CELLULAR BIOMARKER RESPONSES OF THE ASIAN CLAM, *CORBICULA FLUMINEA*, NEAR A COAL ASH POND

by

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ABSTRACT

TRAVIS DANE THOMASON. Cellular biomarker responses of the asian clam, *Corbicula fluminea*, near a coal ash pond effluent. (Under the direction of DR. AMY H. RINGWOOD)

The storage of coal combustion residuals (CCR) in aquatic surface impoundments has been a common practice across the United States for decades. Typically these aquatic surface impoundments are designed to allow the upper portion of the ash-basin water column to be removed and emptied into nearby freshwater or coastal systems. This method of coal-ash storage and disposal creates the potential for chronic point-source inputs of toxic metals into vital aquatic resources. The goal of this study was to investigate the potential ecological impacts of coal-ash associated metals on a freshwater ecosystem adjacent to a recently decommissioned coal-ash basin on Mountain Island Lake, NC. Ecological impacts were quantified through chemical analysis of water and sediments, and the use of the freshwater clam *Corbicula fluminea* as a bioindicator species. Clams were allocated into cages and deployed *in situ* at five sites near the ash basin for a one-month duration. Study sites included two locations within Mountain Island Lake's coal ash effluent mixing zone, two locations further downstream, and one upstream reference site. After exposure, clam soft tissues were assessed for coal-ash associated metal bioaccumulation using Atomic Absorption Spectroscopy (AAS) and Direct Mercury Analysis (DMA), and clam health was assessed using a suite of sub-lethal cellular toxicity assays (lysosomal destabilization, lipid peroxidation, and micronucleus). Site water and sediments were collected from each study site and analyzed for common coal-ash associated metals. One-week laboratory exposures were also conducted using

field collected water and sediments. The use of these integrative methods can provide important insights into the temporal and spatial scales of CCR associated toxicity while also serving to further validate *C. fluminea* as a suitable bioindicator species in freshwater systems.

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CHAPTER 1: INTRODUCTION

1.1 Coal Combustion Residuals

Coal has been an important fuel source throughout recorded history, and to this day it remains the primary source of energy for electricity production worldwide, especially in the southeastern United States. Coal combustion accounts for over half of the electricity production in the United States each year (NRC 2006; Rowe et al. 2002), resulting in the release of massive amounts of CO_2 into the atmosphere and the creation of millions of tons of solid particulates commonly known as coal ash. Each year, over 110 million tons of coal ash is generated in the United States as a by-product of electricity production in coal fired power plants (USEPA 2015). Although the exact composition of coal ash varies depending on the geology of the deposit from which the coal was mined, the ash typically contains high concentrations of a variety of toxic metals and trace elements including arsenic, beryllium, boron, cadmium, chromium, cobalt, lead, manganese, mercury, molybdenum, selenium, strontium, thallium and vanadium (Rowe et al. 2002). When coal is burned, these inorganic metals and trace elements are released, some into the air and some that are concentrated in the ash by-product.

Coal ash is currently the largest industrial waste produced in the United States (Harkness et al. 2016). Storage and disposal of this massive amount of coal ash poses significant environmental and human health hazards. Although a portion of this solid waste can be recycled in materials such as concrete and gypsum wallboard, approximately half will be disposed of in landfills and unlined aquatic surface impoundments (Rowe et al. 2002). This creates the risk for chronic ground and surface

water contamination, particularly when stored in aquatic surface impoundments because of their close proximity to freshwater and coastal resources. These surface impoundments serve as settling basins where larger particles sink to the bottom while the upper portion of the water column is skimmed off and emptied into "mixing zones" of nearby waterways, creating a constant input of dilute toxic metals. Recent studies have highlighted some of the abiotic and biotic mechanisms through which toxic elements are transported from coal ash effluents through nearby ecosystems, but there is still much work to be done in this area (Peltier et al. 2009; Rowe 2014).

The ecological consequences of catastrophic ash-pond dike failure have received increased attention in the past few years, partially due to the devastating spills on the Emory River, near the Tennessee-North Carolina border and the Dan River, near the North Carolina-Virginia border (Otter et al. 2015). Many of the adverse ecological consequences from these large-scale spills are obvious because the contamination is so sudden and drastic. It is also important, however, to consider the chronic effects of the leachates and effluents that these ponds inherently create. The design of these ash-basins and the surface skimming activities associated with them create the potential for long-term point source pollution into adjacent waterways. It was once thought that chronic inputs of ash pond effluents would be diluted enough by the bodies of water that they are pumped into to negate any detrimental environmental impacts. Metal inputs, however, are quickly adsorbed to sediments and absorbed by the biota, so the complexity of metal chemistry and physical environmental factors often create depositional zones where metal contaminants have the potential to accumulate in higher concentrations. Due to the persistent properties of metal contaminants, aquatic ecosystems located near coal ash

basins may experience significant ecological consequences, even for many years after the basins have been decommissioned.

1.2 Metal Toxicity

Because much of the toxicity of CCRs stem from high concentrations of metals and trace elements, the contamination to ecosystems can endure for many years. The persistent nature of metal contamination is largely because at their most fundamental level they are atomic and not molecular complexes. Therefore, metals do not degrade into relatively safe metabolites like some organic pollutants, they persist and cycle through organisms and the environment, often becoming more concentrated as they move up trophic levels. Although environmental conditions sometimes convert metals to less toxic valence states or bind them in less harmful complexes, their elemental character allows them to remain in an ecosystem with the potential to increase suddenly in bioavailability and toxicity when conditions change. Therefore, metal pollutants are often only temporarily bound in sediments creating a repository of contaminants which can readily become re-suspended and bioavailable to both benthic and pelagic organisms (Burton 2010).

Many of the toxicological effects of metals are ultimately a result of oxidative stress caused by metal-induced reactive oxygen species (ROS). Excessive ROS generation can be severely detrimental to biological systems because ROS can damage tissues and cellular components such as membranes, DNA, and proteins (Kelly et al. 1998). Certain transition metals are particularly effective ROS generators because their multiple possible valence states allow them to readily participate in redox reactions, sometimes affecting their toxicity potential. Chromium (Cr), for example, is typically

found in the environment in one of two stable valence states: Cr(III) and Cr(VI). Low doses of Cr(III) serve as an essential dietary mineral; it is found in most foods and required for normal glucose metabolism (Jomova and Valko 2011). Hexavalent Cr, however, is considered to be much more toxic, primarily because it crosses cellular membranes more readily. Once inside cells, Cr(VI) is reduced to Cr(III), forming ROS (Jomova and Valko 2011) and a form that more readily binds to DNA to cause DNA damage. The increase in cellular ROS caused by Cr and other coal ash associated metals has the potential to cause severe oxidative stress and DNA damage in exposed organisms.

In addition to ROS generation, arsenic (As) metabolism has been shown to also generate damaging reactive nitrogen species (RNS), further contributing to oxidative damage of cellular components and macromolecules (Shi et al. 2004). Arsenic exposure also hinders the cell's ability to negate increased ROS and RNS production by inhibiting the synthesis of glutathione, one of the most ubiquitous and powerful cellular antioxidants (Jomova and Valko 2011). Arsenic toxicity is not limited to oxidative damage, however. Various cellular enzymes have been shown to be inhibited via binding of trivalent As to sulphydryl groups (Wang and Rossman 1996). On the other hand, As can also be converted to arseno-betaine which is considered a non-toxic form.

Mercury (Hg) is often considered one of the most toxic metal contaminants in aquatic ecosystems, especially in the form of methylmercury (MeHg). In aquatic systems, Hg typically enters the food web when elemental Hg is biotransformed by microbes into MeHg. This organic form of Hg is readily bioaccumulated by other organisms and has great potential for biomagnification at higher trophic levels. At the molecular level, MeHg toxicity proceeds through similar mechanisms as As. MeHg, like As, has been

shown to covalently bind the sulphydryl groups of proteins, leading to the inhibition of a variety of cellular enzymes. The affinity of MeHg for sulphydryl groups also allows it to bind to the thiol group of glutathione, forming an excretable complex, although most organisms have much lower excretion than accumulation rates for Hg. This can lead to a depletion of glutathione and subsequent increase in oxidative stress. MeHg also has the potential to directly increase ROS generation through interaction with other nucleophilic protein groups and induction of mitochondrial dysfunction (Farina et al. 2011).

