was dissected and three samples (~15 mg each) were placed individually in 1 mL centrifuge tubes, on ice, with 100 µL of SEI buffer solution (sucrose 250 mM, disodium ethylenediaminetetraacetic acid

dihydrate ($Na_2EDTA\ 2H_20$) 10 mM, imidazole 50 mM) for Na^+,K^+-ATPase activity analysis. The remaining whole mussel was bagged and stored frozen ($\geq$-20 °C) for tissue Pt and Pd analysis.

Na^+,K^+-ATPase Activity Assay

The Na^+,K^+-ATPase activity was determined using the method of Mosher (2008), as modified for mussels. The method used by McCormick (1993) to assess Na^+,K^+-ATPase activity in fish gill tissue relied on ouabain to inhibit Na^+,K^+-ATPase activity, however research performed by Mosher (2008) showed that ouabain was not effective at inhibiting Na^+,K^+-ATPase activity in *E. complanata*. Therefore, a K+-free salt solution was used in place of ouabain and effectively inhibited Na^+,K^+-ATPase activity for the assay.

The following procedure was adapted from Mosher (2008). An assay mixture (AM) was prepared fresh each day. Aliquots of phosphoenolpyruvate (PEP) and adenosine diphosphate (ADP) were removed from the -80 ºC freezer and thawed. 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 combined and centrifuged at 12,000 x g for eight min at 4ºC using an Allegra^{TM} 25R Centrifuge, Beckman Coulter, Fullerton, CA, USA. The pellet was re-suspended with imidazole buffer, and added to the graduated cylinder along with 4.7 mL PEP. The volume was brought to 37.5 mL with imidazole buffer, and the completed AM was mixed. Two test solutions were prepared with a 3:1, AM:salt solution ratio. For solution

A, 15 mL of AM was mixed with 5 mL of a salt solution (imidazole 50mM, NaCl 189mM, MgCl 10.5mM, KCl 42mM) and stored on ice. For solution B, 15 mL of AM was mixed with 5 mL of K^+-free salt solution (imidazole 50mM, NaCl 189mM, MgCl 10.5mM) and stored on ice. Solution A is used to measure total ATPase activity and solution B is used to measure ATPase activity minus the activity of K^+ dependent Na^+,K^+-ATPase activity. Mussel gill samples were thawed and immediately homogenized with 25 µL 0.3% SEID (0.0751 g Na deoxycholic acid in 25 mL SEI) by grinding in a centrifuge tube for 20-30 s using 1.5 mL centrifuge tube pellet pestles. All samples were read within 30 min of processing. The homogenates were centrifuged at 5,000 x g for one minute at 4ºC, and 10 µL of supernatant was pipetted into each of four wells per sample on a 96-well plate. Then 200 µL of solution B (without K^+) was added to two of the four wells for each sample, and 200 µL of solution A was added to the other two wells. An ADP standard curve was measured in the first plate of each day running the assay using 4 mM ADP stock solution. ADP stock solution was diluted with imidazole buffer to concentrations of 0, 5, 10 and 20 nMoles ADP/10 µL and 10 µL were pipetted into 3 wells per concentration, and 200 µL of solution A was added to each well. Plates were analyzed at 340 nm for 10 min at 1 min intervals using a Fusion™ Universal Microplate Analyzer (A153600 Meriden, Connecticut) and results were read in milli Optical Density (mOD)/nmole ADP. ATPase activity was calculated from the depletion of ADP over the 10 minute interval as mOD/10 µL /min. The Na^+,K^+-ATPase activity was calculated as the difference between the total ATPase activity measured in the wells with solution A and the ATPase activity with Na^+,K^+-ATPase inhibited in wells with

solution B. A Bradford Protein Assay Kit (Shelton Scientific-IBI; VWR #14221-496) was used to determine protein concentration, and the final Na^+,K^+-ATPase activity was measured as μmoles ADP/mg protein/hour (Bradford 1976). A more detailed description of the necessary mixtures and calculation is included in Appendix 1.

Sample Analysis

All mussel hemolymph and tissue samples were analyzed using a Thermo X-Series II Inductively Coupled Plasma Mass Spectrometer (ICP-MS) at Research Triangle Institute (RTI, Durham, North Carolina, USA) for concentrations of Pt and Pd. Prior to analysis, the instrument was optimized for signal to noise and a system suitability check consisting of 10 replicates of a multi-element standard was run. The Percent Relative Standard Deviation (%RSD) of the 10 replicates was required to be <2% for the instrument to be considered ready for analysis. The ICP-MS was calibrated at the beginning of each analytical run using a series of dilutions prepared from a NIST-traceable stock solution matched to the acid matrix of the samples. The minimum acceptable correlation coefficient for the standard curve was 0.995. A calibration check was performed immediately after the calibration, at an interval of no more than every 10 samples, and at the end of the analysis so that all samples analyzed in a batch were bracketed by calibration checks. The calibration check was prepared from a separately prepared NIST-traceable stock solution than the calibration standards and at a concentration that was not used in the calibration curve. Measured concentrations of the target elements in mussel tissue were expressed as ng/g dry weight and concentrations in hemolymph were expressed as ng/mL.

Mussel Tissue

At the time of processing, mussel samples were removed from the freezer, partially thawed, dissected from their shells and placed into tared, acid-cleaned 50 mL digestion tubes. The samples were then weighed to the nearest 0.1 g wet weight and were frozen for a minimum of 24 h at ≥ -20ºC. After freezing, the samples were placed in a freeze dryer for a minimum of 36 h. After lyophilizing, the samples were reweighed to determine the dry weight of the sample. All samples were coarsely ground in the plastic digestion tubes with plastic spatulas.

The samples were treated with 2.0 mL of concentrated nitric acid (Ultrex®) and 2.0 mL of concentrated hydrochloric acid (Ultrex®) and allowed to stand at room temperature for approximately 1 h. The samples were then placed in a graphite digestion block with a digital temperature control module. The samples were heated on an automated program for 1 h at 50ºC and 1 h at 80ºC. The samples were removed, allowed to cool, and then 0.5 mL each of concentrated nitric and hydrochloric acids and 3 mL of deionized water were added to each sample. The samples were returned to the block digestion unit and heated for 6 h at 101ºC. Once the digestion program was completed, the samples were allowed to cool and brought to a final volume of 40 mL using deionized water. The samples were tightly capped, shaken, and a 4 mL aliquot was taken for analysis.

Mussel Hemolymph

The mussel hemolymph samples were stored and transported to RTI in 1.2 mL cryotubes. A 0.25 mL aliquot of hemolymph was transferred to an acid washed 15 mL plastic centrifuge tube. 4.8 mL of a 2.5% HCl/ 2.5% HNO_3 acid extraction solution was added to each sample and each sample was vortex mixed. All samples were placed in a water bath at 60°C for 30 min. Samples were removed from the bath, vortex mixed, allowed to cool to room temperature, and then centrifuged for 30 min at 2,800 RPM. A 3 mL aliquot of the supernatant liquid was removed for analysis.

Statistical Analysis

Results for Na^+,K^+-ATPase activity were analyzed following a mixed-effects 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, North Carolina). Homogeneity of variances among treatments was tested with Brown Forsythe's test available in SAS (Proc GLM). Residual plots were used to visually analyze statistical assumptions for the analysis of variance: homogeneity, normality, and independence of errors. Response variables that showed increasing variability with increasing response were log transformed (log: base 10) prior to analysis to achieve homogeneity of variances. Means were compared using Dunnett's test.

Results

The relationship between exposure concentration and tissue concentration of Pt and Pd and were highly correlated with exposure concentration and were fit to a power function (Table 2, Figure 1). Accumulation of Pt and Pd from solution was linear and increased continuously throughout the experiment. The relationship between exposure concentration and hemolymph concentration for both metals was also modeled by a power function, but displayed more variation than tissue concentrations and less correlation with exposure concentration (Table 3, Figure 2). Hemolymph uptake was non- linear and for all exposure concentrations, reached a maximum concentration on day 7 or day 14 and decreased by day 28.

All mussel tissue and hemolymph concentrations from the exposed groups were greater than the tissue and hemolymph concentrations from the control groups. By day 28 the mussel tissue concentrations from the lowest exposure concentration group (0.05 µg/L) were 14.7 times greater for Pd and 16.5 times greater for Pt than. Hemolymph concentrations in the 0.05 µg/L group were 8.0 times greater for Pd and 1.9 times greater for Pt than hemolymph concentrations in controls on day 28.

Na^+,K^+-ATPase activity in gill tissue initially decreased during the 5 d acclimation period. The Na^+,K^+-ATPase activity of baseline mussels was 4.0 µmoles ADP/mg protein/hour, but after 5 d of acclimation in laboratory conditions, the Na^+,K^+-ATPase activity in mussels sampled on day 0 had decreased to a mean of 1.63 µmoles ADP/mg protein/hour. Mussels sampled on day 7, 14, 28 displayed a general trend of

increasing Na^+,K^+-ATPase activity with increasing Pt and Pd exposure concentration. On day 7 and day 28, the lowest exposure group (0.05 µg/L) was slightly less than controls, but the difference was not statistically significant. All other Na^+,K^+-ATPase activities were greater than controls and the increase was modeled by a power function (Table 4). Due to high variation within treatment groups, the only comparisons that were statistically different from the same day control, using Dunnett's adjusted probability values ($P<0.05$), were the 5.0 µg/L and 50 µg/L treatment, from the day 28 samples (Figure 3).

Ion concentrations in mussel hemolymph initially responded to increased Pt and Pd contamination by increasing Ca^{2+} and decreasing Na^+ concentration. K^+ concentrations were not decreased until day 28 of the experiment and Cl^- concentrations remained fairly stable. All hemolymph ion relationships were described by logarithmic equations (Table 5).

Hemolymph Ca^{2+} concentrations tended to increase with increasing exposure concentration during the first two weeks of exposure (Figure 4). On day 7, Ca^{2+} concentrations in the two highest exposure treatments, 50 µg/L and 500 µg/L, were statistically different from the controls. By day 14, Ca^{2+} levels in the 50 µg/L group had dropped below concentrations reflecting statistical significance, but concentrations in the 500 ug/L treatment remained significantly higher than controls. Hemolymph Na^+ concentrations tended to decrease with increasing exposure concentration throughout the exposure (Figure 5). By day 28, Na^+ concentration in the 5.0 µg/L and 50 µg/L treatments were significantly decreased compared to control. Hemolymph K^+ ion levels

displayed a shifting trend during this experiment (Figure 6). On day 7, hemolymph K^+ ion levels displayed a decreasing trend with increasing exposure concentration. On day 14, K^+ ion concentrations increased with increasing exposure concentration, and by day 28, the trend was again decreasing with increasing exposure concentration, with the 50 µg/L treatment statistically different from control. Hemolymph Cl^- concentrations were highly decreased in the 500 µg/L treatments on days 7 and 14 and moderately decreased in the 50 µg/L treatment on day 28 (Figure 7).

Discussion

The present study demonstrates that *E. complanata* is able to accumulate Pt and Pd from solution. The uptake of Pt and Pd was linear and increased continuously throughout the 28 d exposure, indicating that the mussels had not reached equilibrium and may have continued to accumulate Pt and Pd for a longer period. In a similar study, zebra mussels continued to accumulate Pd from solution at a concentration of 500 µg/L for 10 weeks without reaching equilibrium and without significant mortality (Frank et al. 2008). Tissue concentration for both Pt and Pd were highly correlated with exposure concentration, indicating that *E. complanata* is a good bioindicator of PGM in solution prior to reaching equilibrium.

Hemolymph concentrations of Pt and Pd were more variable and less correlated with exposure concentration than mussel tissue concentrations. Instead, Pt and Pd concentrations in mussel hemolymph reached their peak on day 7 or day 14 for all treatments, but subsequently declined and were all decreasing by day 28 despite

continuing increases in mussel tissue concentration. The mechanism responsible for this decrease in hemolymph concentrations is unknown. However, the decreased correlation with exposure concentration makes hemolymph Pt and Pd concentrations less useful than whole tissue concentrations as a biomarker of PGM contamination.

Hemolymph ion concentrations were assessed for use as a biomarker of Pd and Pt exposure. In this study mussel Ca^{+} was increased compared to control on day 7 of the exposure in the 50 µg/L, but these concentrations were within reference levels of hemolymph Ca^{+} reported by Gustafson et al. (2005a) and had fallen back to within the control range by day 14. K^{+} was variable throughout the exposure and concentrations were only significantly decreased on day 28 in the 50 µg/L treatment. The most consistent fluctuation in hemolymph ion levels was the decrease in hemolymph Na^{+} concentrations at the higher exposure concentrations. For each sampling period, the concentration of Na^{+} in the hemolymph in the 50 µg/L treatment was less than the lowest hemolymph Na^{+} level (344 mg/L) reported by Gustafson et al. (2005b). A similar pattern of impaired osmoregulation has been described in the freshwater mussel *Anodonta cygnea* exposed to Cd, where an immediate decrease in Na^{+} was compensated for by an increase in Ca^{+} that soon stabilized and was later followed by a drop in K^{+} (Hemelraad 1990a). In a later study, it was suggested that the mechanism responsible for the changes in hemolymph ion levels was attributable to interference with oxidative metabolism within the cells of the gill and those of the kidney, affecting influx and efflux rates of ions (Hemelraad 1990b). If this were true in *E. complanata,* a switch to increased anaerobic metabolism in the cells of the gill could lead to an excess of NH^{4+} ion in the cell, which

has been shown to upregulate Na^+,K^+-ATPase activity in the freshwater shrimp *Macrobrachium olfersi* (Furriel 2004).

The initial decrease in Na^+,K^+-ATPase activity observed between the baseline mussels and the day 0 mussels could be a natural response to acclimation to the laboratory environment. The ASTM soft water used to contain mussels in the laboratory had a greater average specific conductivity (165 µS/cm) compared to the specific conductivity of the water from the Eno River, North Carolina (88 µS/cm), where the source mussels were collected. Na^+,K^+-ATPase activity in mussels from control treatments increased slightly throughout the experiments, but remained within the 95% confidence interval of the day 0 mussels throughout the experiment, and Na^+,K^+-ATPase activity increased in mussels exposed to Pt and Pd. By day 28, the Na^+,K^+-ATPase activity in mussels from the 5 µg/L and the 50 µg/L had increased to the levels observed in the baseline mussels. The increase in Na^+,K^+-ATPase activity observed in this study could represent a compensatory mechanism used by the gill cells to compensate for the negative effects of reduced oxidative metabolism and impaired gill and renal function.

The combination of high variability in Na^+,K^+-ATPase and the lack statistical significant difference in activity levels at environmentally relevant concentrations make the use of Na^+,K^+-ATPase activity questionable as a biomarker of Pt and Pd exposure. The additional confounding issue of activity fluctuation due to fluxes in ambient solute concentrations would make it unusable as a biomarker in the field.

Significant changes in Na^+,K^+-ATPase activity and in ion concentrations were only observed at the highest exposure concentrations, despite significantly elevated levels

of tissue Pt and Pd in all of the exposure groups. Based on the responses measured in this study, the environmentally relevant concentrations of Pt (<2.0 ng/g in mussel tissue) and Pd may not pose an urgent concern. However, this study constitutes only a first step in elucidating the dynamics and effects of PGM in freshwater mussels. The response to PGM exposure should be evaluated in other species and in additional life stages in mussels to ensure that chronic low level PGM contamination does not have an adverse impact on mussel species density and richness at environmentally relevant concentrations.

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Tables

Table 3-1. Average daily exposure of Pt and Pd (µg/L) in test water sampled from aquaria throughout the 28 d exposure period. Three 5 mL samples were collected from three aquaria per concentration at random and combined for analysis. Time (T) is represented in hours after addition of the Pt/Pd test solution and values are an average of the sample concentrations collected at the corresponding time point. % of Target is the percent of the intended exposure concentration of Pt or Pd that was recovered at each time point. The average daily exposure is the calculated by averaging three T 0, three T 48, and one T 72 time points.

Target Pt	Mean Measured Concentration (ug/L)						
Concentration (ug/L)	T 0 (*n=4)	% of Target	T 48 (*n=3)	% of Target	T 72 (*n=4)	% of Target	Average Daily Exposure
Control 0	0.03	n/a	0.01	n/a	0.01	n/a	0.00
0.05	0.04	80.0	0.04	87.3	0.04	77.0	0.04
0.5	0.38	75.3	0.34	68.2	0.32	63.8	0.35
5	4.50	89.9	3.77	75.5	3.55	71.1	4.05
50	51.77	103.5	36.34	72.7	29.37	58.7	41.96
500	454.25	90.9	382.39	76.5	129.77	26.0	377.10

* Each sample is a composite sample from three aquaria

Target Pd	Mean Measured Concentration (ug/L)						
Concentration (ug/L)	T 0 (*n=4)	% of Target	T 48 (*n=3)	% of Target	T 72 (*n=4)	% of Target	Average Daily Exposure
Control 0	0.00	n/a	0.00	n/a	0.00	n/a	0.00
0.05	0.00	-1.0	0.01	10.0	0.01	13.0	0.00
0.5	0.04	8.0	0.09	18.8	0.09	18.8	0.07
5	0.60	12.1	1.02	20.3	1.07	21.4	0.85
50	12.83	25.7	5.72	11.4	7.38	14.8	9.01
500	427.26	85.5	125.94	25.2	95.80	19.2	250.77

* Each sample is a composite sample from three aquaria

Table 3-2. Regression of Pt and Pd concentrations (ng/g) in mussel tissue as a power function of dissolved Pt and Pd exposure concentration at 7, 14 and 28 d of exposure (x= exposure concentration (ng/L)+1, y= tissue concentration (ng/g)).

Element	Source	Day	Equation	R^2
Platinum	Tissue	7	$y=0.53x^{0.76}$	0.99
Platinum	Tissue	14	$y=0.63x^{0.79}$	0.99
Platinum	Tissue	28	$y=0.82x^{0.79}$	0.99
Palladium	Tissue	7	$y=1.07x^{0.62}$	0.99
Palladium	Tissue	14	$y=2.02x^{0.61}$	0.92
Palladium	Tissue	28	$y=0.79x^{0.82}$	0.92

Table 3-3. Regression of Pt and Pd concentrations in mussel hemolymph as a power function of dissolved Pt and Pd exposure concentration at 7, 14 and 28 d of exposure (x= exposure concentration (ng/L)+1, y= hemolymph concentration (ng/g)).

Element	Source	Day	Equation	R^2
Platinum	Hemolymph	7	$y=0.19x^{0.20}$	0.83
Platinum	Hemolymph	14	$y=0.29x^{0.20}$	0.84
Platinum	Hemolymph	28	$y=0.15x^{0.24}$	0.89
Palladium	Hemolymph	7	$y=0.25x^{0.16}$	0.54
Palladium	Hemolymph	14	$y=0.44x^{0.18}$	0.59
Palladium	Hemolymph	28	$y=0.65x^{0.35}$	0.87

Table 3-4. Regression of Na^+,K^+-ATPase activity in mussel gill tissue as a power function of dissolved Pt and Pd exposure concentration at 7, 14 and 28 d of exposure (x= exposure concentration (ng/L)+1, y= Na^+,K^+-ATPase activity (µmoles ADP/mg protein/hour)).

Day	Equation	R^2
7	$y=1.10x^{0.02}$	0.05
14	$y=2.46x^{0.02}$	0.78
28	$y=2.13x^{0.05}$	0.72

Table 3-5. Regression of Pt and Pd concentrations in mussel hemolymph as a logarithmic function of dissolved Pt and Pd exposure concentration at 7, 14 and 28 d of exposure (x= exposure concentration (ng/L)+1, y=hemolymph ion (mg/L)).

Ion	Day	Equation	R^2
Na^+	7	y= -6.29 ln(x)+416.45	0.34
Na^+	14	y= -5.77 ln(x)+363.34	0.36
Na^+	28	y= -11.20 ln(x)+366.68	0.73
Ca^+	7	y= 6.80 ln(x)+117.24	0.43
Ca^+	14	y= 3.57 ln(x)+133.60	0.25
Ca^+	28	y= 4.63 ln(x)+104.76	0.45
K^+	7	y= -0.72 ln(x)+22.27	0.35
K^+	14	y= 0.44 ln(x)+17.55	0.64
K^+	28	y= -0.94 ln(x)+24.99	0.67
Cl^-	7	y= 2.33 ln(x)+324.27	0.08
Cl^-	14	y= -5.81 ln(x)+334.91	0.79
Cl^-	28	y= -4.03 ln(x)+255.08	0.12

Figures

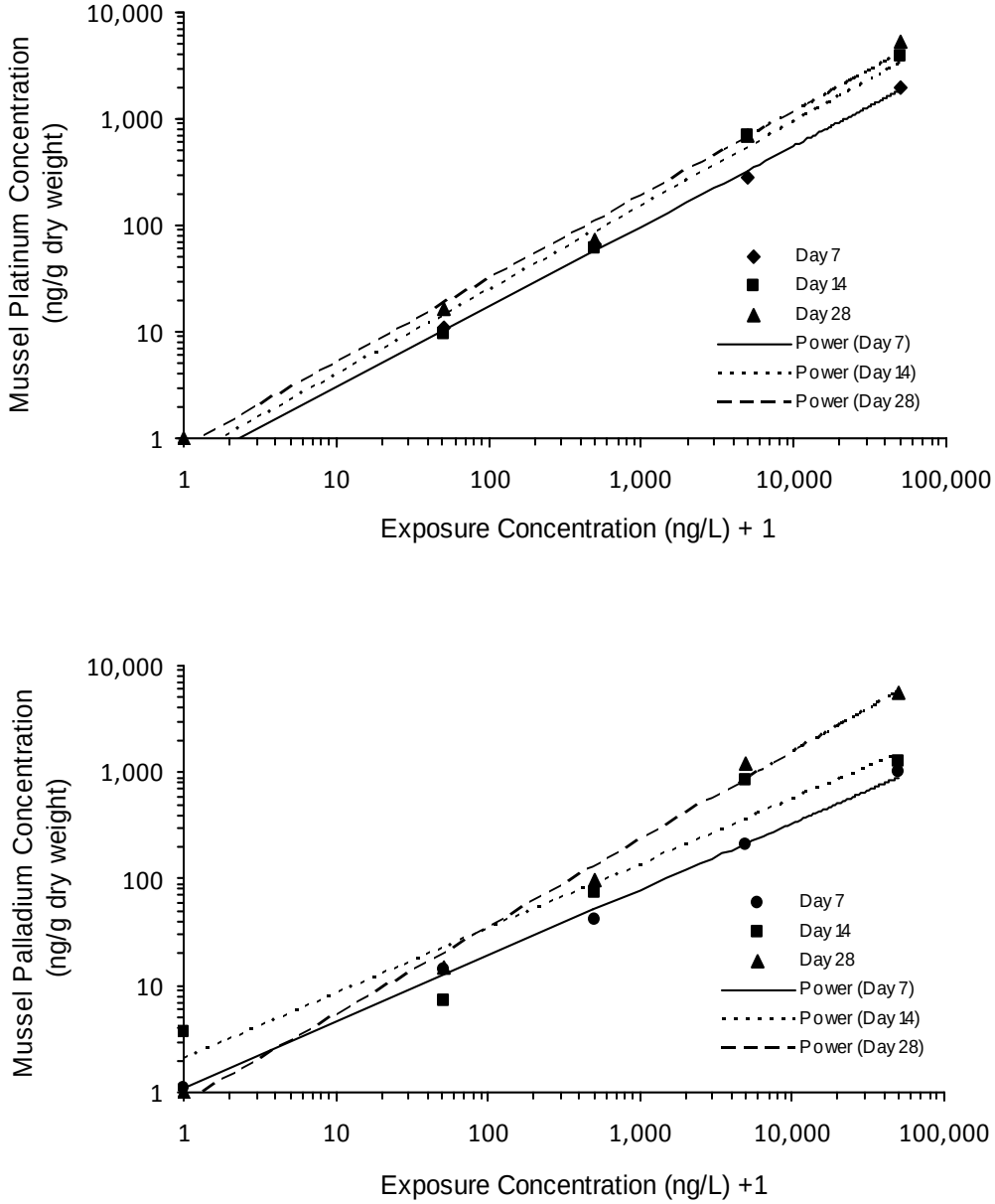

Figure 3-1. Relationship between Pt and Pd concentrations (ng/g) in mussel tissue and dissolved Pt and Pd exposure concentration (ng/g) +1 at 7, 14 and 28 d of exposure.

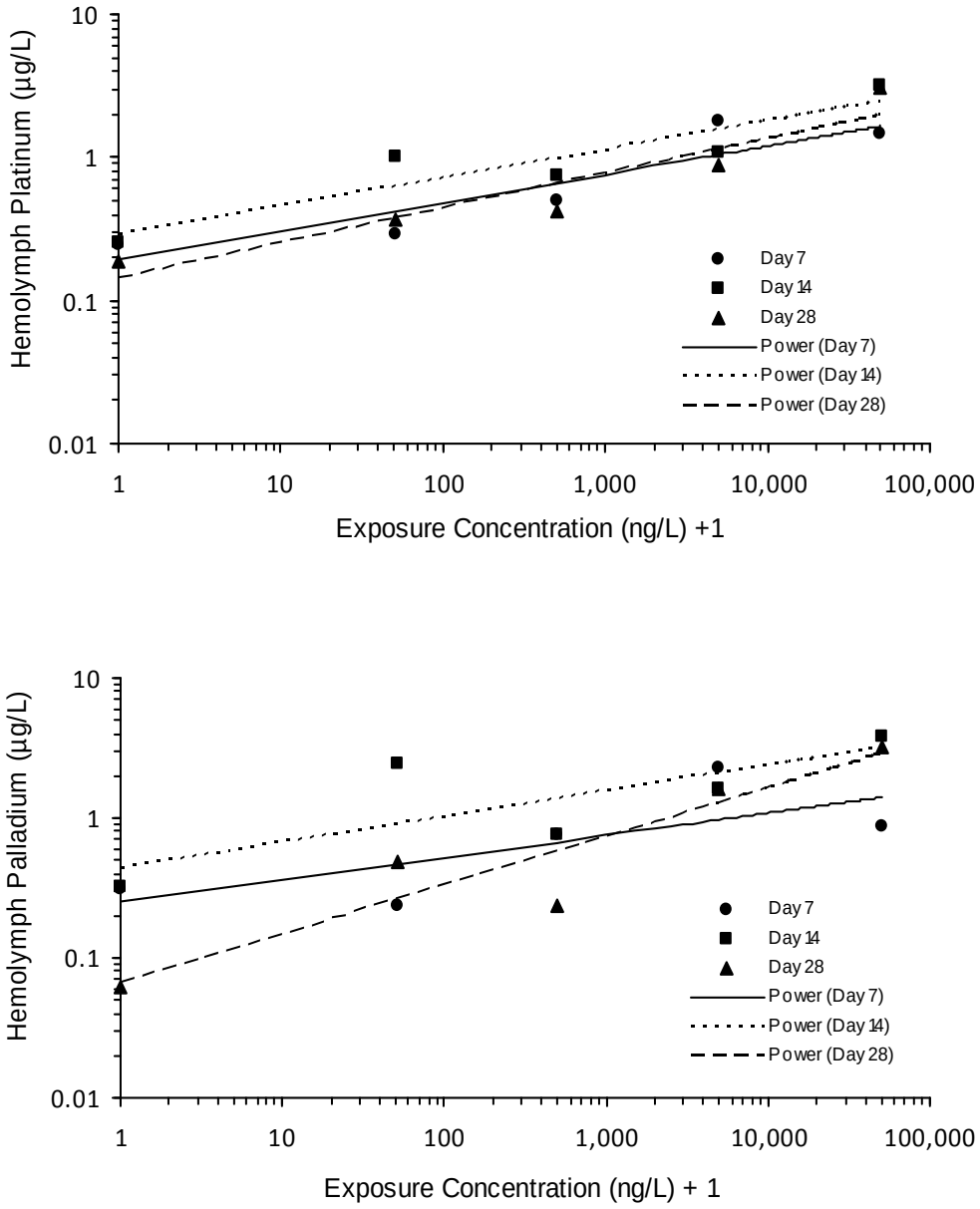

Figure 3-2. Relationship between Pt and Pd concentrations (µg/L) in mussel hemolymph and dissolved Pt and Pd exposure concentration (ng/L) +1 at 7, 14 and 28 d of exposure.

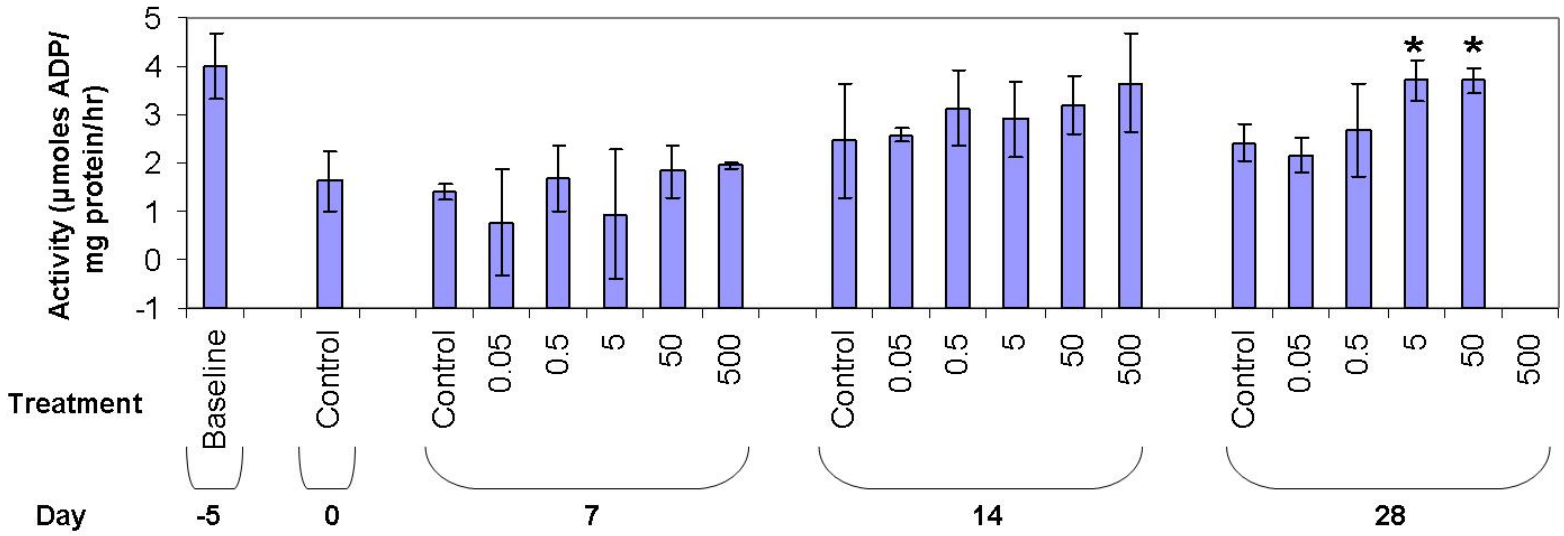

Figure 3-3. Na^+,K^+-ATPase activity (µmoles ADP/mg protein/hr) measured in mussel gill tissue collected on day -5 from the source location (baseline), day 0 pre-exposure control mussels, and from exposed mussels on days 7, 14, and 28. Error bars represent ±1 standard deviation. Asterisks indicate results are statistically different from control sampled on the same day.

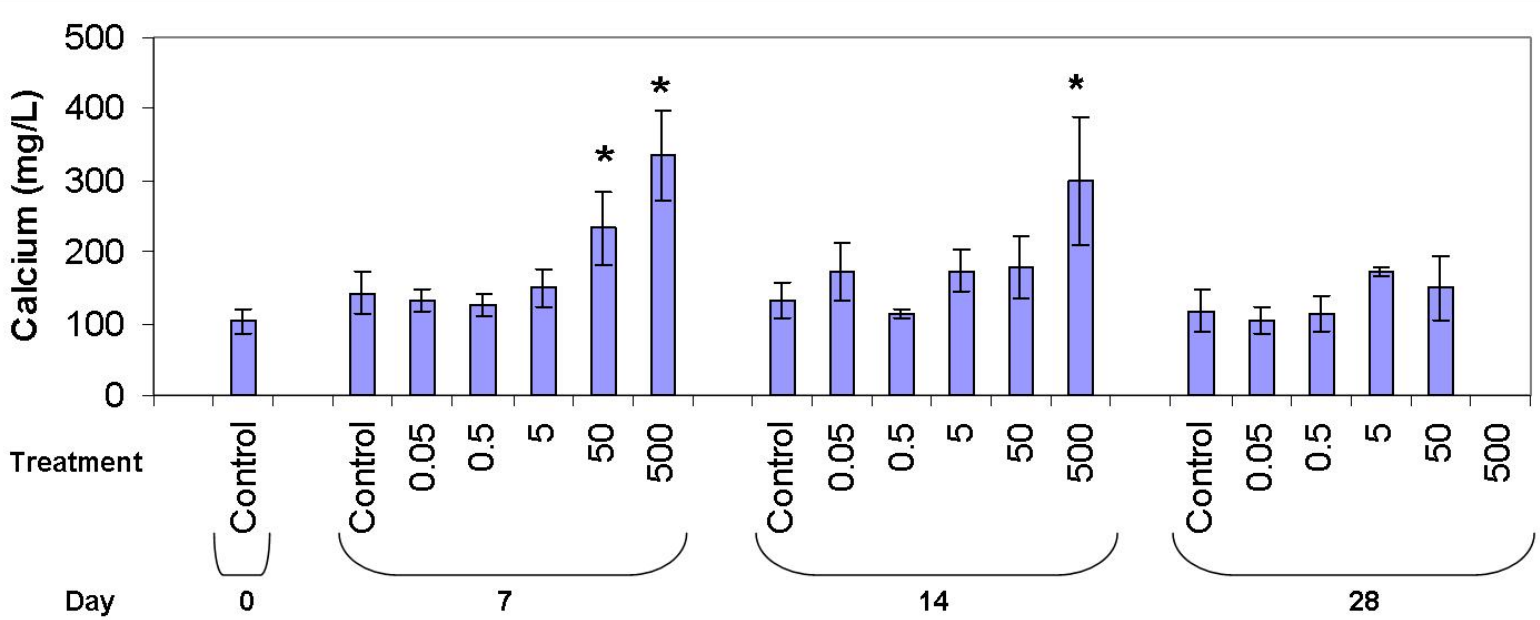

Figure 3-4. Calcium (Ca^{2+}) concentration measured in mussel hemolymph collected from day 0 pre-exposure control mussels, and from exposed mussels on days 7, 14, and 28. Error bars represent $\pm$1 standard deviation. Asterisks indicate results are statistically different ($P<0.05$) from control sampled on the same day.