Selenoproteins are one of the most common targets of MeHg. While selenoproteins are major MeHg targets, selenium (Se) in molar excess of MeHg has been shown to have a protective effect against Hg toxicity. The antagonism between Se and Hg toxicity is one aspect of the complexity of Se contamination. Although Se is a constituent of the amino acid selenocysteine and is essential for a wide range of biological functions, exposure to excessive levels of Se can have toxic effects. Most essential metals behave in this manner, becoming toxic when present in either too high or too low concentrations, but Se is thought to have one of the narrowest ranges of essentiality of all essential metals (Luoma and Rainbow 2008). Furthermore, Se has been shown to bioaccumulate in a variety of aquatic organisms, and a number of major fish kills have been linked to Se contamination (Hamilton 2004). Therefore, there is a delicate balance between Se toxicity and amelioration of Hg toxicity.

1.3 Biomarkers and Bioindicators

Most current regulations and monitoring procedures for metal contamination focus mainly on water column concentrations, giving a severely limited view of the potential environmental effects of chronic low-level inputs such as ash-basin skimming

and dewatering. Although guidelines exist for acceptable sediment concentrations for some toxic metals, they are largely only recommendations and are consequently unenforceable and often somewhat vague. Further complicating matters is the complexity of metal-sediment interactions. Release of stored toxic metals into the water column from sediments is influenced by a multitude of physical factors including temperature, pH, water currents, bioturbation, and sediment particle composition. The complexity of metal chemistry and ecosystem interactions has made it somewhat difficult to determine the potential biological effects of chronic coal-ash basin discharge on the organisms in and around the mixing zones, or in potential deposition zones such as coves or turns along the river channel.

Measurable changes in biochemistry and cellular functions, e.g. "biomarkers," and their effectiveness as valuable approaches for assessing potential contaminant effects has been validated throughout the world, especially in estuarine and marine ecosystems. It has been well documented that a variety of cellular stress responses are valuable, sensitive indicators of sublethal pollutant exposure for many aquatic organisms (Ringwood et al. 1999). A variety of sensitive cellular assays can be used as tools to provide more direct links to biological effects and examine overall ecosystem contamination. Approaches that integrate results from sublethal biomarker assays with sediment and water chemistry along with spatial analyses are essential for protecting valuable freshwater resources.

Bivalves are regarded as especially valuable bioindicator species due to their relatively sessile lifestyle and filter feeding behavior (Ringwood et al 1999). They are readily maintained for laboratory studies, and very amenable to caging methods that

facilitate *in situ* field studies. A variety of biomarker assays are routinely used to assess cellular stress responses related to tissue and sediment contamination levels (Ringwood et al. 1999).

While these approaches have been used extensively with marine bivalve species as bioindicators of ecosystem health, these valuable approaches are only recently being used with freshwater species (Bonnail et al. 2016). One problem has been that freshwater bivalves have suffered high rates of extinction so large sampleable populations are relatively rare, especially in potentially polluted habitats. Therefore in the freshwater arena, invasive bivalves such as zebra mussels (*Dressenia sp*.) and Asian clams (*Corbicula sp.*) are being developed as bioindicator species. *Corbicula fluminea* is a valuable freshwater bioindicator species due their high filtering and growth rates, and their infaunal behavior is also ideal for these types of studies, because they burrow into the shallow sediments and filter feed at the sediment/water interface where the flux of metals between the sediments and the water column is most pronounced. Recent laboratory and field studies with *Corbicula* have established it as one of the few freshwater bivalve species suitable for these types of assays (Fournier et al. 2005; Santos et al. 2007).

1.4 Study Area

Mountain Island Lake, a 2,788-acre lake located northwest of Charlotte on the Catawba River system in North Carolina was a primary focus of these studies. It supplies drinking water to over 750,000 people in Charlotte and surrounding areas as well as supporting a variety of freshwater recreational activities such as boating, fishing, and swimming. Because much of the area surrounding Mountain Island Lake remains

undeveloped, many aquatic and terrestrial species depend on it for suitable habitat and freshwater resources. Mountain Island Lake is also home to Duke Energy's Riverbend Steam Station and its two coal-ash settling basins. Although Riverbend Steam Station was decommissioned on April 1, 2013, the two ash-basins still contain the ash that was generated when the plant was still operational, between 1929 and 2013. Dewatering and skimming processes are currently underway in preparation for the eventual excavation and relocation of the coal-ash.

1.5 Purpose and Hypotheses

The purpose of this project was to assess the cellular toxicity and metal bioaccumulation of *C. fluminea* downstream of a coal ash effluent. This was accomplished using a combination of field and laboratory exposures and a suite of cellular biomarker toxicity assays designed to test the following hypotheses:

H_1: Clams deployed near the coal ash effluent will exhibit the highest levels of lysosomal destabilization, micronucleus (MN) formation, and lipid peroxidation with levels decreasing as sites become further downstream of the effluent

H_2: Sediments and tissues of clams deployed near the coal ash effluent will exhibit the highest concentrations of toxic metals, with concentrations decreasing as sites become further downstream of the effluent.

This study will help clarify some of the potential impacts of conventional coal ash storage and disposal methods on freshwater systems, and serve to further validate *C. fluminea* as a suitable bioindicator species in freshwater habitats.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animal Care and Maintenance

Asian clams (*Corbicula fluminea)* were collected from an undeveloped location in Lake Wylie, NC and transported to the lab in aerated site water. Clams were then maintained in aquaria containing chlorine-free well-water in the lab for a period of at least 2 weeks prior to field deployment or lab exposure. Aquaria were carbon filtered and kept under constant aeration for the duration of the pre-exposure period. Carbon filters were removed twice per week and clams were fed stock cultures of *Ankistrodesmus sp.* purchased from Carolina Biological Supply Company and grown in Aquatic Eco-Systems INC Proline F/2 Algae Food media.

2.2 Field Studies

Clams were transported from the UNCC lab to Mountain Island Lake, NC in aerated aquaria water, where they were randomly allocated into cages and deployed *in situ* at 5 sites on the lake. Deployment sites included 2 sites inside the coal ash effluent mixing zone (Outflow and Cove Mouth), 2 sites at varying distances downstream of the mixing zone (Midway and Latta), and 1 upstream reference site (Upstream) (Figure 1). Cages were designed to sit on the lake bottom in order to allow clams to exhibit their natural infaunal behavior. Each cage was constructed using one-half of a vinyl-coated minnow trap (Bass Pro Shops) with each end covered by black HDPE mesh attached with nylon cable ties. One cage containing 40 randomly-allocated clams was deployed at each site for a one-month duration beginning October 17th 2017 and ending November 17th 2017. Each cage was anchored in place by attaching it to a 4-inch cinder block with a 6-foot length of polypropylene rope. After the one-month deployment period, cages were

retrieved from each site and transported back to the lab in a bucket of water from their respective sites, and aerated overnight for post-exposure processing the next day.

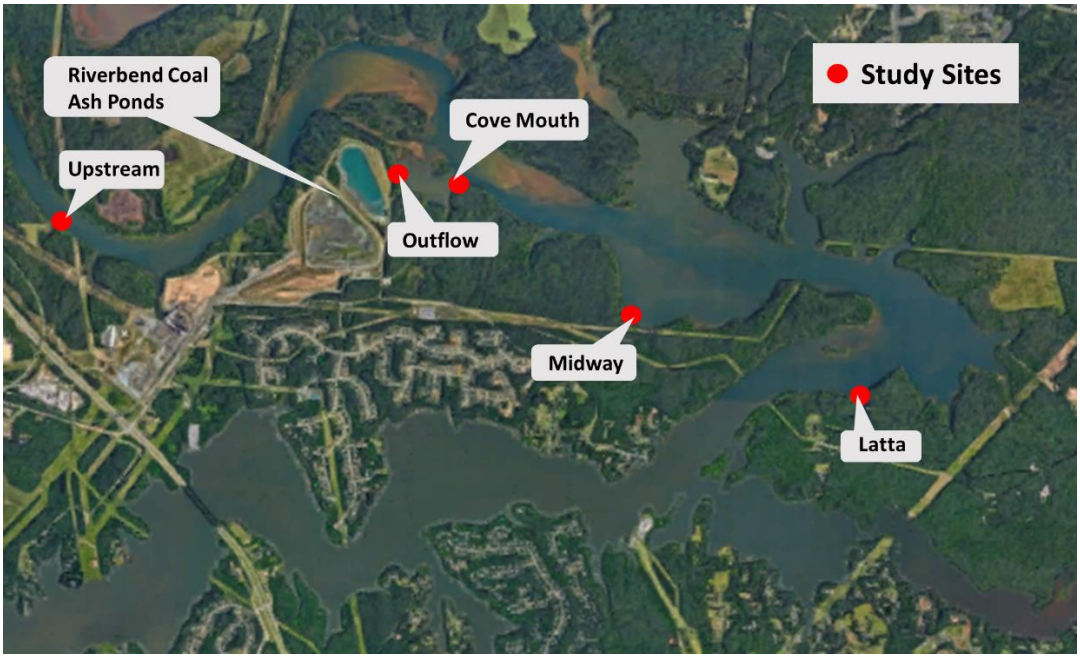

Figure 1: Aerial view of Mountain Island Lake NC showing coal ash ponds and study site locations.