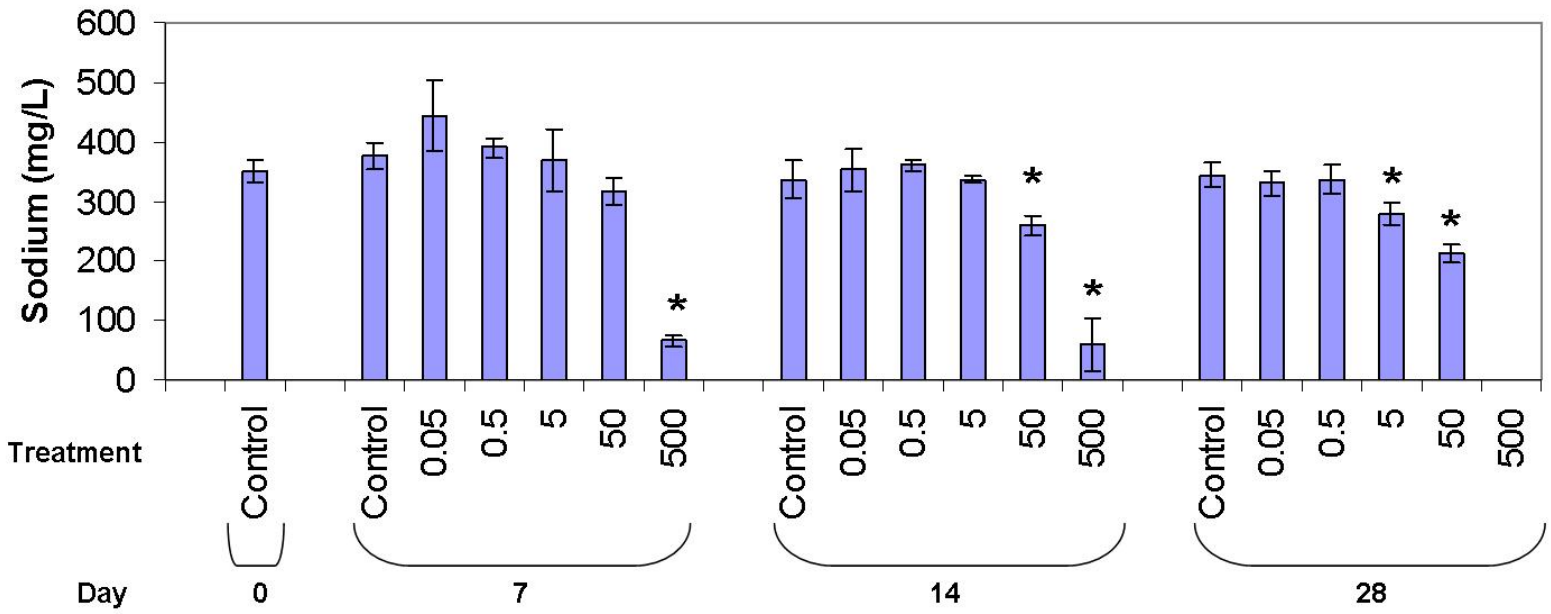

Figure 3-5. Sodium (Na^+) concentration measured in mussel hemolymph collected from day 0 pre-exposure control mussels, and from exposed mussels on days 7, 14, and 28. Error bars represent $\pm$1 standard deviation. Asterisks indicate results are statistically different ($P<0.05$) from control sampled on the same day.

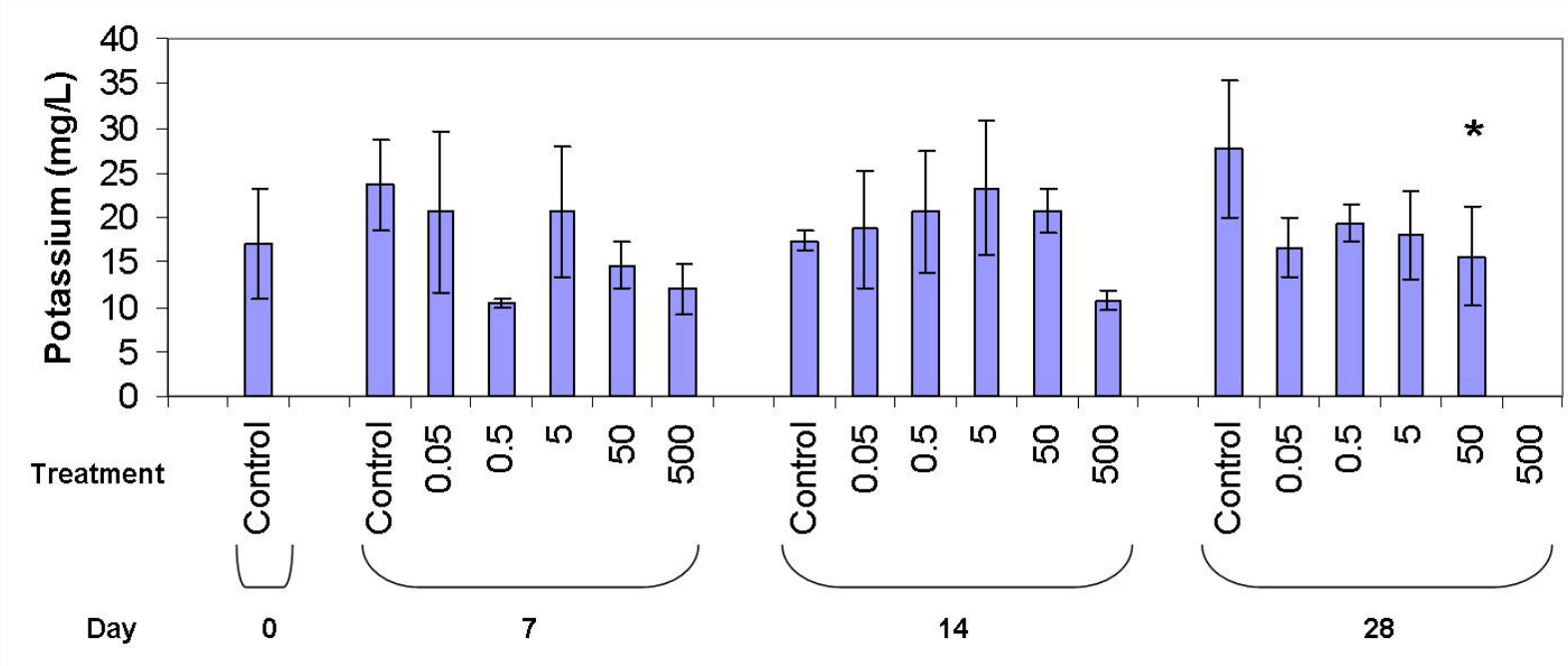

Figure 3-6. Potassium (K^+) concentration measured in mussel hemolymph collected from day 0 pre-exposure control mussels, and from exposed mussels on days 7, 14, and 28. Error bars represent $\pm$1 standard deviation. Asterisks indicate results are statistically different ($P<0.05$) from control sampled on the same day.

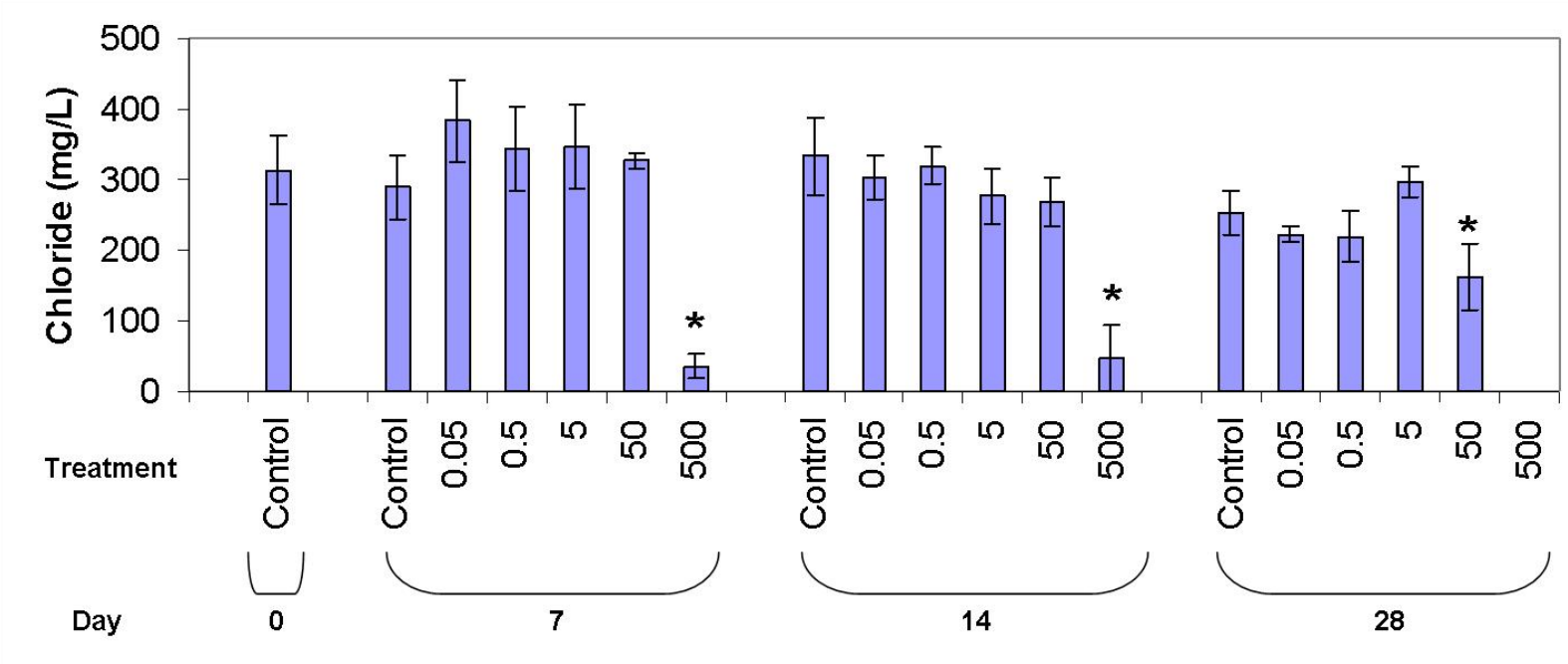

Figure 3-7. Chloride (Cl^-) concentration measured in mussel hemolymph collected from day 0 pre-exposure control mussels, and from exposed mussels on days 7, 14, and 28. Error bars represent $\pm$1 standard deviation. Asterisks indicate results are statistically different ($P<0.05$) from control sampled on the same day.

Appendix

Na^+, K^+-ATPase Assay – Microplate Assay

I. Buffer preparation and reaction mixtures

Salt Solution

Imidazole (50 mM)	1.702 g
NaCl (189 mM)	5.52 g
$MgCl_2 \cdot 6H_2O$ (10.5 mM)	1.02 g
KCl (42 mM)	3.14 g

Add 450 mL deionized water, adjust to pH 7.5 with HCl, qs to 500 mL. Store up to 3 months at 4°C.

Salt Solution ($-K^+$)

Imidazole (50 mM)	1.702 g
NaCl (189 mM)	5.52 g
$MgCl_2 \cdot 6H_2O$ (10.5 mM)	1.02 g

Add 450 mL deionized water, adjust to pH 7.5 with HCl, qs to 500 mL. Store up to 3 months at 4°C.

SEI Buffer

Sucrose (250 mM)	42.79 g
Na2EDTA (10 mM)$\cdot 2H_2O$	1.86 g
Imidazole (50 mM)	1.70 g

Add 450 ml deionized water, adjust to pH 7.3 with HCl, qs to 500 mL. Store up to 1 month at 4°C.

0.3% SEID (3X concentrate)

0.1 g Na deoxycholic acid in 33.3 mL SEI, (0.0751g in 25 mL). Store up to 1 week at 25°C.

Assay Mixture (AM) Reagents

Sigma #	Buffer, Abbreviation, Molecular Weight	Stock Conc.	Final Conc.	Conc. in AM
I-2399	**Imidazole Buffer (IB; MW = 68.08)** 1.702 g Imidazole in 475 mL DiH_2O, adjust to pH 7.5 with HCl, qs to 500 mL, store up to 3 months at 4°C	50 mM	50 mM	50 mM
P-7252	**Phosphoenolpyruvate (PEP; MW = 465.6)** 0.978 g in 100 mL IB, divide into 10 or 20 mL aliquots, store up to 6 months at -80°C	21 mM	2 mM	2.8 mM
N-6005	**NADH- reduced (MW = 709.4)** Add to assay mixture, make fresh with each batch of AM	–	0.16 mM	0.22 mM
A-3377	**Adenosine Triphosphate (ATP; MW = 551.1)** Add to assay mixture, make fresh with each batch of AM	–	0.5 mM	0.7 mM
L-2500	**Lactic Dehydrogenase (LDH)** Spin for 8 minutes at 12,000g at 4°C; a distinct pellet should result, remove supernatant, suspend pellet in several mL IB; add to assay mixture, make fresh with each batch of AM	–	3.3 U/ml	4.6 U/ml
P-1506	**Pyruvate Kinase (PK)** Spin for 8 minutes at 12,000g at 4°C; a distinct pellet should result, remove supernatant, suspend pellet in several mL IB; add to assay mixture, make fresh with each batch of AM (centrifuge LDH and PK together)	–	3.6 U/ml	5.1 U/ml

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

II. Assay Standard

ADP Standard (MW = 427.2)
4mM Stock Solution:
0.0427g in 25 mL Na Acetate (57 mM)
(0.4627g Na Acetate in 100 mL deionized water, pH 6.8)
Store in 500 µL aliquots at –80°C. On day of assay, thaw aliquot and dilute:

nmoles/10 µL	IB	4 mM ADP Stock
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 Solution is added. Standard curve slope should be in the range of 17-19 mOD/nmole.

III. Assay Mixture Recipe

Solution	4 microplates (96 assays)	8 microplates (192 assays)
PK	30.6 µL *	61.2 µL *
LDH	24.3 µL *	48.6 µL *
NADH	10.9 mg	21.8 mg
PEP	9.33 mL	18.66 mL
ATP	27 mg	54 mg
IB	qs to 75 mL	qs to 150 mL
	Divide into 35 mL halves	Divide into 70 mL halves
	Add 11.66 mL Salt Solution to one (A)	Add 23.32 mL Salt Solution to one (A)
	Add 11.66 mL Salt Solution ($-K^+$) to the other (B)	Add 11.66 mL Salt Solution ($-K^+$) to the other (B)
	Make AM fresh every day to ensure quality	

* 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

1. Run standard curves for ADP

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.

2. Sample preparation and assay protocol

a. Gill tissue is stored in 100 μL SEI buffer at –20^{o}C for up to 2 months and –80^{o}C for 6 months
b. Thaw samples immediately prior to assay and add 50 μL SEID (3X concentrate).
c. Homogenize in tube (20 – 30 seconds), ensuring all tissue is homogenized.
d. Centrifuge at 5,000g for 1 minute at 4°C to remove insoluble material.
e. Pipette 10 μL of sample into 4 wells (2 for Solution A and 2 for Solution B). Uses a total of 40μL homogenate.
f. With AM/Salt solutions still in ice bath, add 200 μL 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. Pipette 90 µL of Salt solution into centrifuge tubes for samples.
3. Pipette 10 µL 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^{o}C, and transfer to 1 mL microcuvetes.
7. This is an endpoint assay read at a wavelength of 595 nm

Preparation of standards:

ug/10uL	BSA standard	Salt Solution
0	--	100 µL
5	5 µL	95 µL
10	10 µL	90 µL
15	15 µL	85 µL
20	20 µL	80 µL

VI. ATPase Activity Calculation

The ATPase standard curve should be read in mOD/nmole ADP
The ATPase acitivy measurements should be read in mOD/10□L /minute

Sample calculation:

Standard curve = 20 mOD/nmole ADP
ATPase (Solution A) = 30.0 mOD/10µL /minute
ATPase (Solution B (-K^{+})) = 13.5 mOD/10µL /minute
Na+,K+-ATPase = 30.0 – 13.5 = 16.5 mOD/10µL/minute
Protein reading = 8.5 µg/10µL

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

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

(0.097)(60min) = 5.82 µmoles ADP/mg protein/hour

VII. References

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Chapter 4: Evaluation of Na^+,K^+-ATPase activity and hemolymph ion concentrations as biomarkers of Pb exposure in freshwater mussels

Abstract

We 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^+,K^+-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, we 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^+,K^+-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 ($R^2 = 0.82$; $P = 0.0131$, at day 28). Ion results correlated as expected with Na^+,K^+-ATPase activity, with reduced hemolymph Na^+ at the greatest exposure, while enzyme activity was at 30% of controls. Hemolymph Ca^{2+} 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. We conclude that Na^+,K^+-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 we evaluated several possible biomarkers for Pb exposure and effect utilizing mussel hemolymph.

The two non-lethal biomarkers assessed were direct Pb concentrations, as well as ion (Na^+, K^+, Cl^-, and 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 Na^+ and Cl^- levels in rainbow trout [17] attributed to reduced Na^+,K^+-ATPase activity, as well as glucose and ion disruption in rainbow trout and whitefish [18, 19], ion disruption in crabs [20] and 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], we hypothesized the disruption of ion transporting enzymes, hemolymph ion concentration and chemistry as a consequence of Pb exposure in Eastern elliptio.

Na^+,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 Na^+,K^+-ATPase has been shown to be significantly reduced [17, 25-27] and correlated with Pb tissue concentration by noncompetitive inhibition. While no Na^+,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 Na^+,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, we 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 Φ 240 (Beckman Instruments, Fullerton, CA, USA) calibrated meter. Alkalinity was determined by titration of 0.02 N H_2SO_4 to pH 4.5 and hardness by titration with 0.01 M ethylenediaminetetra-acitic 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^+, K^+, Cl^-, Ca^{2+}) 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® 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 (Na^+, K^+, Cl^-, Ca^{2+}) and Pb analysis. These mussels were then dissected and three samples of gill tissue (~ 15 mg each) were removed per mussel for Na^+,K^+-ATPase activity assessment, and each placed in 1 mL centrifuge tubes, on ice, with 100 µL of SEI buffer solution. Ion hemolymph samples along with gill samples were then frozen at -80°C. Mussels were bagged and frozen along with Pb hemolymph samples at -20°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 µg 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 µg/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 μL 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 $CaCO_3$ (range 28 – 32), and hardness 42 mg/L (range 40 – 44).