Water and sediment samples were collected concurrent with cage deployments to be analyzed and used for subsequent laboratory exposures. Sediment samples were collected using a Wildco Fieldmaster mighty grab sampler and placed into labeled one-gallon Ziploc bags for transport. Water samples were collected in 1 L Thermo Scientific Nalgene HDPE bottles (EPA certified for trace metal analysis). All water and sediment samples were returned to the lab on ice and stored at 4° C until use.

2.3 Laboratory Exposures

A series of two laboratory exposures were conducted using the field-collected sediment and water samples. For each exposure, sediments were first dry sieved through

a 500 µm brass/stainless steel sieve and water samples were filtered through a 1 µm filter to remove unwanted debris. Laboratory exposures were then conducted by adding 50 mL of sediment and 500 mL of water from each site to replicate 1 L beakers. Control beakers were also set up using 500 mL of chlorine-free well-water. Four randomly allocated clams were added to each beaker and placed on aeration for an exposure period of one week. Clams were fed every 48 hours by adding 10 mL of *Ankistrodesmus sp.* (approximately 650,000 cells/mL) to each beaker. Two to four replicate beakers were used for each treatment.

2.4 Post Exposure Processing

Both laboratory and field exposed clams were washed with tap and deionized water to remove extraneous sediment particles before further processing. Thirty field-exposed clams from each site were then frozen whole for subsequent metal analysis. The remaining 10 field-exposed clams from each site were then measured and processed for biomarker analyses. A notch was cut in the ventral surface of each clam to allow access to the foot. A 1 mL syringe containing 200 µL of CaMg-free-saline (CMFS) at 20 ppt was then used to extract hemolymph from the foot of each clam. Extracted hemolymph was then split between two 1.5 mL micro-centrifuge tubes and kept on ice for hemocyte lysosomal destabilization and MN assays. Hemolymph samples designated for MN determination were centrifuged at 800 g for five minutes and the supernatant was decanted. These hemocytes were then fixed by resuspending them in 1 mL of Carnoy's solution and stored at 4° C. Hemolymph samples designated for lysosomal destabilization determination were immediately processed. After hemolymph extraction clams were

dissected and individual soft tissues were frozen at -80° C for lipid peroxidation determination.

2.5 Lysosomal Destabilization Assays

Lysosomal destabilization assays based on well-established methods (Ringwood et al 2005) were used to assess cellular damage. Hemolymph samples were centrifuged at 800 g for five minutes and the supernatant was decanted. Hemocyte pellets were then resuspended in 50 µL of Neutral Red Dye (NR) (concentration of 0.04 mg/mL CMFS) and incubated in the dark for a 60-minute period. After incubation, a 15 µL aliquot was placed on a slide and examined using standard light microscopy at 400x magnification. At least 50 hemocytes from each sample were scored as either stable (NR contained within the lysosomes) or destabilized (NR leaching through the lysosomal membrane into the cytosol), and the number of destabilized cells were expressed as a percentage of total cells scored. Eight clams were analyzed per site for field exposures (n=8). Sample sizes for each treatment varied from 8 to 16 for laboratory exposures due to sampling issues.

2.6 Micronucleus Assays

Micronucleus assays were used to identify DNA damage related to strand breaks. This is a commonly used DNA damage assay for a variety of aquatic organisms (Bolognesi and Hayashi 2011). Carnoy-fixed hemocytes were centrifuged at 1000g for 10 minutes and the Carnoy fixative was decanted. Hemocyte pellets were then resuspended in 50 µL 4’,6-diamidino-2-phenylindole (DAPI) nuclear-dye (concentration of 1 µg/mL in phosphate-buffered saline, PBS) by gently pipetting up and down. A 10 µL aliquot was then placed on a microscope slide and the coverslip was sealed with clear nail polish. Mounted slides were then incubated in the dark for 15 minutes and slides were examined

using differential interference contrast (DIC) microscopy on an epifluorescent microscope (Zeiss Axio Observer, 630x magnification with Zeiss filter set 25). Hemocytes were scored for six animals from each site for both laboratory and field exposed clams. Two slides were counted per sample, with 1000 cells per slide scored as either normal or MN. Micronucleated cells were defined as intact hemocytes with distinct cellular and nuclear membranes which possessed both a main nucleus and an additional distinct MN less than 1/3 the size of the main nucleus. Micronucleus values were expressed as number of MN cells per 1000 total cells. A total of six clams were analyzed for each treatment for both laboratory and field exposed clams (n=6).

2.7 Lipid Peroxidation Assays

Lipid peroxidation levels were quantified for hepatopancreas (field exposures) and gill (field and laboratory exposures) tissues by measuring malondialdehyde (MDA) levels using the thiobarbituric acid (TBA) test (Gutteridge and Halliwell 1990; Khan et al. 2018; Ringwood et al 1999). Tissues were weighed and homogenized in 50 mM potassium phosphate buffer (pH 7.0). For gill tissues, 5 volumes of buffer per tissue weight was used; for hepatopancreas tissue, 4 volumes of buffer per tissue weight was used. Homogenized tissues were then centrifuged for 5 minutes at 13,000 g and 4° C. Standards of known MDA concentrations (800, 400, 200, 100, 50, and 25 µM) were prepared from a 10 mM MDA stock solution. A 50 µL subsample of each standard or sample supernatant was then combined with 700µL of a 0.375% thiobarbituric acid solution dissolved in 15% trichloroacetic acid, and 7 µL of 2% butylated hydroxytoluene dissolved in absolute alcohol. Samples and standards were heated in a 100° C water bath for 15 minutes, centrifuged at 13,000g for 5 minutes at room temperature, and 200 µL

aliquots of supernatant were transferred in triplicate to a 96-well microplate. Absorbance values were measured at 532 nm (Thermo Fisher Scientific Multiskan GO microplate reader). A standard curve was generated using absorbance values for known MDA standard concentrations and used to calculate MDA concentrations for each sample. Final MDA concentrations were expressed as nmol/g wet tissue weight. Gill and hepatopancreas tissues were analyzed for 10 field exposed clams from each site (n=10 for each tissue). For laboratory exposed clams, gills from two clams were pooled due to small size, and four pooled samples were analyzed for each site (n=4).

2.8 Mercury Analysis of Tissues and Sediments

Clam tissues and sediments were dried via lyophilization for 24 hours. Dry weights were recorded and Hg concentrations were determined using direct analysis by thermal decomposition on a Milestone Inc DMA-80 direct Hg analyzer, located in the Center for the Environment at Catawba College, Salisbury, NC. Four individual clams (n=4) and three subsamples of sediment (n=3) were analyzed from each site. Mercury concentration values were expressed as ng Hg per g dry weight (ppb).

2.9 Tissue, Sediment, and Water Metal Analyses by Atomic Absorption Spectroscopy

The soft tissues of 10 frozen field-exposed clams from each site were lyophilized, weighed, crushed into small pieces with a glass rod, mixed with 1:1 trace metal grade nitric acid, and heated to digest the tissues for metal analyses. Samples were diluted with Milli Q ultrapure water so that the final acid concentration was between 1 and 2%. Subsamples from two clam digestions were pooled for analyses (n=5). Similarly, sediment samples were lyophilized, mixed, and a subsample (approximately 0.5 g) was digested in ultrapure nitric acid for analysis (n=1). Graphite furnace Atomic Absorption

Spectroscopy (Perkin Elmer AAS 200) was used to analyze for Cr and As in tissue, Cr in sediment samples, as well as blanks that were processed without tissue or sediments through the processing steps. Standard Oyster Tissue (NIST 1566b) samples were also processed as described above and analyzed with each sample batch to insure quality control analyses of analyte recoveries and different analysis days.

Water samples from each site were acidified to 1.4% final acid concentration by mixing 200 µL of 35% trace metal grade nitric acid with 4.8 mL of water (1 µm filtered) from each respective site, and analyzed by AAS.

2.10 Statistical Analyses

For biomarkers and metal concentration analyses, means and standard deviations were calculated, and treatment effects (laboratory treatments or sites) were analyzed using ANOVA. Checks for normality and equal variance were conducted, and data were transformed if necessary. Post-hoc analyzes (Tukey) were used for pair-wise comparisons. If data could not be normalized by transformation, then non-parametric tests were used (Kruskal-Wallis and Steel-Dwass). Regression analyses were used to evaluate relationships between biomarker responses. All data were analyzed using JMP Pro 13 or Sigma Stat 2.0 software.

CHAPTER 3: RESULTS

3.1 Exposure pH

Metal bioavailability in aquatic systems is affected by pH, therefore pH measurements were made for each treatment before and after laboratory exposures as well as during *in situ* deployment (Table 1).

Table 1: Summary of pH measurements for each laboratory exposure conducted with sediments and water from each field site; and pH measurements taken at the field sites at the time of deployment of the clams (NA = Not Applicable; ND = Not Determined due to equipment malfunction).

Treatment	Lab Exposure #1		Lab Exposure #2		Field
	Start pH	End pH	Start pH	End pH	Deployment pH
Lab Control	6.55	6.62	6.64	6.65	NA
Upstream	6.68	6.70	6.75	6.72	ND
Latta	6.73	6.69	6.75	6.73	6.70
Midway	6.82	6.80	6.76	6.78	6.87
Cove Mouth	6.84	6.79	6.78	6.74	ND
Outflow	6.92	6.90	6.85	6.87	6.95

3.2 Lysosomal Destabilization Assays

Microscopic analysis of field-deployed clam hemocytes showed a significant difference in percentage of cells with destabilized lysosomes between study sites (ANOVA, $p<0.0001$) (Figure 2). Examples of hemocyte cells scored as stable or destabilized are shown in Figure 3. Post-hoc pairwise comparisons indicated that Outflow and Cove Mouth sites were significantly higher than Upstream, Latta, and Midway ($p<0.0001$) sites, and that Midway was significantly higher than Latta ($p=0.05$). Similarly, laboratory exposed clams were found to have significantly higher lysosomal

destabilization when exposed to water and sediment collected from the Outflow site when compared to any other site ($p<0.01$) (Figure 4). Both field and laboratory exposed clams showed the highest levels of lysosomal destabilization at sites inside the mixing zone, and a general trend of decreasing lysosomal destabilization as site distance from the coal ash effluents increased. Overall these results indicate that the coal ash effluents are likely creating adverse cellular and physiological impacts, especially near the coal ash pond, but also extending into nearby downstream areas.

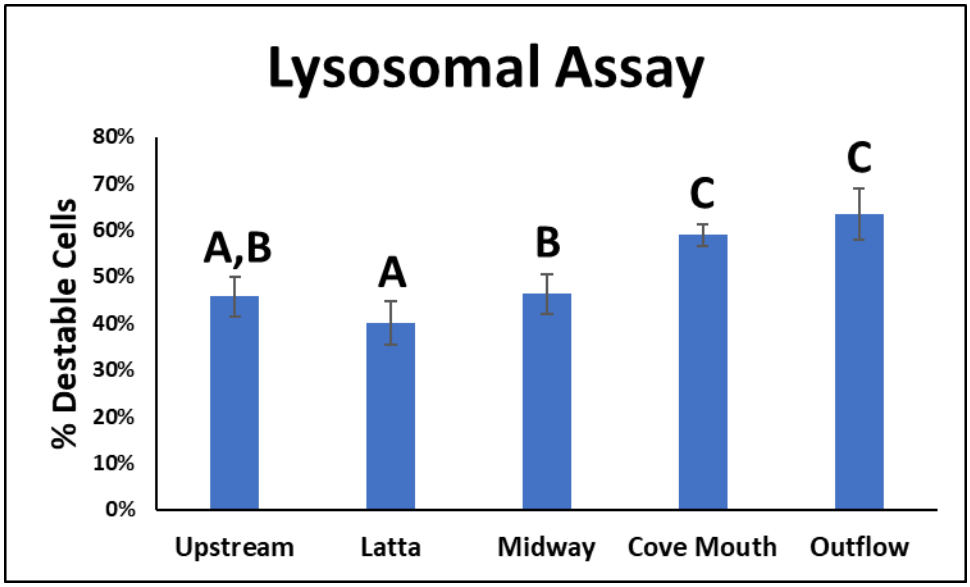

Figure 2: Mean percentage of hemocytes with destabilized lysosomes for field deployed clams from each site. Error bars represent standard deviations. Different letters indicate significant differences between sites.

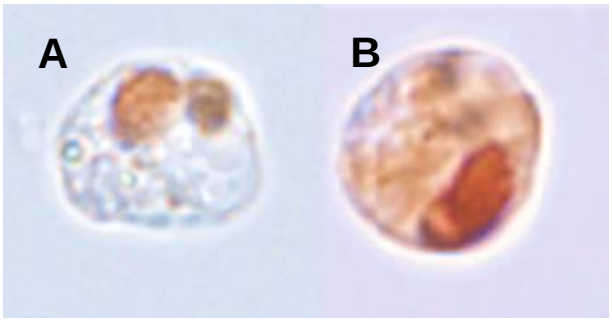

Figure 3: Examples of neutral red stained hemocytes containing (A) stabilized and (B) destabilized lysosomes using standard light microscopy (400x magnification).

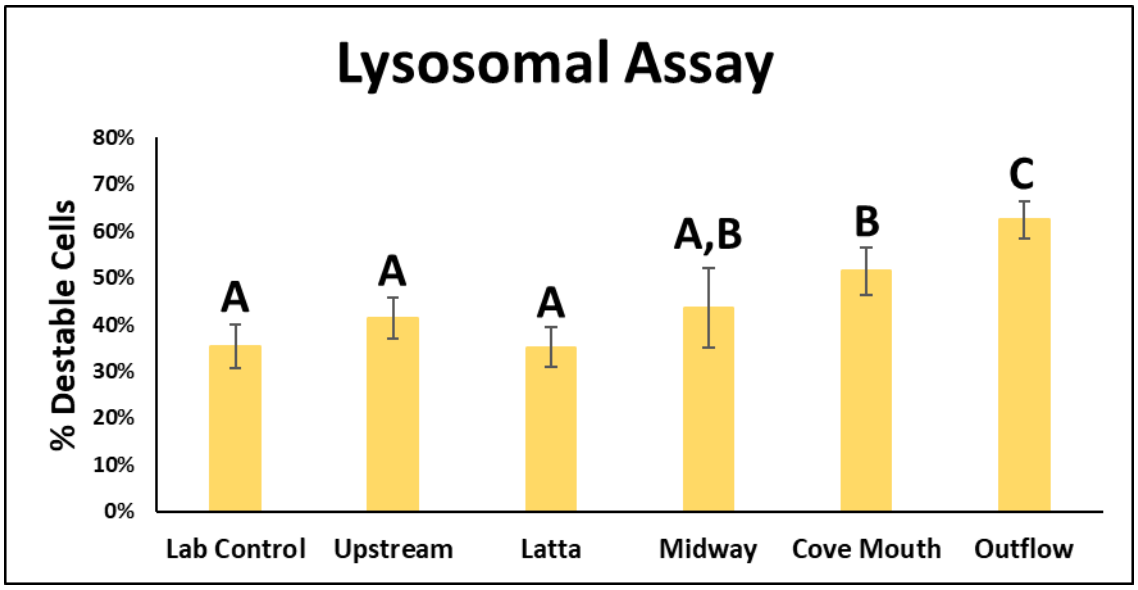

Figure 4: Mean percentage of hemocytes with destabilized lysosomes for laboratory exposed clams. Error bars represent standard deviations. Different letters indicate significant differences between sites.