Na^+,K^+-ATPase Activity Assay

The Na^+,K^+-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^+,K^+-ATPase activity by binding to the alpha-subunit of the enzyme after it has released $3Na^+$ and before it binds $2K^+$, thus stopping the cycle [24]. However ouabain proved insufficient in inhibiting Na^+,K^+-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^+,K^+-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^+,K^+-ATPase, when mutated, will reduce ouabain sensitivity [38]. Therefore, we used a K^+-free salt solution for inhibition instead of ouabain and much stronger and more consistent results were achieved. This method of detection has been used in conjunction with ouabain inhibition to verify that Na^+,K^+-ATPase activity was not present in measurable amounts in the mussel *Anodonta cataracta*, with both salt depletion and ouabain showing negative results [28]. This method has also been used to detect Na^+,K^+-ATPase activity in the gills of goldfish which are insensitive to ouabain,

requiring an extremely high concentration (10mM) for inhibition [39]. Chasiotis and Kelly [39] found that inhibition with a K^+-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 we found high activity, about 50% of total ATPase in *Elliptio complanata* gill tissue, we 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^{TM} 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 Na^+,K^+-ATPase activity, 15 mL of AM was mixed with 5 mL of the 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 µL of 4 mM ADP and 200, 175, 150 and 100 µL imidazole buffer, respectively, were added for 0, 5, 10 and 20 nMoles ADP/10 µL concentrations. A Fusion™ 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 µL 0.3% SEID (3X concentrate; 0.0751 g in 25 mL SEI (sucrose 250 mM, disodium ethylenediaminetetraacetic acid dihydrate (Na2EDTA $2H_20$) 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 µL 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 µL per 3 wells per concentration was pipetted into the plate, and 200 µL of ice cold A w/salt was added to each well. The ADP standard curve rapidly decreases in the first 2 – 3 minutes, but then stabilizes, and is read in mOD/nmole ADP. Then 200 µL of ice cold B w/salt (-K^+) was added to two of the four wells for each sample, and 200 µL of ice cold A w/salt

was added to the other two wells. The plate was then promptly read on the plate reader at 340 nm for 10 minutes at 1 minute intervals. ATPase activity was measured as mOD/10 µl /minute. The Bradford Protein Assay [41] of Kit (IBI/Shelton Scientific; VWR #14221-496) was used to determine protein concentration, and the final Na^+,K^+-ATPase activity as µmoles 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 Na^+,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 µg/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 µg 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 µg/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 µg/L in the 245 µg/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 µg/L treatments all increased over the 28 day study, though not significantly (Figure 3). The hemolymph in mussels from the 245 µg/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 µg/L Pb treatment group (**Error! Reference source not found.**).

Ion analysis of the hemolymph revealed that while calcium (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), Ca^{2+} levels increased significantly in the hemolymph from mussels at the highest treatment (245 µg/L). Chloride (Cl^-) levels were reduced significantly with time in all treatment groups, including the controls, compared

to the baseline (Figure 5) mussel samples. Hemolymph Cl^- was also reduced in the controls with each time point, with day 28 controls significantly less than day 0 controls. On day 28, 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 (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 µg/L treatment significantly reduced Na^+ below the controls at that time point, while the 1 µg/L treatment on day 7 and the 57 µg/L treatment on day 14 were significantly greater than their respective time point controls. Potassium (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 µg/L treatment group on day 7 which was significantly lower at the $\alpha = 0.10$ level.

The average Na^+,K^+-ATPase activity for each treatment group at each time point ranged from 0.95 to 3.46 µMoles 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 µg/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 µg/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 µg/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 µg/L treatment group significantly reduced Na^+,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 µg/L treatment group. When the average Na^+,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' Na^+,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 µg/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, we 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 µg Pb/L. At the greatest exposure of 245 µg Pb/L, however, 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 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 Ca^{2+}/L for this experiment was similar to the median of 175 mg Ca^{2+}/L from other observations with *Elliptio complanata* [12] and well within the 95% confidence interval (CI) of 131 to 237 mg Ca^{2+}/L as derived by Gustafson, et al. [48]. Acclimation

of the mussels to ASTM soft water brought the Ca^{2+} 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^{2+} 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, we conclude that high concentrations of Pb significantly increased hemolymph Ca^{2+} concentrations. The high concentration of Pb required to obtain this effect, however, makes Ca^{2+} 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^- 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^+ and Pb exposure, differences were only significantly higher from the control at the greatest exposure, and only at the 0.10 α 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^+ 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, we 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 Na^+,K^+-ATPase activity was explained by the concentration of Pb in the tissue. The mussels at the lowest exposure concentration of 1 μg/L of Pb had an average Na^+,K^+-ATPase activity 18% lower compared to the controls (**Error! Reference source not found.**), and because Na^+,K^+-ATPase is responsible for maintaining Na^+,K^+ transmembrane gradients, it has been suggested that inhibition may lead to gill tissue damage [53]. In addition, disruption of Na^+ levels in the cell would affect Na^+/H^+ exchange altering pH levels, and thereby affecting shell formation and dissolution [40] along with Ca^{2+} and HCO_3^- levels. And as Pb will compete for Ca^{2+} uptake sites [17], its presence will directly affect ion concentrations and shell formation and dissolution as well.

The use of Na^+,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. We conclude that current measured environmental concentrations of Pb from this study in North Carolina may be sufficient to cause reductions of Na^+,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 Na^+,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^+,K^+-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 4-1. 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	Mean Measured Concentration (ug/L)						
Concentration (ug/L)	T0 (*n=10)	% of Target	T48 (*n=5)	% of Target	T72 (*n=3)	% of Target	Average Daily Exposure
Control (0)	0.0	n/a	0.0	n/a	0.0	n/a	0.0
2	2.1	105.5	0.1	4.4	0.1	5.2	1.0
8	8.3	104.2	0.3	3.4	0.3	3.6	3.7
31	31.1	100.3	1.0	3.2	1.3	4.2	13.9
125	121.0	96.8	9.3	7.5	6.0	4.8	56.7
500	457.5	91.5	88.9	17.8	73.1	14.6	244.6
	* Each 'n' represents 3 pooled samples.						

Table 4-2. The results of tissue Pb analysis as the average of the three mussels for each treatment group at every timepoint.

Treatment	Day 7		Day 14		Day 28	
Group (ug/g)	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
	Average	SD				
Baseline	0.30	0.18				
d0 Control	0.24	0.08				

Table 4-3. The results for hemolymph Pb concentrations as the average of the three mussels for each treatment group and timepoint, and the standard deviation.

Treatment	Day 7		Day 14		Day 28	
Group (ug/L)	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
	Average	SD				
Baseline	0.71	0.80				
0 Control	0.52	0.68				

Table 4-4. The averagte N+,K+-ATPase activity for each treatment group at every timepoint, as well as the Baseline and day 0 Control averages. Activity is expressed as µmoles of ADP/mg protein/hour.

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

Table 4-5. The Na+,K+-ATPase activity for each treatment group as a percentage of the Control for day 28 of the study.

Inhibition of Na+,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

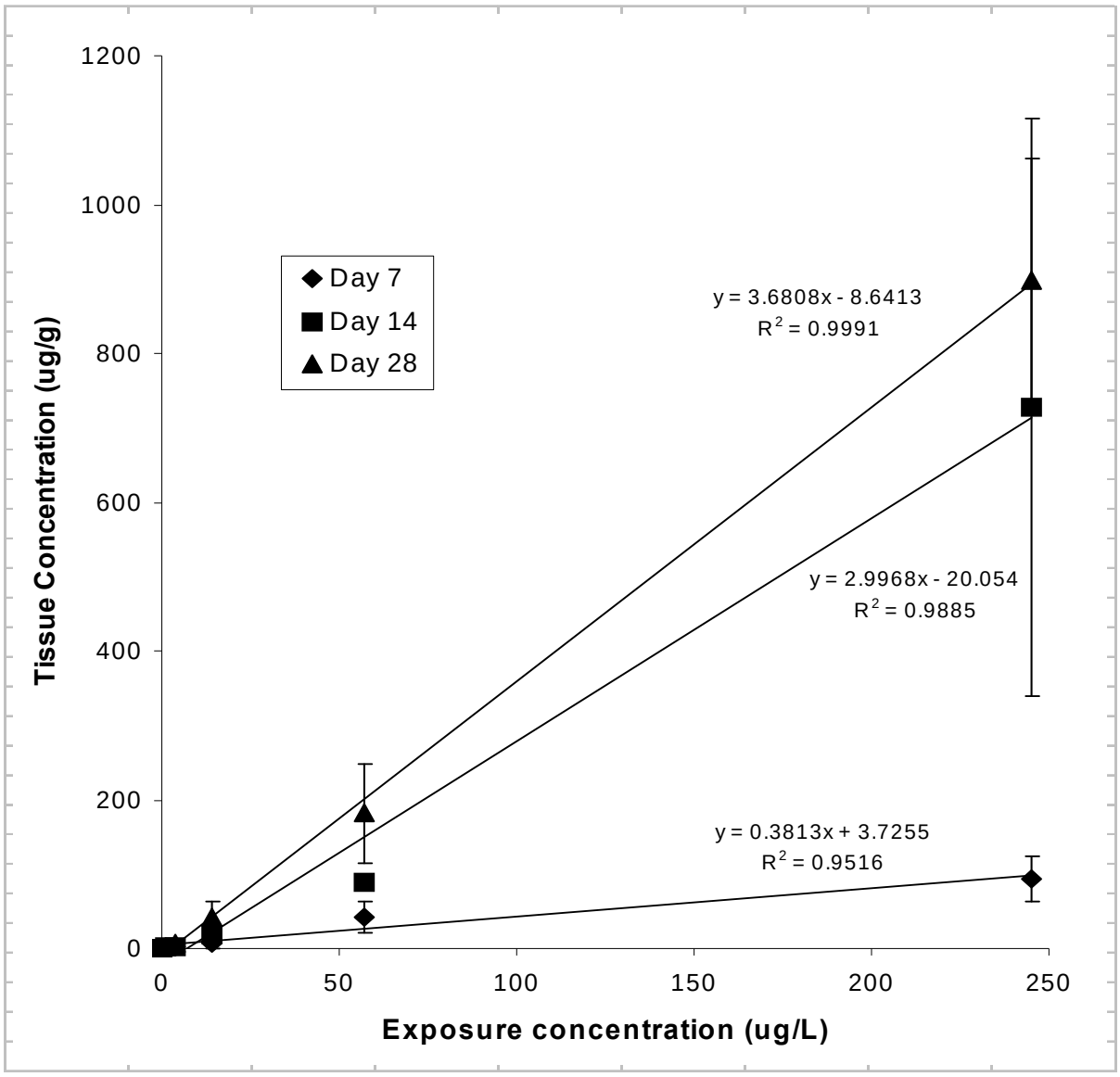

Figure 4-1. Correlations between mussel tissue Pb concentration and exposure concentration on days 7, 14 and 28. Error bars represent 95% confidence intervals.

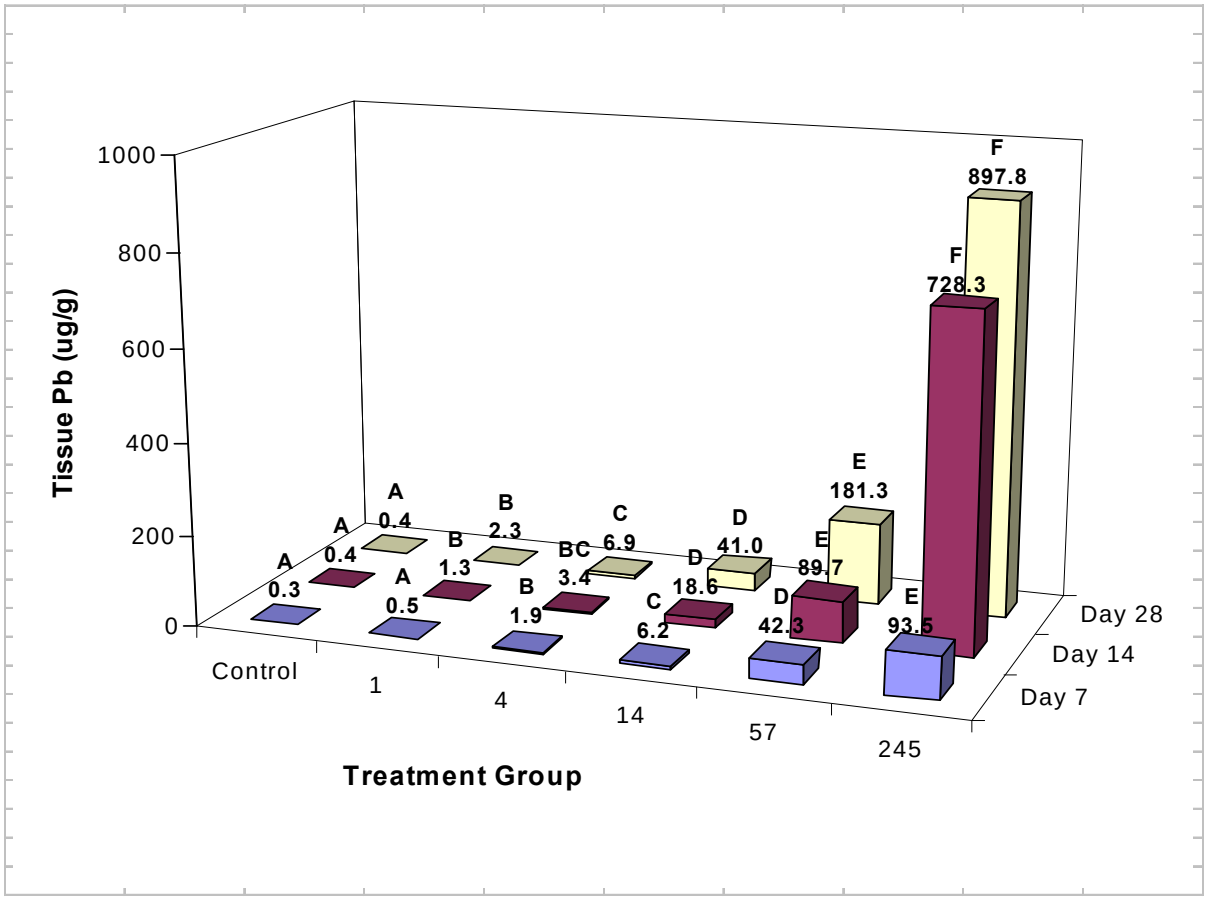

Figure 4-2. The average tissue Pb concentration for each treatment group, over the 28 day study. Values with the same letters are not significantly different.

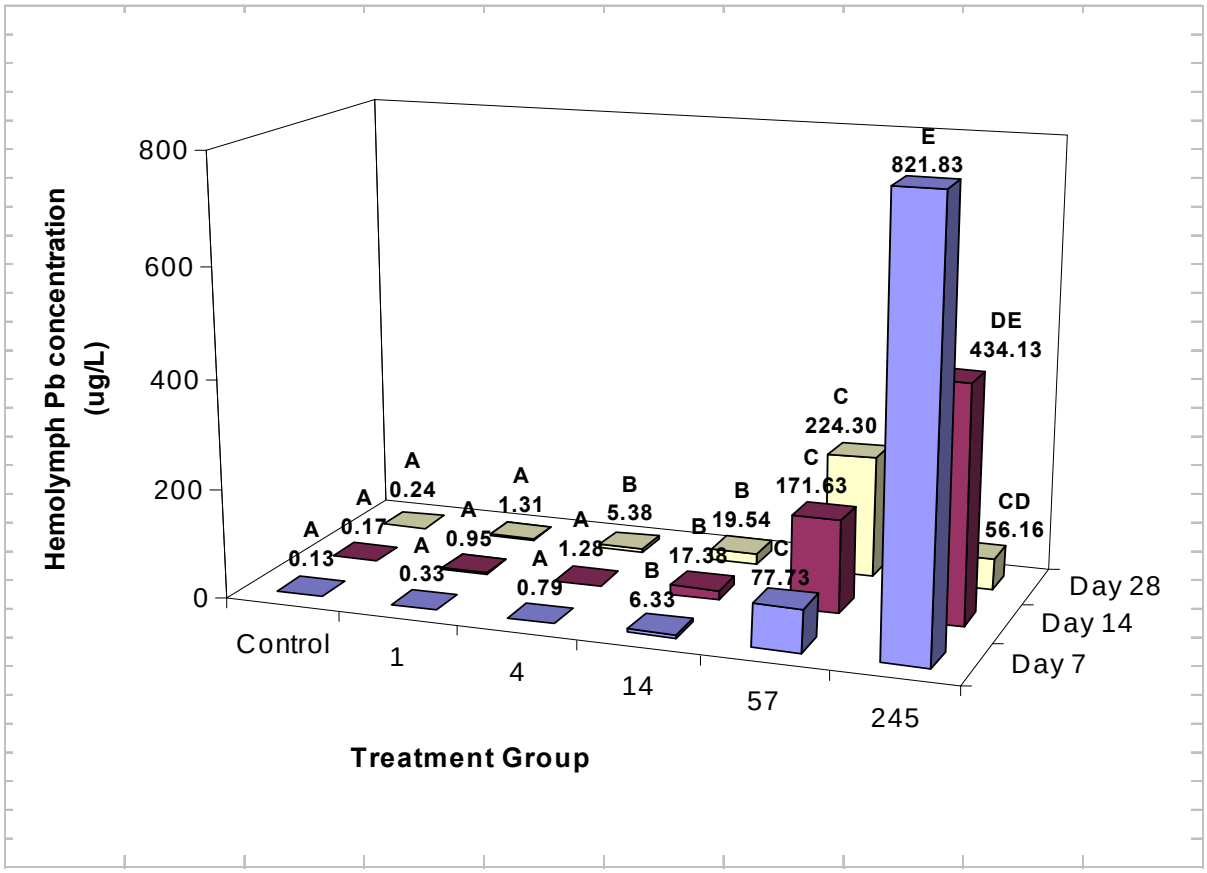

Figure 4-3. The average hemolymph Pb concentration, for each treatment group, over the 28 day study. Values with the same letters are not significantly different.