3.3 Micronucleus Assays

In addition to increased lysosomal destabilization, hemocytes of field deployed clams also showed a significant increase in MN formation when deployed *in situ* within the coal ash effluent mixing zone (Figure 5). Examples of cells with no MN and MN are shown in Figure 6. It is likely that this observed increase in DNA damage is the result of

exposure to genotoxic metal contaminants commonly found in coal ash effluents. Laboratory exposed clams followed a similar trend, although overall incidence of micronuclei was somewhat lower when compared to field exposed clams from the same respective site (Figure 7). This lower occurrence of micronuclei formation during laboratory exposure is likely a result of the shorter exposure duration, as the MN assay is generally a better indicator of chronic exposure to genotoxins.

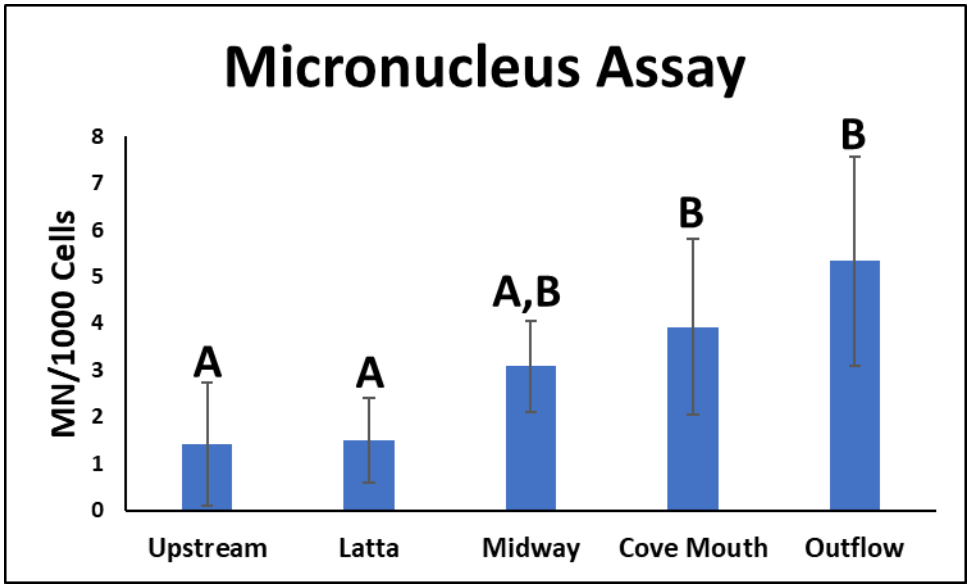

Figure 5: Mean number of micronucleated clam hemocytes per 1000 for field deployed clams for each site. Error bars represent standard deviations. Letters indicate significant differences between sites.

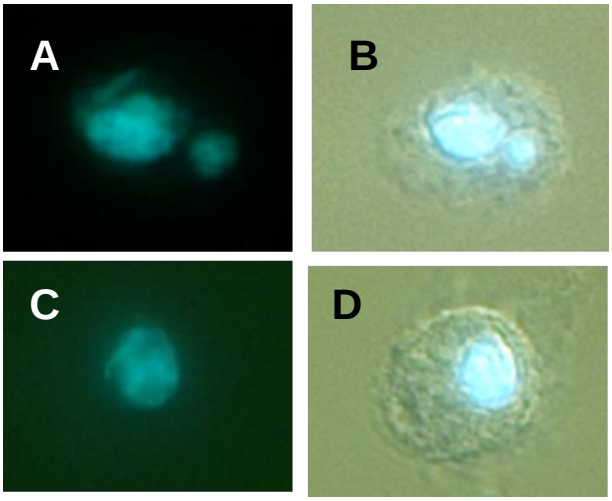

Figure 6: Examples of DAPI stained hemocytes containing: (A) MN cell with fluorescent light only, (B) MN cell with fluorescent and brightfield light, (C) normal cell with fluorescent light only, and (D) normal cell with fluorescent and brightfield light (DIC microscopy, 630x magnification, Zeiss filter set 25)

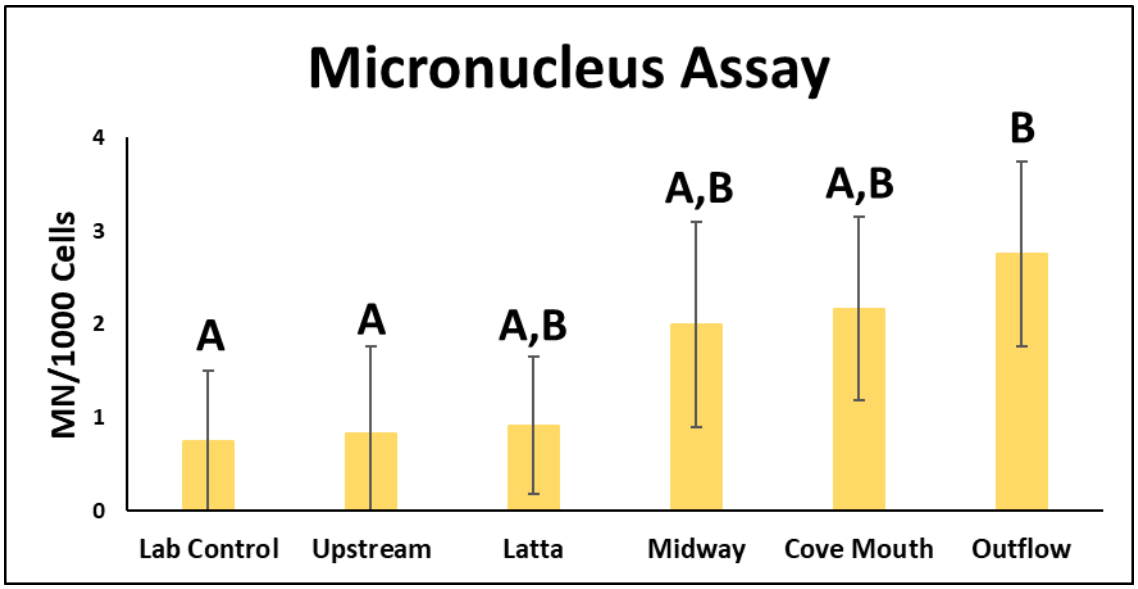

Figure 7: Mean number of micronucleated clam hemocytes per 1000 for laboratory exposed clams for each site. Error bars represent standard deviations. Letters indicate significant differences between sites.

Because the hemocytes for the lysosomal destabilization and MN assays were taken from the same cellular preparations for each clam, regression analysis was

conducted to evaluate the relationship between these two biomarker responses (Figure 8). Regression analysis indicated a significant correlation between these biomarkers in hemocytes, suggesting that the cellular damage impacts could be coupled, or at least indicate impacts on multiple damage pathways. Furthermore, the significant correlation between these biomarkers serves to further validate the efficacy of each biomarker for this type of environmental monitoring program.