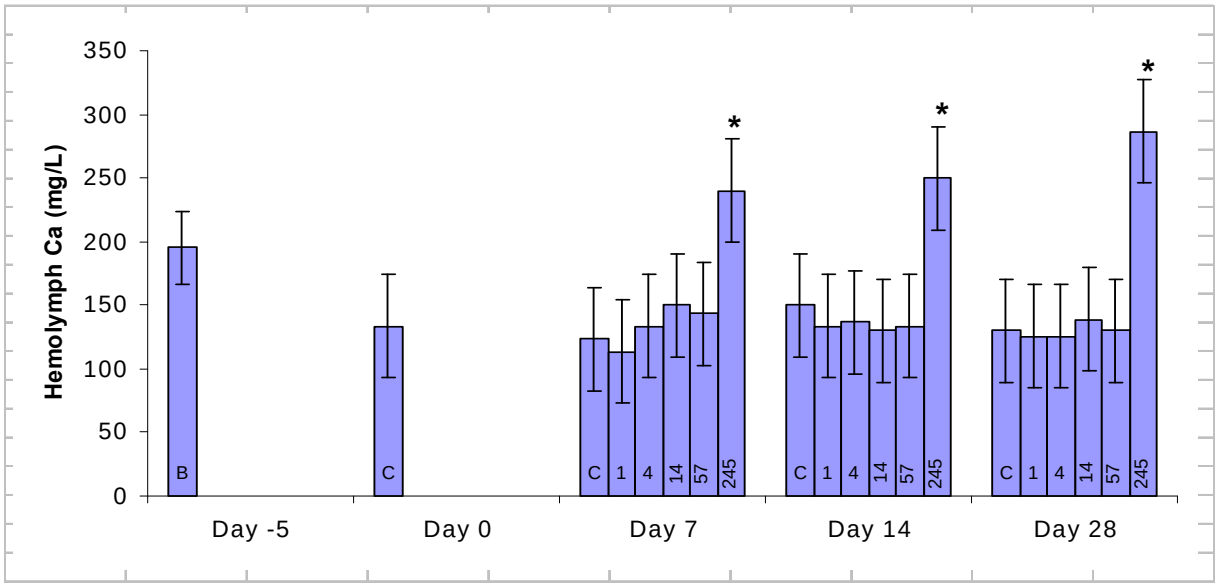

Figure 4-4. Hemolymph Calcium levels as mg/L during the second 28 day exposure for each treatment group and time point. All Controls were significantly lower than the Baseline. The asterix ' * ' signifies values significantly greater than the Baseline. Error bars represent 95% confidence intervals.

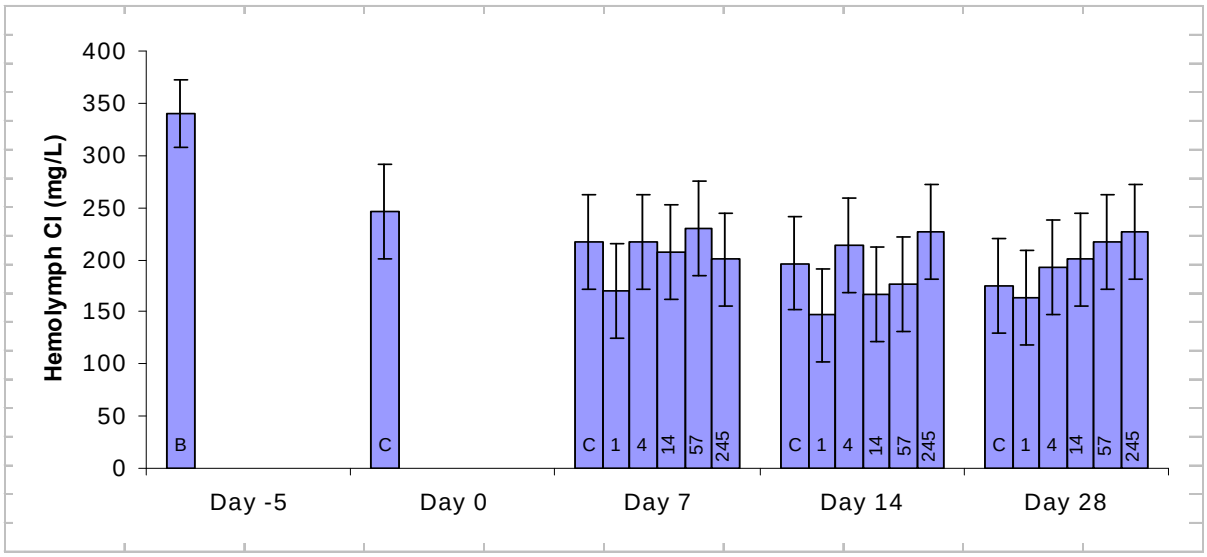

Figure 4-5. Hemolymph Chloride levels as mg/L during the second 28 day exposure for each treatment group and time point. All treatment groups were significantly less than the Baseline. Controls decrased with each time point, with the day 28 Control significantly lower than the day 0 Control. Error bars represent 95% confidence intervals.

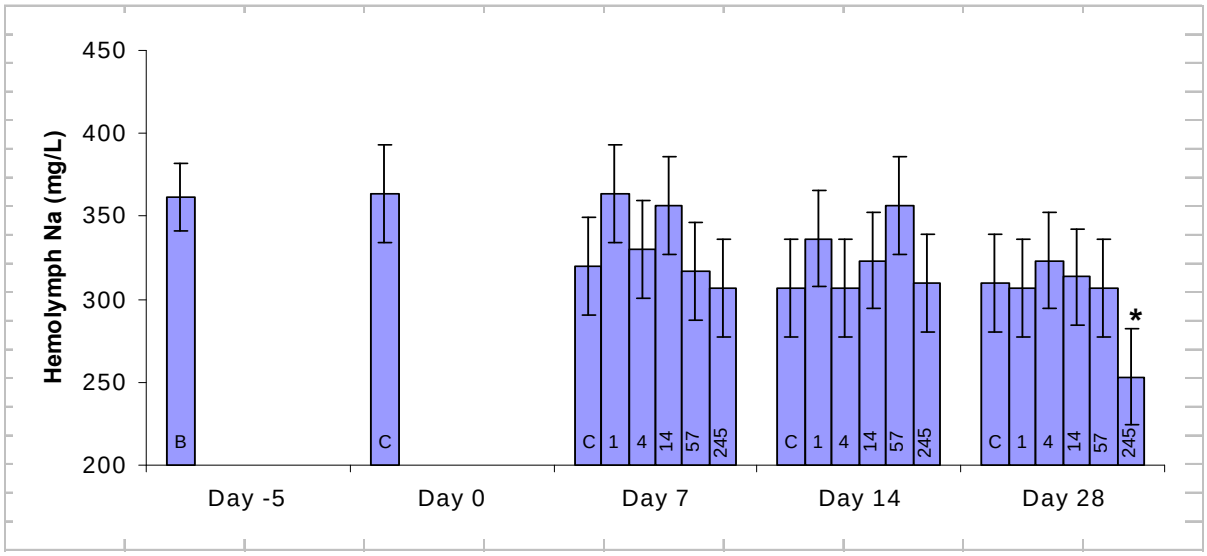

Figure 4-6. Hemolymph Sodium levels as mg/L during the second 28 day exposure for each treatment group and time point. The asterix ' * ' signifies values significantly lower than that time point's Control. Controls on day 7,14 and 28 are significantly lower than the Baseline and day 0 Control. Error bars represent 95% confidence intervals.

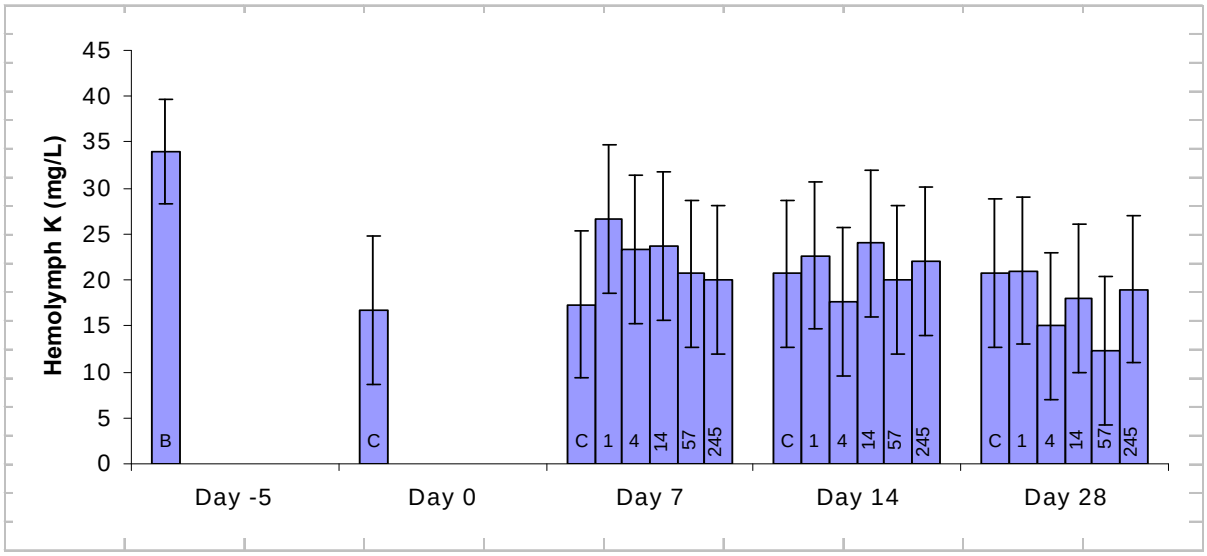

Figure 4-7. Hemolymph Potassium levels as mg/L during the second 28 day exposure for each treatment group and time point. All treatment groups were significantly lower than the Baselinewith an alpha = 0.05 level, except day 7: 2 which is significant at the alpha = 0.1 level. Error bars represent 95% confidence intervals.

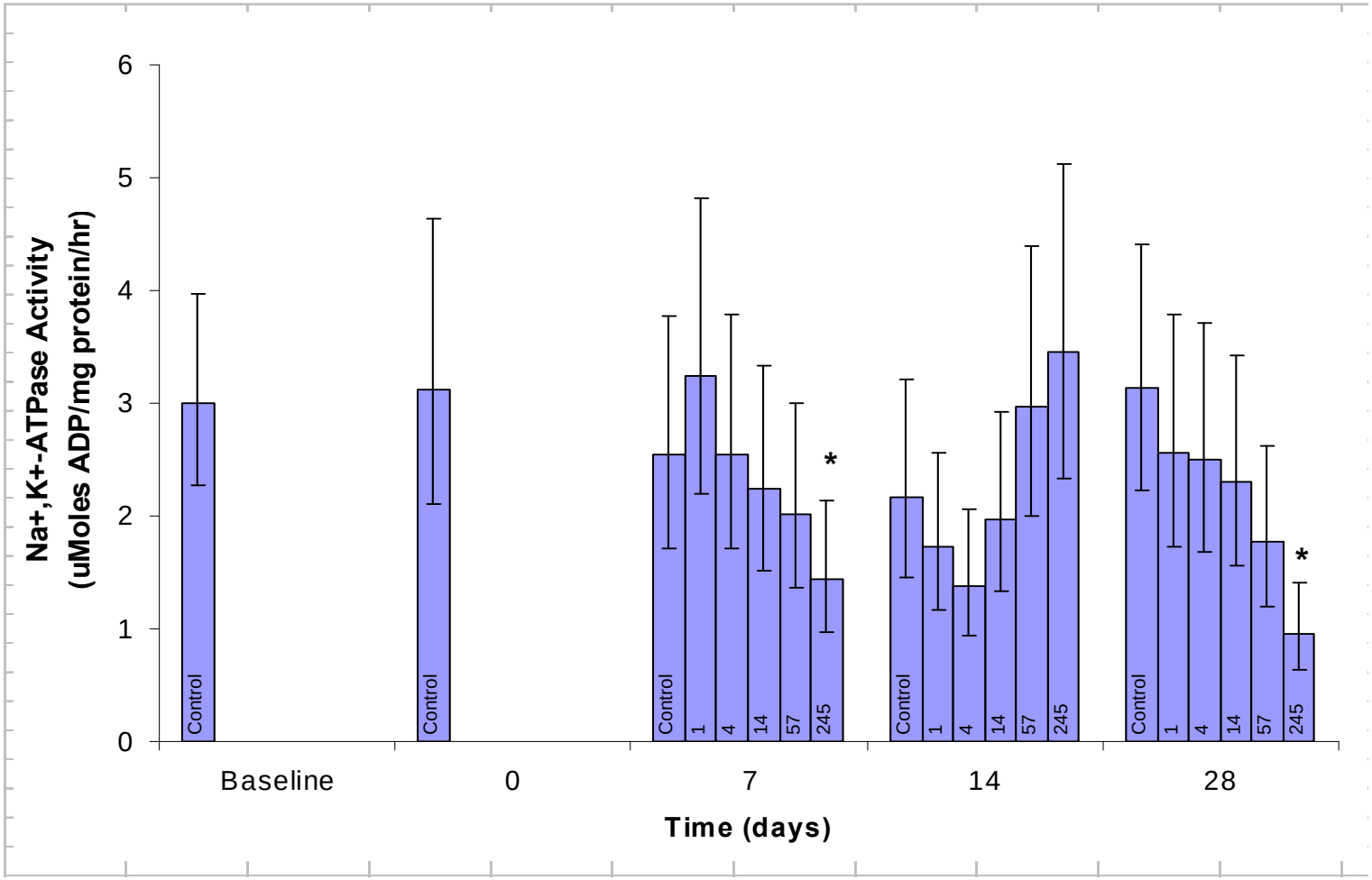

Figure 4-8. The average Na+,K+-ATPase activity for each treatment group over the 28 day study. Bars represent 95% confidence intervals. The " * " indicates values which are significantly different from the Control of that time point, as well as the baseline and day 0 values.

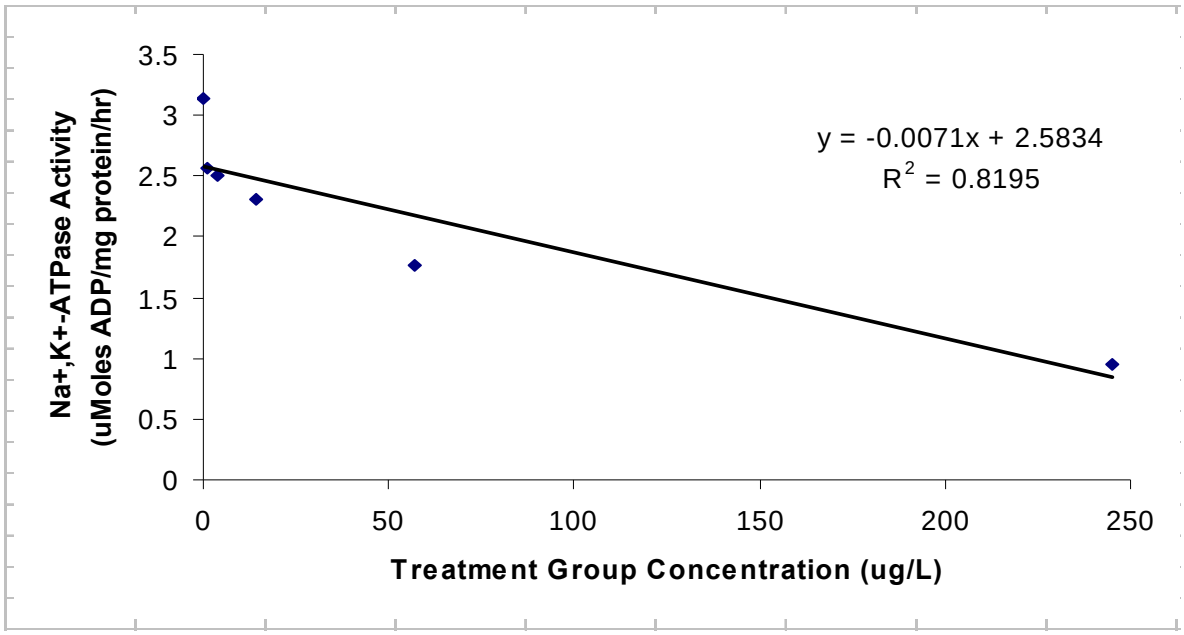

Figure 4-9. Na+,K+-ATPase activity correlations with treatment groups as it relates on day 28 of the study.

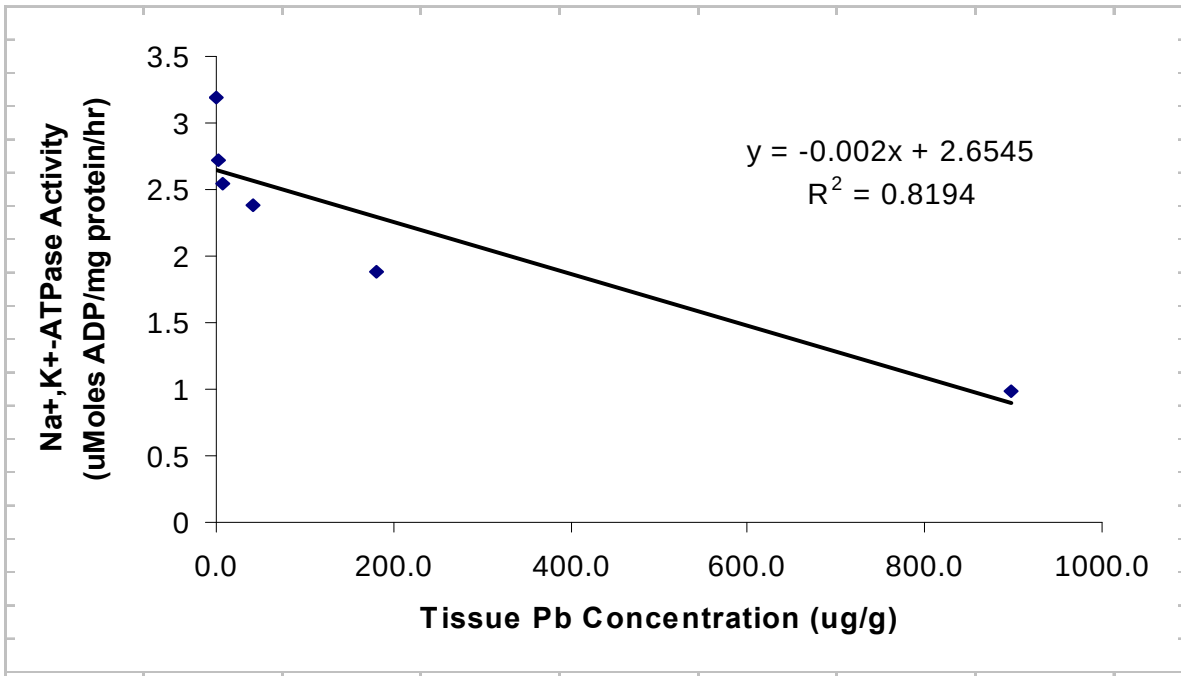

Figure 4-10. The correlation of average Na+,K+-ATPase activity and average tissue Pb concentration, per treatment group, on day 28.

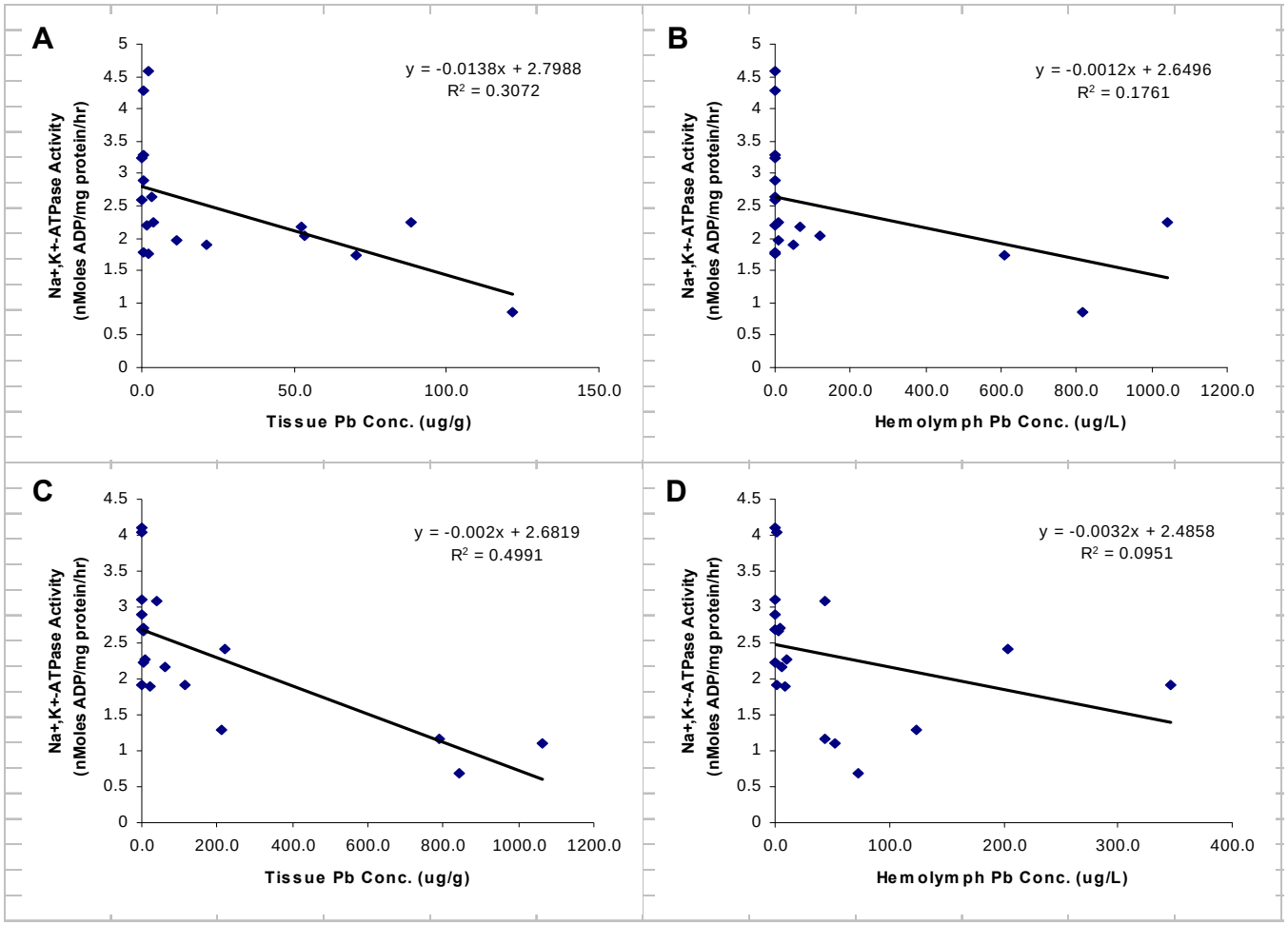

Figure 4-11. Na+,K+-ATPase activity correlations: A) with tissue Pb concentration on day 7, B) with hemolymph Pb concentration on day 7, C) with tissue Pb concentration on day 28, D) with hemolymph Pb concentration on day 28.

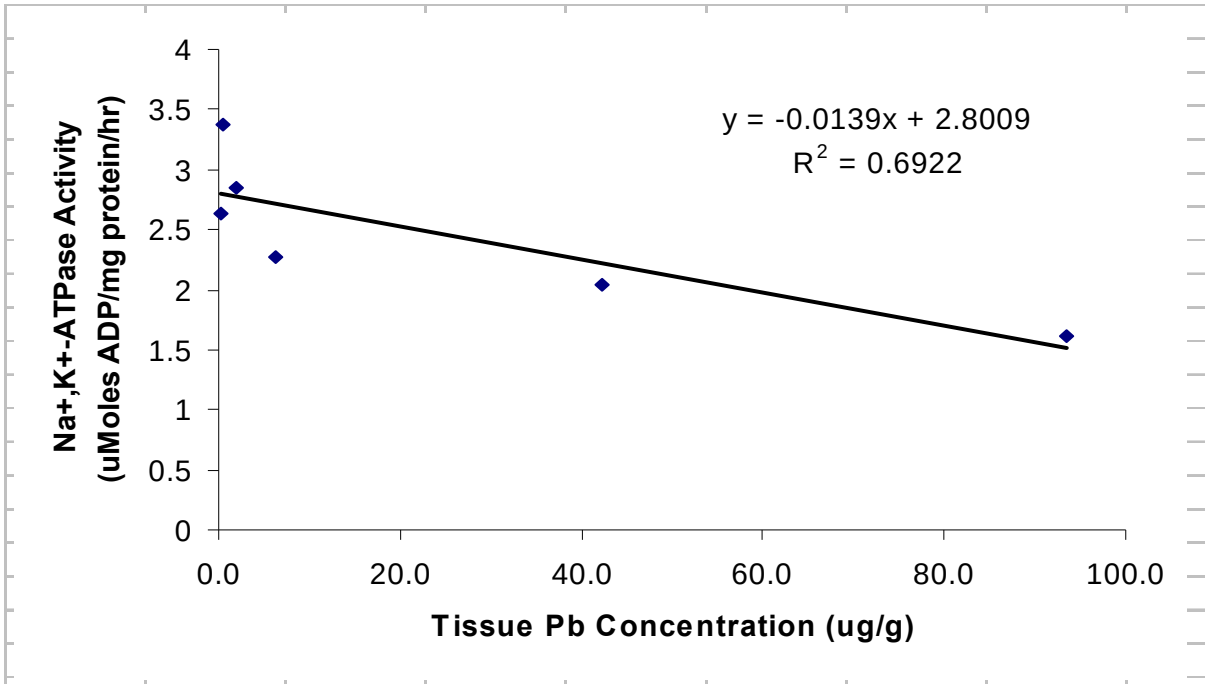

Figure 4-12. The correlation of average Na+,K+-ATPase activity and average tissue Pb concentration, per treatment group, for day 7.

Appendix

<u>Na^+, K^+-ATPase Assay – Microplate Assay</u>

I. Buffer preparation and reaction mixtures

Salt Solution

Imidazole (50 mM)	1.702 g
NaCl (189 mM)	5.52 g
$MgCl_2 \cdot 6H_2O$ (10.5 mM)	1.02 g
KCl (42 mM)	3.14 g

Add 450 mL deionized water, adjust to pH 7.5 with HCl, qs to 500 mL. Store up to 3 months at 4^oC.

Salt Solution ($-K^+$)

Imidazole (50 mM)	1.702 g
NaCl (189 mM)	5.52 g
$MgCl_2 \cdot 6H_2O$ (10.5 mM)	1.02 g

Add 450 mL deionized water, adjust to pH 7.5 with HCl, qs to 500 mL. Store up to 3 months at 4^oC.

SEI Buffer

Sucrose (250 mM)	42.79 g
Na2EDTA (10 mM)$\cdot 2H_2O$	1.86 g
Imidazole (50 mM)	1.70 g

Add 450 ml deionized water, adjust to pH 7.3 with HCl, qs to 500 mL. Store up to 1 month at 4^oC.

0.3% SEID (3X concentrate)

0.1 g Na deoxycholic acid in 33.3 mL SEI, (0.0751g in 25 mL). Store up to 1 week at 25ºC.

Assay Mixture (AM) Reagents

Sigma #	Buffer, Abbreviation, Molecular Weight	Stock Conc.	Final Conc.	Conc. in AM
I-2399	**Imidazole Buffer (IB; MW = 68.08)** 1.702 g Imidazole in 475 mL DiH_2O, adjust to pH 7.5 with HCl, qs to 500 mL, store up to 3 months at 4°C	50 mM	50 mM	50 mM
P-7252	**Phosphoenolpyruvate (PEP; MW = 465.6)** 0.978 g in 100 mL IB, divide into 10 or 20 mL aliquots, store up to 6 months at -80°C	21 mM	2 mM	2.8 mM
N-6005	**NADH- reduced (MW = 709.4)** Add to assay mixture, make fresh with each batch of AM	–	0.16 mM	0.22 mM
A-3377	**Adenosine Triphosphate (ATP; MW = 551.1)** Add to assay mixture, make fresh with each batch of AM	–	0.5 mM	0.7 mM
L-2500	**Lactic Dehydrogenase (LDH)** Spin for 8 minutes at 12,000g at 4°C; a distinct pellet should result, remove supernatant, suspend pellet in several mL IB; add to assay mixture, make fresh with each batch of AM	–	3.3 U/ml	4.6 U/ml
P-1506	**Pyruvate Kinase (PK)** Spin for 8 minutes at 12,000g at 4°C; a distinct pellet should result, remove supernatant, suspend pellet in several mL IB; add to assay mixture, make fresh with each batch of AM (centrifuge LDH and PK together)	–	3.6 U/ml	5.1 U/ml

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

II. Assay Standard

ADP Standard (MW = 427.2)
4mM Stock Solution:
0.0427g in 25 mL Na Acetate (57 mM)
(0.4627g Na Acetate in 100 mL deionized water, pH 6.8)
Store in 500 μL aliquots at –80°C. On day of assay, thaw aliquot and dilute:

nmoles/10 μL	IB	4 mM ADP Stock
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 Solution is added. Standard curve slope should be in the range of 17-19 mOD/nmole.

III. Assay Mixture Recipe

Solution	4 microplates (96 assays)	8 microplates (192 assays)
PK	30.6 μL *	61.2 μL *
LDH	24.3 μL *	48.6 μL *
NADH	10.9 mg	21.8 mg
PEP	9.33 mL	18.66 mL
ATP	27 mg	54 mg
IB	qs to 75 mL	qs to 150 mL
	Divide into 35 mL halves	Divide into 70 mL halves
	Add 11.66 mL Salt Solution to one (A)	Add 23.32 mL Salt Solution to one (A)
	Add 11.66 mL Salt Solution ($-K^+$) to the other (B)	Add 11.66 mL Salt Solution ($-K^+$) to the other (B)
	Make AM fresh every day to ensure quality	

* 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

1. Run standard curves for ADP

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.

2. Sample preparation and assay protocol

a. Gill tissue is stored in 100 μL SEI buffer at –20^{o}C for up to 2 months and –80^{o}C for 6 months
b. Thaw samples immediately prior to assay and add 50 μL SEID (3X concentrate).
c. Homogenize in tube (20 – 30 seconds), ensuring all tissue is homogenized.
d. Centrifuge at 5,000g for 1 minute at 4°C to remove insoluble material.
e. Pipette 10 μL of sample into 4 wells (2 for Solution A and 2 for Solution B). Uses a total of 40μL homogenate.
f. With AM/Salt solutions still in ice bath, add 200 μL 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. Pipette 90 µL of Salt solution into centrifuge tubes for samples.
3. Pipette 10 µL 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^{o}C, and transfer to 1 mL microcuvetes.
7. This is an endpoint assay read at a wavelength of 595 nm

Preparation of standards:

ug/10uL	BSA standard	Salt Solution
0	--	100 µL
5	5 µL	95 µL
10	10 µL	90 µL
15	15 µL	85 µL
20	20 µL	80 µL

VI. ATPase Activity Calculation

The ATPase standard curve should be read in mOD/nmole ADP
The ATPase acitivy measurements should be read in mOD/10□L /minute

Sample calculation:

Standard curve = 20 mOD/nmole ADP
ATPase (Solution A) = 30.0 mOD/10µL /minute
ATPase (Solution B (-K^{+})) = 13.5 mOD/10µL /minute
Na+,K+-ATPase = 30.0 – 13.5 = 16.5 mOD/10µL/minute
Protein reading = 8.5 µg/10µL
$\frac{16.5 \text{ mOD/10µL/minute}}{20 \text{ mOD/nmole ADP}}$ = 0.825 nmoles ADP/10µL/minute
$\frac{0.825 \text{ nmoles ADP/10µL/minute}}{8.5 \text{ µg/10µL protein}}$ = 0.097 µmoles ADP/mg protein/minute
(0.097)(60min) = 5.82 µmoles ADP/mg protein/hour

VII. References

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Note: This section is formatted for publication in the journal Ecotoxicology and Environmental Safety

Chapter 5: Assessing accumulation and sublethal effects of lead in a freshwater mussel

Abstract

We 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 μg/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 μg/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, Ca^{2+} levels were reduced below the 95% CI of the baseline range for Ca^{2+} in low Pb exposures (≤ 3 μg/L), and above it for high lead exposures (11, 66 and 251 μg/L), indicating potential adverse effects of Pb on 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 ≤ 11 μg/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, we found that ALAD proved to be an unsuitable biomarker for Pb exposure in the freshwater mussel *Elliptio complanata*, Ca^{2+} 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 μm), 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 we 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 δ-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], the gastropod *Biomphalaria glabrata* [27] and the freshwater bivalve *Corbicula fluminea* [28]. 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 assessed was ion (Na^+, K^+, Cl^-, and Ca^{2+}) concentrations in mussel hemolymph. Even with high renal ion absorption resulting in an excretory fluid concentration half that of hemolymph [29], 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 [30] (36 mOsm for the zebra mussel [31]) to reduce loss and expended energy for active ion uptake [32].

Because Pb has been shown to cause disruption to protein and glucosamine in unionids [33], and other heavy metals such as Cd will cause osmotic imbalances in unionids [34], we 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 [35] with modifications for mussel adult testing. For the 28 day lead exposure study, we 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 [36] 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 [37] were used for all measurements

of water-quality parameters. All test water was measured for pH with a Beckman Model Φ 240 (Beckman Instruments, Fullerton, CA, USA) calibrated meter. Alkalinity was determined by titration of 0.02 N H_2SO_4 to pH 4.5 and hardness by titration with 0.01 M ethylenediaminetetra-acitic 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® 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® 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^+, K^+, Cl^-, Ca^{2+}) 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 $CaCO_3$ (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 the freshwater bivalve *Corbicula fluminea* as well as in gastropod tissues and hemolymph, therefore, we 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 µL 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.

Following this experiment, a second assay was conducted to compare levels of ALAD activity from Corbicula mussel whole-body tissue to Elliptio mussel whole-body tissue, as well as a fish control. Methods for this test were the same as described above,

except samples were allowed to incubate for 4 hours instead of 1 to allow for higher sensitivity and a lower detection limit.