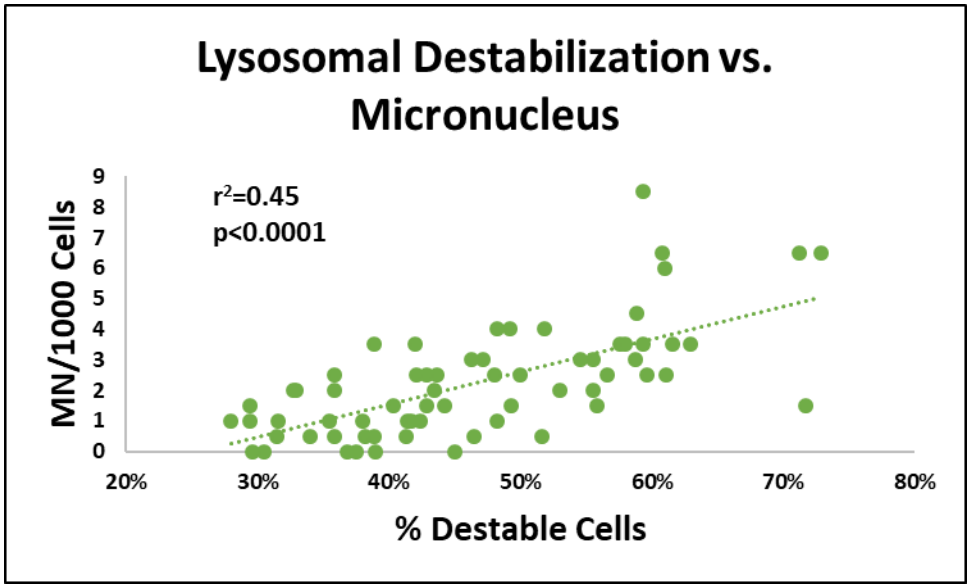

Figure 8: Regression analysis showing the significant correlation between lysosomal destabilization and MN assays for individual clams from both field and laboratory exposures (r^2=0.45, p<0.0001).

3.4 Lipid Peroxidation Assays

Although hepatopancreas tissues of clams deployed within the mixing zone had higher mean lipid peroxidation levels, these differences were not found to be significantly different (Kruskal-Wallis, p=0.21) (Figure 9). It should be noted, however, that hepatopancreas tissues of field exposed clams did exhibit a clear trend of decreasing

variability in lipid peroxidation as distance downstream of the coal ash effluent increased. The coefficient of variation for sites within the mixing zone were over twice as high as for the upstream reference site (Upstream CV=12.9%, Cove Mouth CV=26.1%, Outflow CV=30.4%). This increased variation within the input and mixing zone areas affected our overall statistical power for the data analysis, thereby diminishing our ability to detect significant differences when they exist, and could be indicative of an effect in itself.

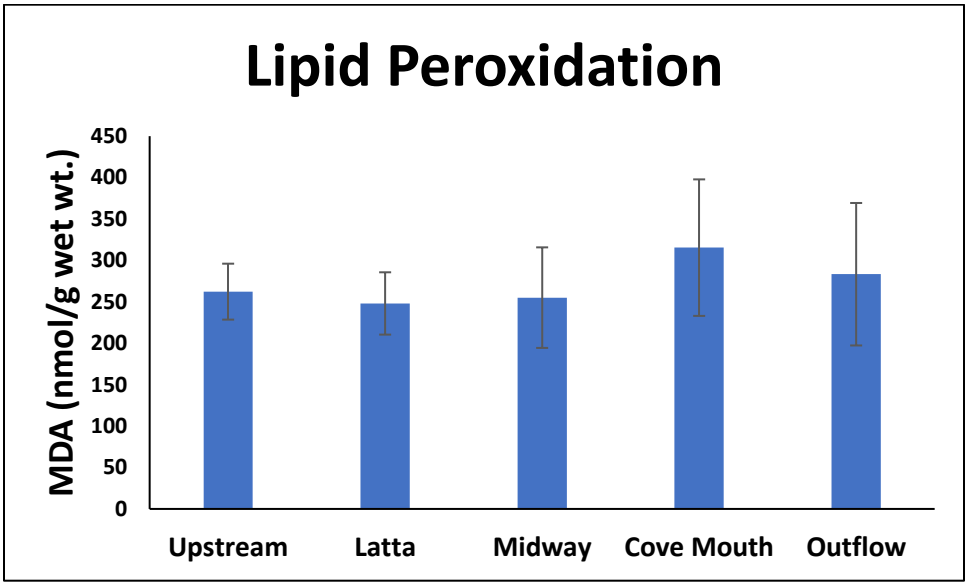

Figure 9: Mean MDA concentrations (nmol/g wet wt.) for hepatopancreas tissues of field deployed clams for each site. Error bars represent standard deviations.

Analysis of gill tissues of field deployed clams also indicated no significant differences in MDA concentrations between treatments (ANOVA, p=0.60) (Figure 10). Similarly, analysis of gill tissues of laboratory exposed clams also indicated no significant differences in MDA concentrations between treatments (ANOVA, p=0.92) (Figure: 11). Overall, MDA concentrations in gill tissues showed no trend and were more consistent between sites within each exposure type than hepatopancreas tissue,

suggesting that for this study system hepatopancreas tissue is likely the better indicator of oxidative stress. Indeed, in oysters and other bivalves, gills are often first affected during exposures to metal contaminated sediments, and hepatopancreas tissues are typically the most affected by longer term exposures (Khan and Ringwood 2016).

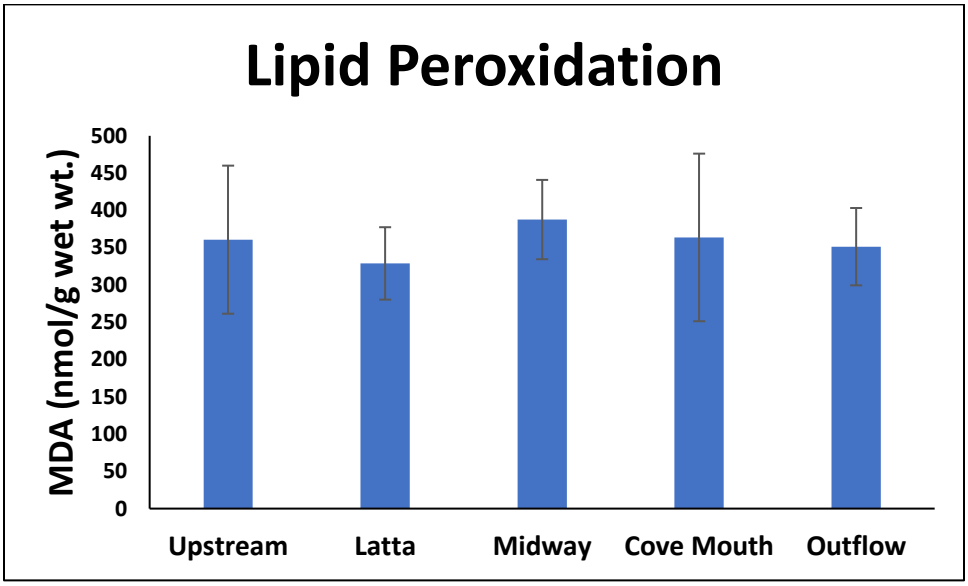

Figure 10: Mean MDA concentrations (nmol/g wet wt.) for gill tissues of field deployed clams for each site. Error bars represent standard deviations.

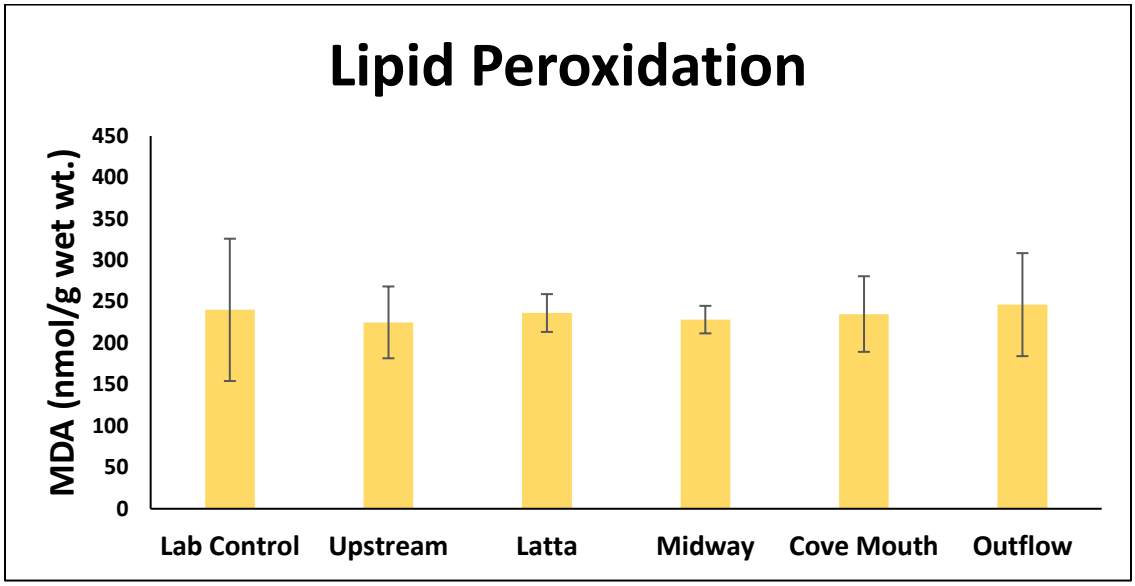

Figure 11: Mean MDA concentrations (nmol/g wet wt.) for gill tissues of laboratory exposed clams for each site. Error bars represent standard deviations.