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 µg/L. After the 48 hour time intervals post renewal, the lower concentrations of 1.95 and 3.9 µg/L were below detection limit, 7.8 to 62.5 µg/L were 8% of target, and the higher concentrations of 125 and 500 µg/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 ≤ 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, we 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^{2+} 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^{2+} 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, we 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 [39, 40].

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. We 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 Ca^{2+} is adversely affected by Pb exposure. There is mechanistic plausibility for Ca-Pb interactions [41], but more data are needed to validate this relation.

Lastly, our 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 [42, 43] depending on the genera. And while the absence of iron hemoglobin in hemolymph does not necessarily negate its presence in the tissue of bivalves [43], we 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, 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 Ca^{2+} concentrations in response to various stressors before such a monitoring assessment could be made.

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Tables

Table 5-1. 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	Mean Measured Concentration (ug/L)						
Concentration (ug/L)	T0 (*n=3)	% of Target	T48 (*n=2)	% of Target	T72 (*n=3)	% of Target	Average Daily Exposure
Control	0.0	n/a	0.0	n/a	0.0	n/a	0.0
2	1.8	90.2	0.0	0.0	0.0	0.0	0.9
4	2.6	65.5	0.0	0.0	0.0	0.0	1.3
8	5.2	65.2	1.7	20.7	0.0	0.0	3.2
16	12.2	76.2	0.5	3.2	1.0	6.4	6.4
31	19.2	61.9	1.2	4.0	2.8	9.0	10.5
63	48.4	76.8	3.2	5.2	4.0	6.3	25.9
125	101.0	80.8	33.4	26.7	27.8	22.3	66.3
500	395.9	79.2	121.1	24.2	75.2	15.0	250.8
	* Each 'n' represents 3 samples pooled.						

Table 5-2. 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	Average Tissue Pb	
Concentration (ug/L)	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 5-3. The pooled hemolymph Pb concentration as µg/L. The stared " * " Day 28 represents the individual hemolymph Pb concentration of the non-sampled replicate 5 mussels.

Treatment	Day of Exposure					
Concentration (ug/L)	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 5-1. Jar setup with aeration for 28 day Pb exposure study.

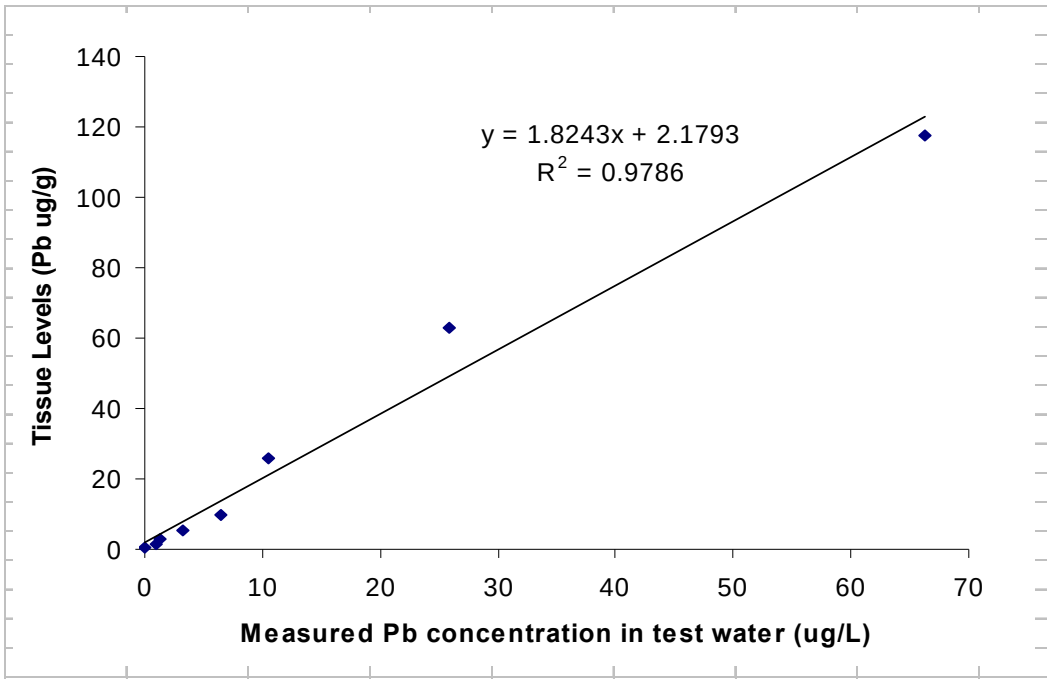

Figure 5-2. Correlation between Pb concentrations in mussels and their exposure concentrations, from the control to 66 µg/L.

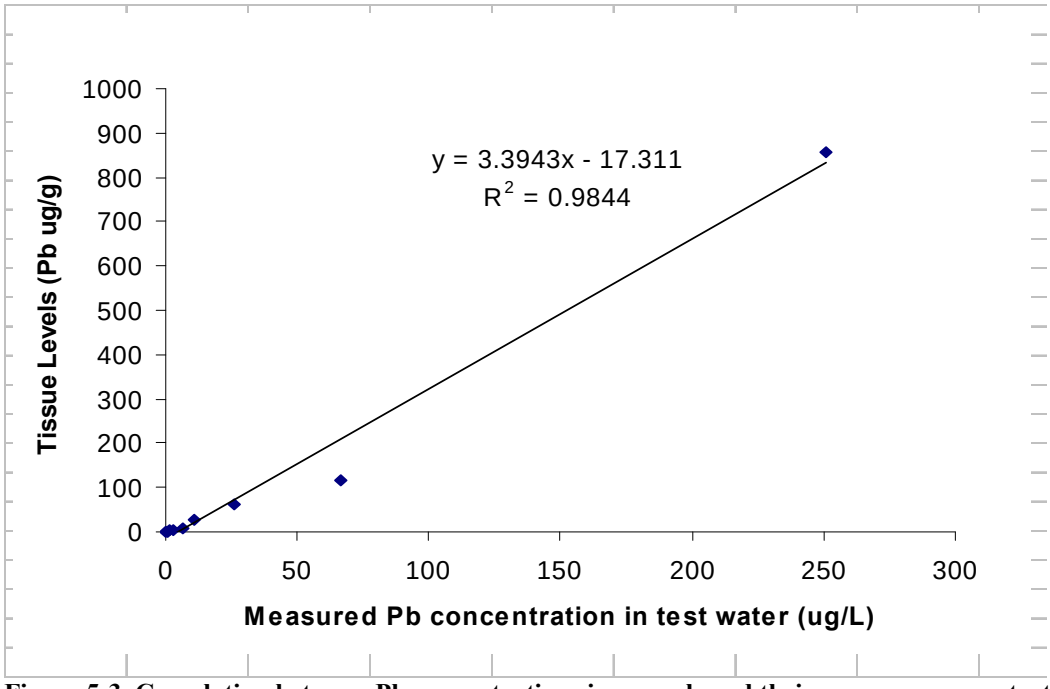

Figure 5-3. Correlation between Pb concentrations in mussels and their exposure concentrations, from the control to 251 µg/L.

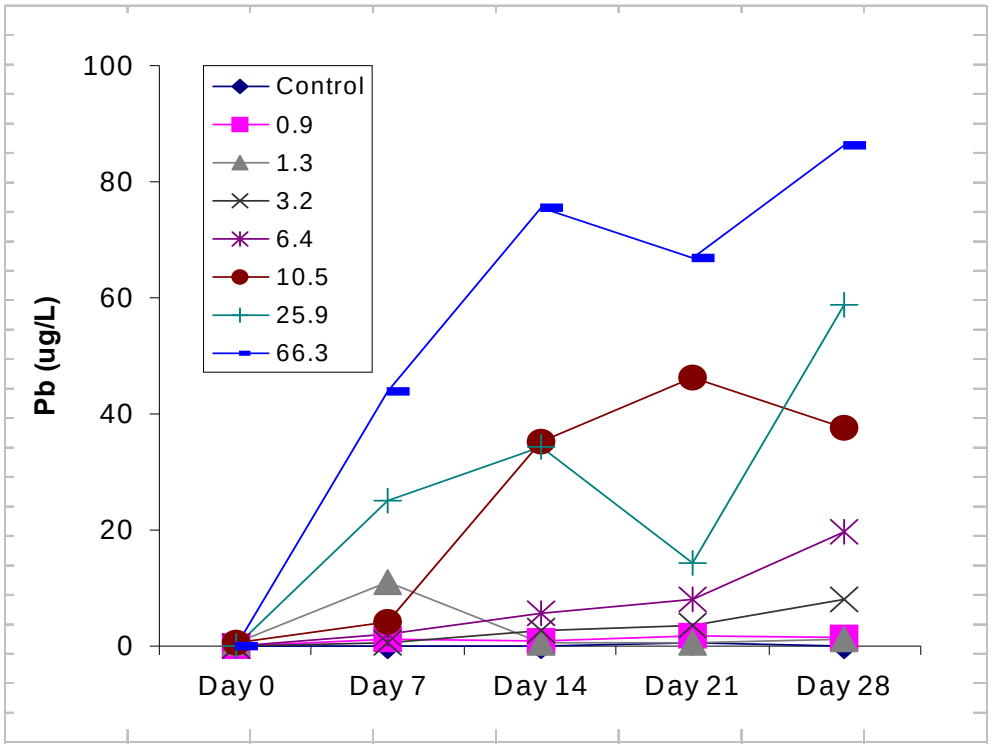

Figure 5-4. The hemolymph Pb concentrations over the 28 day study, as they relate to each treatment group excluding the 251 µg/L treatment.

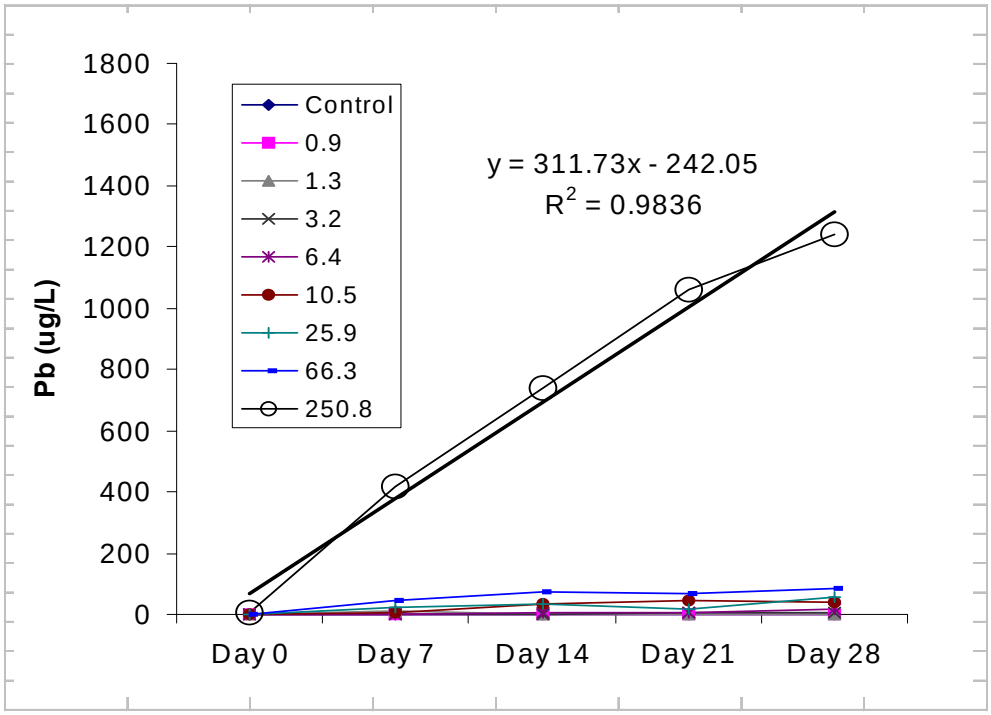

Figure 5-5. The hemolymph Pb concentrations over the 28 day study, as they relate to each treatment group including the 251 µg/L treatment.

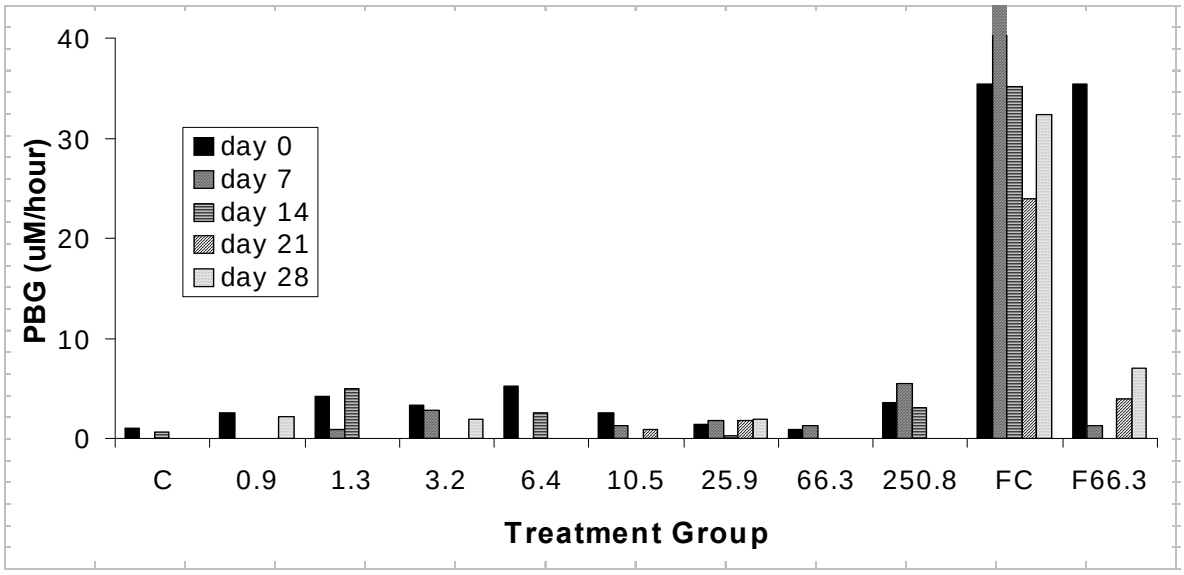

Figure 5-6. Results of the Pb ALAD activity inhibition assay for each mussel treatment group, including the fathead minnow control and 66 µg/L group, over the 28 day exposure.

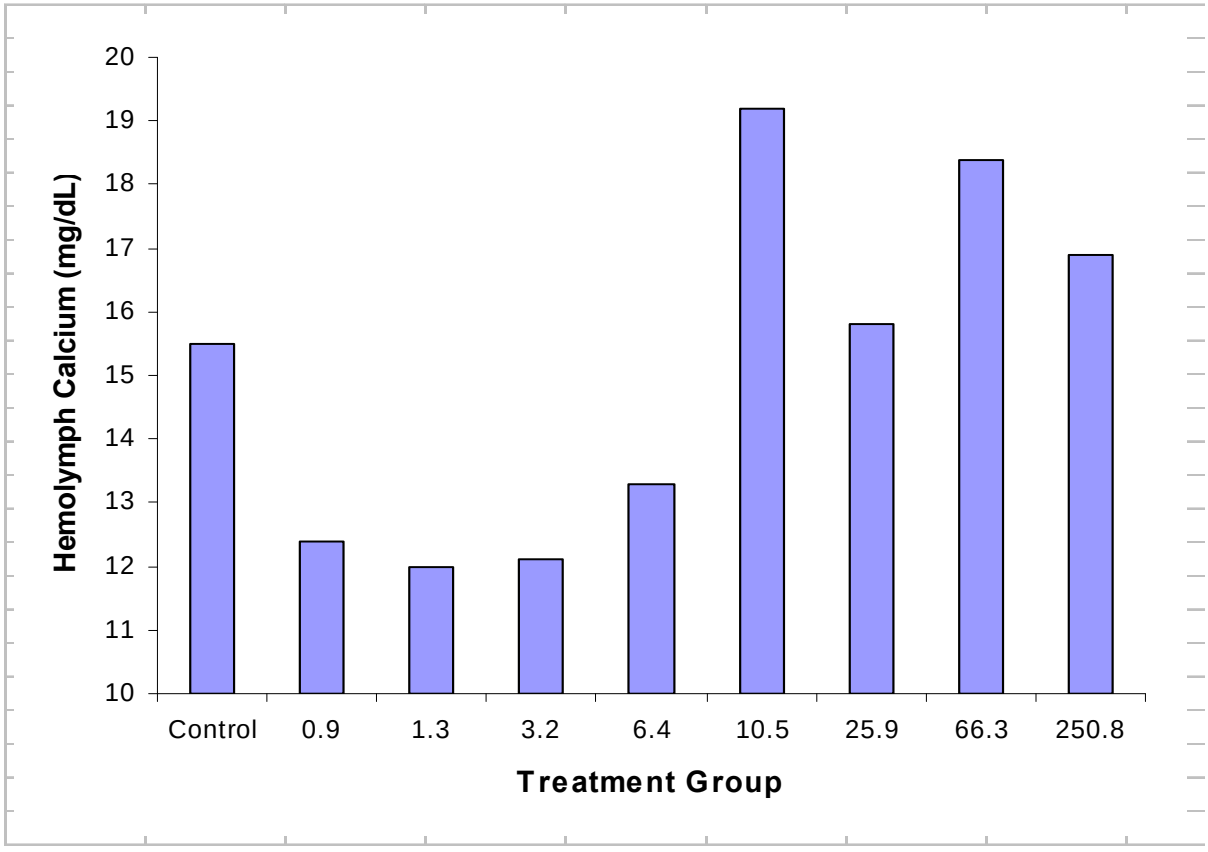

Figure 5-7. Calcium levels in hemolymph from the non-repeatedly sampled replicate 5 mussels, each treatment represents one mussel.

Appendix

Microplate Assay for δ-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_20.
Solution B – Dissolve 5.36 g sodium phosphate dibasic in 100 mL H_20.
Combine: 81.5 mL solution A,
18.5 mL solution B
70 mL H_20.

Adjust pH to 6.2 and bring to final volume of 200 mL with H_20. Store at 4ºC.

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 µL Triton X-100 to 50 mL with phosphate buffer. Store at room temperature.

ALA Buffer (670 µg ALA·HCl/mL) :

Dissolve 10 mg ALA in 15 ml of Assay Buffer. Store at 4ºC 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_20. 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_20
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 6.2×10^4. Aliquot 300 µL of this standard into 33 cryotubes and store in foil-wrapped cryocase at -80ºC.

Weekly Stock Solution:
Dilute the *Long Term Stock Solution* for use in the standard curve as follows: Dissolve 50 µL of the *Long Term Stock Solution* in 500 µL Assay Buffer (yields 221 µM PBG).

Standard Curve Serial Dilution:
Thaw one cryotube of the PBG *Weekly Stock Solution* (221 µM PBG). Transfer 150 µL to a 1 mL snap-cap centrifuge tube. Prepare a 1:1 dilution series of 7 tubes in Assay Buffer transferring 75 µL 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°C for at least 1 hour. Turn on the 37ºC 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 µL Assay Buffer.
  To the ALA replicate tubes, add 25 µL ALA Buffer.
  To the Control replicate tubes, add 75 µL Assay Buffer.
  To the PBG Standard Curve Serial Dilution tube #1, add 75 µL 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 µL of blood into a 1.8 mL cryotube. Record the mass. Add an equal volume of ice cold dH_20 to the sample (e.g. if the blood samples has a mass of 148 mg, add 148 µL of dH_20). Samples are then sonicated with hemolymph for 10 minutes to limit clotting.

Add 50 µL 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 37ºC 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 37ºC water bath. Terminate the reaction with the addition of 200 µL 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 µL 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 µL Ehrlich's reagent to each well. Place on plate shaker for 15 min of color development. Read absorbance at 540 nm.

CONCLUSIONS

Chapter 6: Conclusions

Distribution of Pb in a Stream

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, we 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 (Amiard et al. 1995; Marigómez et al. 2002), 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, δ-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 Ca^{2+} might be adversely affected by Pb exposure, and the findings from the second laboratory

study confirm this. Hemolymph 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 Ca^{2+} (Grosell et al. 2006). Sodium concentrations were also reduced by the end of the study in the greatest exposure, as expected from disruption of Na^+,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 Na^+,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 Na^+,K^+-ATPase activity suggest its potential use as a biomarker of Pb exposure in mussels as has been shown for fish (Rogers et al. 2005). However, other inhibitors and confounding factors of Na^+,K^+-ATPase need to be taken into consideration before its considered as a definitive biomarker of Pb exposure. For example, if sulfydryl donors and L-cysteine are present, they can reduce Pb inhibition of Na^+,K^+-ATPase by as much as an order of magnitude (Rajanna et al. 1991; Krinulović and Vasić 2006). Other inhibitors shown to reduce Na^+,K^+-ATPase activity include the pesticide fenitrothion (Sancho et al. 1997) as well as the metals mercury and copper (Anner et al. 1992; Krstić et al. 2005), but not cadmium (Pivovarova et al. 1992). Because Pb accumulation in mussel tissue on day 28 was not significantly greater than on day 14, we 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 (Brown et al. 1996; Markich et al. 2001), 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 (Levine et al. 2005) or Pb in sediment (Latimer et al. 1990) is not apparent. Erosion and remobilization of Pb from roadside sediment has been shown to be a current problem in Hawaii (Sutherland and Tolosa 2000). After 28 days, the lowest Pb exposure concentrations of 0.9 µg/L in the first laboratory study, and 1.0 µg/L in the second laboratory study, both resulted in mussel tissue Pb concentrations (1.5 and 2.3 µg/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 Na^+,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 µg/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^+,K^+-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^+ when inhibited to 30% of controls (or 1 μMole 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 (Palacios et al. 2000). 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 (Zereini et al. 2007), 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^+,K^+-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 (Angelo et al. 2007), it is critical to continue monitoring aquatic ecosystems and avoid further contamination of the environment.

Distribution of PGM in a Stream

This study has demonstrated for the first time that platinum (Pt) and palladium (Pd), platinum group metals (PGM), are accumulated from solution by *Elliptio complanata* and that Pt is enriched in *E. complanata* soft tissues at road crossings in North Carolina. However, the results of this study did not demonstrate a correlation between the number of daily vehicle crossings on a highway waterway crossing structure and the downstream enrichment of mussel tissue or sediment with PGM. The nonpoint source deposition of PGM from automobile catalytic converters along linear highway corridors was expected to behave like a point source discharge by concentrating road deposited sediments and allowing their transport, via stormwater, to a common point of discharge into the stream environment near highway stream crossings. The correlation between PGM in the roadside environment and the density and conditions of automobile traffic is well documented (Helmers 1997, Morcelli et al. 2005, Limbeck et al. 2007, Zereini et al. 2007). Transport from the roadside environment to the stream environment has been shown to occur where road runoff reaches waterbodies (Tuit et al. 2000, Essumang et al. 2007). In this study, we have demonstrated that enrichment of PGM in sediment and mussel tissue is occurring, and that the level of enrichment in the watershed is related to the number of people living in the watershed, and by extension the number of vehicles operating within the watershed.

Our conclusions on the lack of observable correlation with traffic fall into two general categories. First, emission of PGM from automobiles is primarily in a metallic form that is not immediately bioavailable, and chemical weathering of PGM in organic sediments to a bioavialable form favors a dietary route of exposure for mussels. Second, hydraulic alterations of the watershed and stream channel can have an influence on the location of deposition of PGM in the stream sediments, production of the mussel's primary food source, and the location and density of mussels in the stream; all three variables influence the distribution of PGM in sediment and in mussel tissue.

The emission of PGM from automobiles is primarily in a metallic form attached to an alumina substrate (Ravindra et al. 2003). The fraction of this emission that is immediately soluble is small, perhaps less than 5% (Palacios et al. 2000). Soluble ions of PGM are unstable in solution and tend to be rapidly adsorbed to organic molecules in the water column or to sediments (Turner et al. 2006). As shown in the laboratory portion of this study, mussels are able to accumulate Pt and Pd from solution, but the concentration of PGM in solution has been shown to be very low (<0.1 ng/L) even when sediments are enriched to concentrations more than 5 times the sediment concentration observed in our field study (Rauch and Morrison 1999). It has been demonstrated that particulate PGM displays increased solubility in environmental matrices through interaction with organic acids, similar to the environmental conditions encountered in soils and sediments with decaying vegetation, and that complexation with organic molecules increases its mobility (Bowles and Gize 2005). The deposition of PGM particles in organically enriched stream sediments would result in increased solubility of PGM at the site of production of food sources utilized by the mussels.

Mussels are known to feed on suspended particulate organic matter and detritus, apparently preferentially incorporating the living algal and bacterial fraction (Raikow and Hamilton 2001). The sulfate-reducing bacterium *Desulfovibrio desulfuricans* has been shown to accumulate PGM. This bacterium is similar to bacteria naturally found in decaying detritus in aquatic systems (Yong et al. 2002). Marine algae have also been shown to accumulate PGM from solution (Turner et al. 2007). Bacteria facilitating the breakdown of allocthanous organic materials that are contaminated with catalyst emitted particles would be exposed to bioavialable PGM, creating a dietary route.

Another route of exposure could be deposit feeding on biodeposited and other organic sediment components. Mussels are known to biodeposit unused nutrients in the form of pseudofeces and feces (Vaughn and Hakencamp 2001). Recent studies have shown that mussels may derive a substantial portion of their energy by deposit feeding on surrounding organic sources (Raikow and Hamilton 2001). Although it has not been demonstrated that mussels are able to feed on pseudofeces, it seems possible that the organic material in biodeposited pseudofeces may contribute to material available for deposit feeding. Deposit feeding could be accomplished by movement of food particles to the mouth by cilliary action or, more recently suggested, direct absorption of dissolved organic carbon (DOC). Studies with zebra mussels have shown that they are able to utilize dissolved organic carbon from the water column as a food source. In the presence of metals that readily complex with DOC, this form of carbon sequestration increases the metal concentration factors in exposed mussels. It was concluded that the method of uptake was absorption rather than uptake by filtration and that this route of uptake could supply up to half of the mussels no-growth energy requirements (Roditi et al. 2000).

Complexation with dissolved organic carbon (DOC) has been shown to increase the amount of PGM that is able to remain in solution (Turner et al. 2006). Sures and Zimmermann (2007) demonstrated that the presence of humic substances increased the uptake of Pt, but hampered the uptake of Pd in zebra mussels. Observations that complexation with humic acids and bile increase the lipid solubility of PGM (Zimmermann et al. 2003) introduces the possibility that unionid freshwater mussels may accumulate PGM through deposit feeding and absorption of DOC.

Mussels in dense beds are thought behave as ecosystem engineers, by biodeopsiting nutrients and stabilization of the substrate (Vaughn et al. 2007). The pore water of carbon enriched sediments in mussel beds is likely to have more dissolved organic carbon than the overlying surface water. Additionally, the increased organic activity of sediments in mussel beds may increase the solubility of deposited catalyst derived PGM. Mussels aggregated in dense beds may facilitate increased uptake of PGM and other pollutants by sequestration and feeding on contaminated organic matter that was not incorporated in the first pass of filtration, compared to solitary counterparts.

Creation of mussel beds requires that the flow of water across the bed is adequate to provide the mussels an adequate supply of suspended food particles, but not so great that it destabilizes the bed during high flow. In places where the hydrology allows for long-term biodeposition of organic material in a mussel bed, the efficiency of PGM uptake may be enhanced. In contrast, where mussels are in a more hydraulically active area of stream, such as a riffle, where pseudofeces and other organic materials may not accumulate, the bioaccumulation of PGM may be limited to only what is incorporated in the first pass of filtration. If the bioaccumulation of PGM is dependent on the

transformation of catalyst derived PGM in the sediment and its complexation with the mussels food source, then the behavior of the deposition of these small particles will likely be important in their pattern of distribution in mussel tissues, as shown in our data collected from New Hope Creek, North Carolina.

If hydrology of the stream plays in important role in PGM deposition, speciation, and incorporation into mussels, then alterations to the natural hydrology of the stream could alter patterns of PGM abundance. The field portion of our investigation was largely limited to the areas of stream immediately surrounding a highway crossing of a stream. Bridges and culverts are known to cause alteration of the area immediately downstream and sometimes for some distance upstream of the crossing. Bridges over North Carolina streams are almost never long enough to span the entire floodplain of the stream. Where the structure does span the floodplain, bridge bents are necessarily placed in the stream or in the floodplain to achieve adequate span. A structure that constricts the natural floodplain invariably causes hydraulic alteration. The exact nature of this alteration is based on many factors and is site specific. Our general observation of piedmont streams in North Carolina is that bridges commonly cause stream scour immediately downstream of the structure by increasing the maximum velocity of the water that passes under the structure during high water events. Often this is accompanied by bank destabilization and alteration of the substrate (Johnson 2006). Importantly, the increased range of stream velocity immediately downstream of the bridge tends to scour the substrate, keeping it relatively free of organics and often leading to deposition of sand. This periodic cleaning of the substrate also causes biodeposited and other fine

organics to be washed away, potentially prohibiting the establishment of dense mussel beds.

In the final phase of our field experiment, multiple samples were collected from New Hope Creek in Orange County, North Carolina, in an effort to estimate the variability of the composite samples of mussels collected at other sites. The coefficient of variation at six sites ranged from 12 to 70%. Of these samples, the coefficient of variation of the tissue concentrations of Pt were much higher immediately downstream of the I-40 crossing of New Hope Creek. The wide, multichannel box culvert under I-40 allowed for a well established and dense mussel bed approximately 150 m downstream of the crossing. Farther downstream (150 m), NC Hwy 86 crossed New Hope Creek. This bridge was a short span bridge, common on secondary roads in North Carolina, and caused considerable floodplain constriction. Downstream of NC Hwy 86 (150 m), mussel density was considerably lower and mussels were primarily confined to the more stable areas along banks. Mean tissue Pt concentrations immediately downstream of NC 86 were one-third of the concentrations immediately downstream of I-40 despite being separated by only 300 m. One kilometer downstream of NC Hwy 86, mussel densities remained low and tissue Pt was similar to concentrations observed immediately downstream of NC Hwy 86. At sites 2.5 km and 4.5 km downstream of the I-40 and NC 86 crossings, mussel densities were high and hydrologic conditions permitted a well established mussels bed, and tissue Pt concentrations were highly elevated compared to samples from upstream of I-40 and immediately downstream of the I-40 crossing, despite no apparent source of significant additional input of PGM between sites. At a site 6.7 km downstream of the I-40 crossing, levels of Pt in mussel tissue and sediment had decreased

to within the range observed upstream of I-40, which we consider to represent a background concentration for this part of the watershed.

Our interpretation of the distribution of mussel tissue Pt enrichment in New Hope Creek is that PGM metal particles from I-40 and NC 86 are being unevenly distributed in the area downstream of the sources, largely dependent on hydraulic characteristics. Due to the process of transformation in the sediment and subsequent transport on food particles, the peak of PGM accumulation in mussel tissue is not observed for several km downstream from the primary source. Based on the available data, the area of maximum enrichment from the I-40 and NC 86 crossing is somewhere between the sites located between 2.5 km and 4.5 km downstream of the primary source (i.e., I-40) (Figure 5. Chapter 1).

The implication of this observation for transportation planning, in areas that support protected species of mussels, is that the impact of road derived pollutants is projected for a considerable distance from a road crossing (i.e., a longitudinal lag). A typical distance for consideration of adverse effects to protected mussels is 400 m. Based on the data gathered in this project, it may be necessary to consider impacts from transportation projects for several kilometers downstream of a crossing.

Potential Threat to Freshwater Mussels

This study represents the first information available on the concentrations of PGM in unionid freshwater mussels. Based on the data that we gathered, the concentration of PGM in freshwater mussels is low relative to other common metal pollutants (i.e., Cd and Hg), but the enrichment in mussels over background levels and the short time these

metals have been appreciably emitted into the environment elevate them as an environmental concern.

Reported natural background concentrations in the earth's crust are 0.4 ng/g Pt and Pd (Whedepol 1995). In this study, the observed maximum sediment concentrations were 1.86 ng/g; approximately 4.7 times the background level. Due to the inert form of emission of PGM and slow transformation to its bioavailable form, it is possible that a considerable portion of the PGM emitted within the last three decades remains available for transformation. If this is true, we would expect to see the concentrations of PGM in the mussel tissues to increase disproportionately to its emission from automobiles. The streams included in this study were limited to streams that were able to support mussel fauna and are generally among the least impacted in the region. The enrichment of heavily degraded streams is likely to be greater than in streams that support mussels.

It has been our experience that in streams in the piedmont region of North Carolina supporting species rich mussel assemblages are restricted to relatively undisturbed watersheds. Mussels are rarely found in streams that drain semi-urban areas and never in streams that are primarily urban drainage. The decline of a healthy mussel assemblage in free flowing streams in this region generally follows the pattern of the loss of species until only *E. complanata* remains. *E. complanata* far out numbers all other species in the Atlantic Slope of North Carolina. This common pattern suggests that *E. complanata* is more tolerant of common anthropogenic pollution than species with which it commonly co-occurs, making extrapolation of effects to other more sensitive species difficult.

In the laboratory portion of this study, observable physiological changes and mortality were limited to the highest exposure concentrations. The exposure concentrations that produced significant physiological changes were associated with tissue concentrations that are far in excess of tissue concentrations that we observed in mussels collected from the field. The potential for adverse effects to *E. complanata* adults from present ambient concentrations of PGM appear to be low, but the potential for interactive or synergistic effects may exist.

The potential that mussels aggregated in dense beds are able to incorporate more PGM could have negative implications for juveniles and other mussel species dependent on mussel bed habitat. Young mussels have been reported to be more susceptible to some pollutants than adults (Wang et al. 2007). If mussel beds are established because the engineered habitat increases recruitment, then the concentration of PGM and other pollutants in the bed sediments could lead to reduction in recruitment. Reduced recruitment would eventually lead to a reduction in mussel densities that are necessary to maintain the mussel bed, leading to the loss of function of the mussel bed habitat causing adverse effects to mussels in general.

Avenues for Future Study

Many aspects of the fate and effects of PGM in the environment remain unknown. The effects of combinations of pollutants, the effects to other life stages of *E. complanata,* and effects to other less tolerant species are unknown and should be evaluated. Even though concentrations of PGM are presently low, the long term effects of low level chronic exposure could have detrimental effects on populations of mussels.

The prospect that dense aggregation of mussels in beds may facilitate the concentration of pollutants is an interesting avenue for study. Mussel beds are often made up of multispecies communities that may have some commensal relationship. Mussel bed communities are often dominated by one or a few common species with fewer numbers of less common species. The recent loss of many of the rare species may be due to reliance on the mussel bed habitat. Reductions in recruitment of the dominant species could cause the loss of the bed habitat, reducing the opportunities for dependent species to persist in a stream.

An undesirable weakness in the field portion of this study was an inability to distinguish between sediment PGM in metallic form and that in a bioavailable form. Due to the low ambient concentrations, the technique used for analysis required complete digestion of the samples and did not allow for elucidation of the chemical species. Studies on the chemical species of PGM present in different sediment types, especially focused on sediments in dense mussel beds, would be able to test for enrichment by mussel biodeposition.

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Table

Table 6-1. The Na+,K+-ATPase activity for each treatment group as a percentage of the Control for day 28 of the study.

Inhibition of Na+,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