3.5 Metal Analysis of Tissue, Sediment, and Water

Analysis of sediment Hg concentrations indicated statistically significant differences between study sites (ANOVA, $p<0.0001$) (Figure 12). Post-hoc pairwise comparisons indicated that the Upstream site was significantly lower than all other sites, while the Outflow and Midway sites were significantly higher than other sites; in fact both of these sites were over three times higher than the Upstream reference site. The elevated levels observed at the Outflow site are likely due to the extreme close proximity to the coal ash effluent, and the elevated Hg levels in the Midway site are likely a product of the water flow characteristics of Mountain Island Lake. The input of water from McDowell Creek combined with the shape of the cove in which the Midway site is located appears to create a depositional zone near this downstream site from the Outflow area.

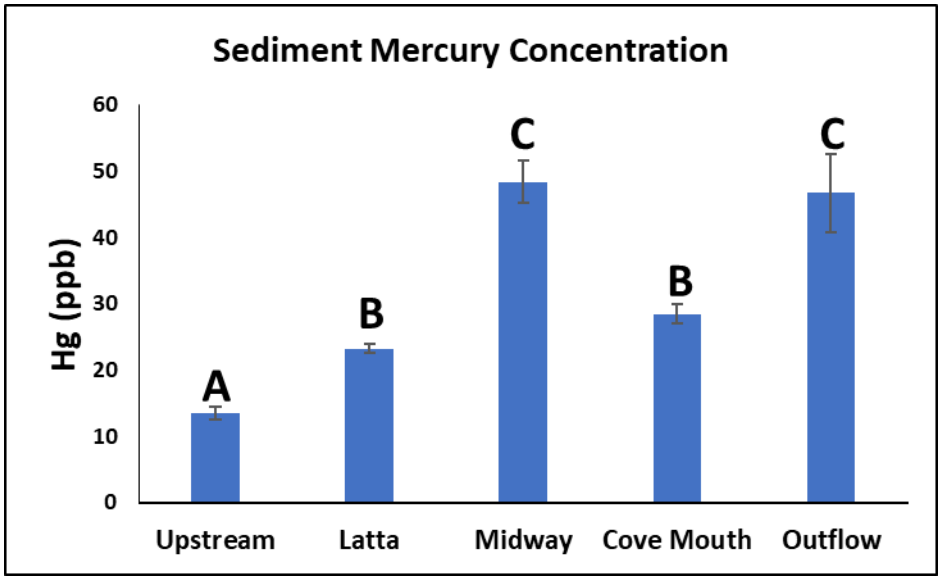

Figure 12: Mean mercury concentrations (ng/g) for dried sediments from each site. Error bars represent standard deviations; n=3 subsamples collected from each site. Letters indicate significant differences between sites.

Analysis of Hg levels in clam tissues indicated significant differences between study sites (ANOVA, $p=0.028$) (Figure 13), but post-hoc pairwise comparisons only showed significant differences between the Upstream site and the Outflow site ($p=0.022$). No significant differences were detected between clam tissues from any other pairs of sites ($p > 0.1$), and Hg concentrations in clam tissues actually showed the inverse trend of sediment Hg concentrations. Although some of this trend could possibly be attributed to differences in clam feeding behavior between sites, it should be noted that tissue Hg concentrations for the three sites closest to the effluent are actually below the levels observed in Lab Control clams. It is possible that this inverse trend in tissue Hg compared to sediment Hg is a result of Se antagonism, another common metal found in coal ash. The antagonistic relationship between Hg and Se has been well documented, and similar

results have been observed in other studies of this nature (Bjerregaard et al. 1999; Hamilton 2004; Luoma and Rainbow 2008; Peltier et al. 2009).

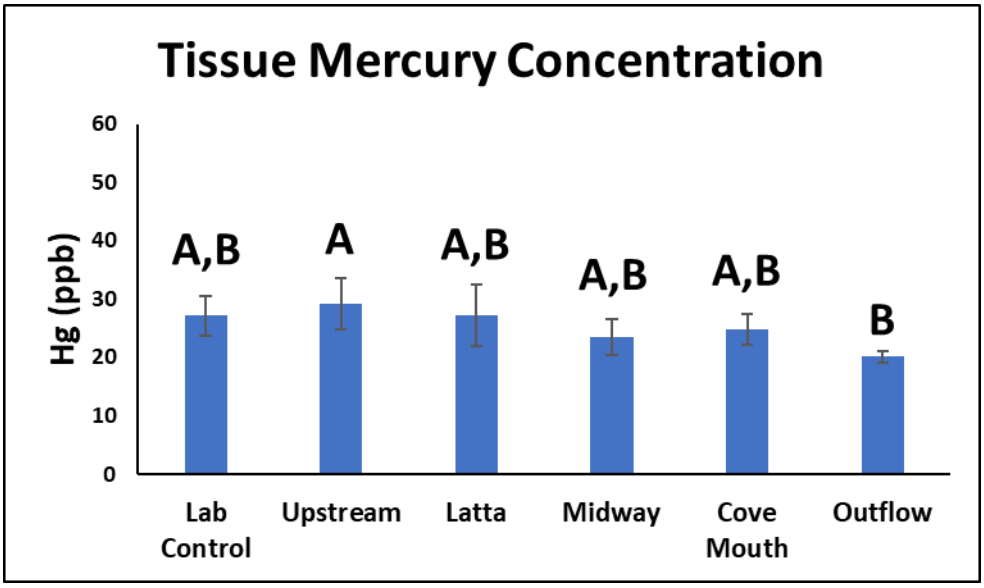

Figure 13: Mean mercury concentrations (ng/g) of dried tissues of clams deployed at each site. Error bars represent standard deviations. Letters indicate significant differences between sites.

Water samples from all sites had Cr levels below detection limits (<2 ug/L) but sediment Cr concentrations for each site decreased in the following pattern Upstream<Midway<Cove Mouth<Latta<Outflow and were over twice as high at the Outflow site as any other site (Figure 14). The elevated concentrations observed at the Outflow site are likely due to Cr loading of the sediments due to the chronic input of coal ash effluents. Unlike Hg, no elevations in Cr were observed for the Midway sites.

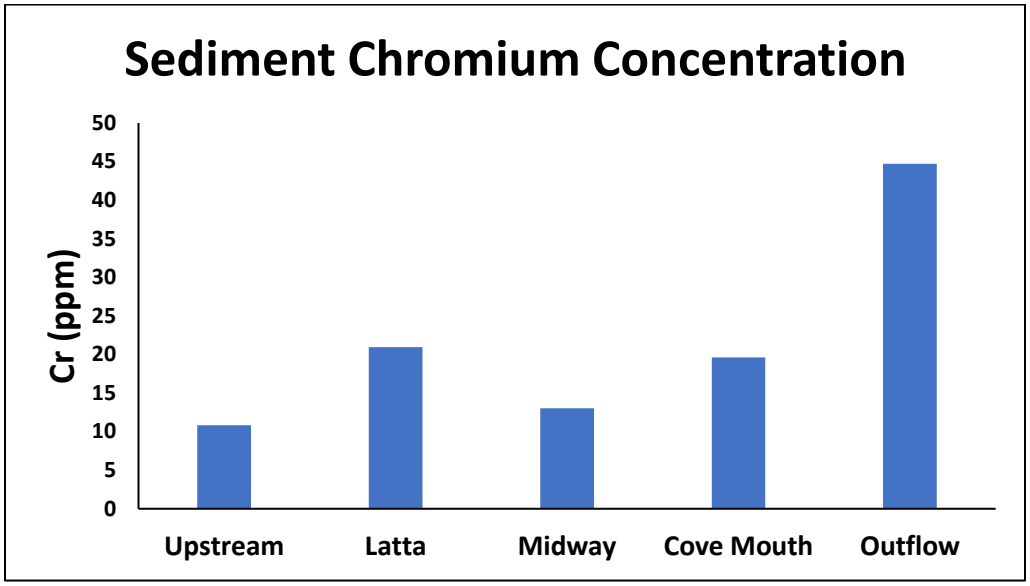

Figure 14: Chromium concentrations (ug/g) of dried sediments collected from each study site. Multiple sediment samples were mixed and a single composite sample was analyzed for each site.

Analysis of Cr concentrations in clam tissues indicated significant differences between sites (ANOVA, $p<0.0001$) (Figure 15), but tissue concentrations of Cr also followed a different trend than sediment. Tissue concentrations of Cr were lowest in Lab Control clams and clams deployed furthest downstream at the Latta site, while clams deployed at the Midway and Cove Mouth sites had the highest concentrations of Cr in their tissues. Tissues from clams deployed at the Outflow site had the third highest mean concentration of Cr, despite the high levels found in sediment from that site.

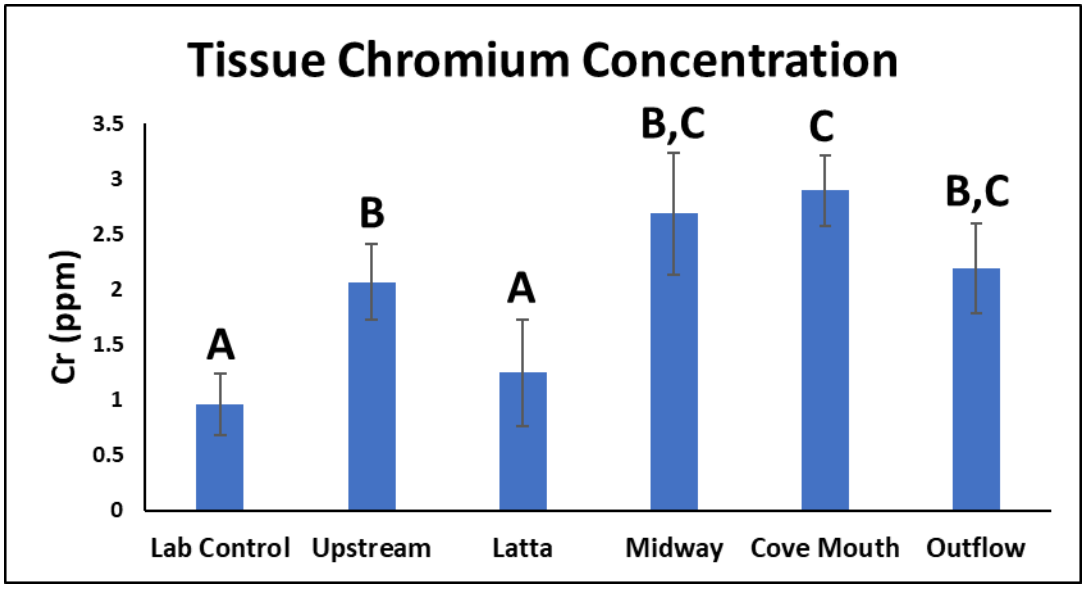

Figure 15: Mean chromium concentrations (ug/g) of dried tissues of clams deployed at each site. Error bars represent standard deviations. Letters indicate significant differences between sites.

Analysis of As concentrations in clam tissues indicated no significant differences between sites (ANOVA, $p=0.06$) (Figure 16), although the p-value was just above the $p<0.05$ alpha level suggesting a potential difference between sites. Furthermore, mean As tissue concentrations show a trend of decreasing as sites become further removed from the coal ash effluents, with the exception of the Outflow site which was similar to the Upstream site.

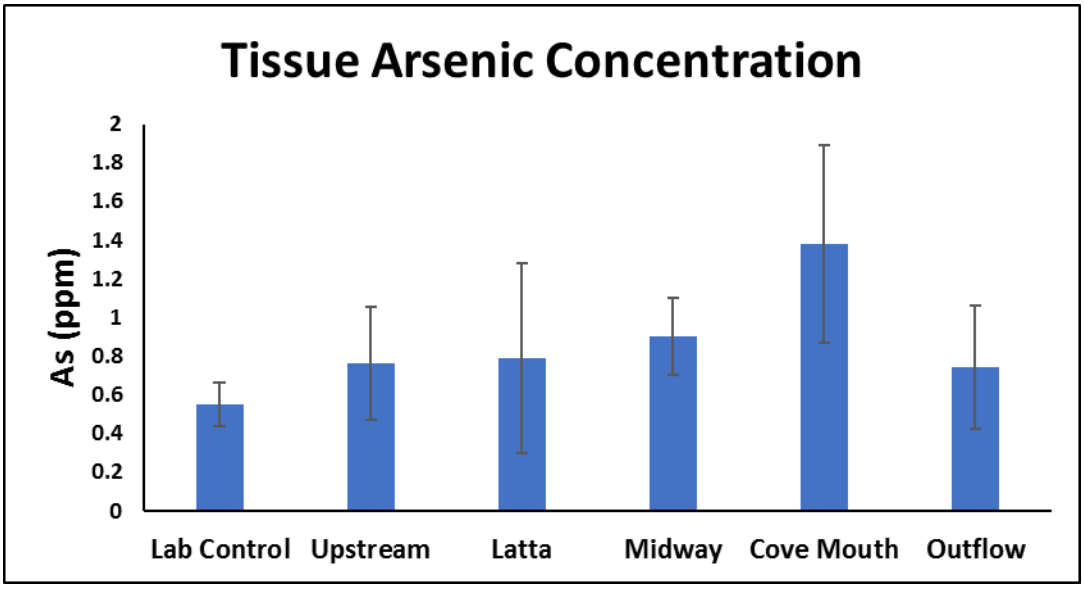

Figure 16: Mean arsenic concentrations (ug/g) of dried tissues of clams deployed at each site. Error bars represent standard deviations.

CHAPTER 4: DISCUSSION

The overall purpose of these studies was to investigate the potential impacts of coal ash storage and disposal practices on nearby freshwater ecosystems. The major focus of this project was to evaluate the potential toxicity of coal ash contaminants in Mtn. Island Lake in a freshwater bivalve, *Corbicula fluminea*. This was accomplished through a combination of field and laboratory exposures, and the use of well-documented stress biomarkers and metal bioaccumulation assays. While marine bivalves have been used extensively as valuable bioindicator organisms throughout the world, these approaches have only recently been explored in freshwater bivalves. Therefore, another important purpose of this work was to further validate a freshwater clam as a suitable bioindicator of ecosystem health, and demonstrate the value of these approaches. When used in conjunction with more traditional environmental monitoring programs, these bioindicator approaches facilitate a better understanding of the potential sublethal effects of ecosystem contamination.

Most existing water quality monitoring programs focus primarily on water column concentrations, collecting samples from locations of interest at specified intervals, often monthly, therefore representing only a single instant in time. Sediment samples are often collected and analyzed also, albeit less frequently, which can give a better estimate of long-term contaminant loading. Sediment quality guidelines (SQG) can be variable based on habitat type and are not enforceable criteria, partially because sediment composition can differ greatly based on the geologic processes which created it. Other approaches such as fish tissue sampling and benthic macroinvertebrate characterization are sometimes used as well, but can suffer from drawbacks of their own.

While it is not our intention to denigrate the value of these existing methods, augmenting them with bivalve bioindicator approaches can potentially give a more comprehensive analysis of organismal health and spatial extent, and ultimately ecosystem health.

In these studies, for instance, metal concentrations of water samples were consistently below detection limits. Sediment analysis indicated that Cr concentrations at the Outflow site were 44.7 ppm, which is above the threshold effect level (TEL) SQG of 37.3 ppm, but below the probable effect level (PEL) SQG of 90 ppm (Burton 2002). This suggests that sediment concentrations of Cr from this site could be high enough to cause moderate toxicity by itself. Although the two highest sediment Hg concentrations observed (Midway and Outflow; 48.4 ppb and 46.7 ppb, respectively) were below the TEL SQG of 170 ppb, it should be noted that each of these sites had significantly higher sediment Hg concentrations than all other sites, and these concentrations were over three times higher than the Upstream reference site.

The increased sediment concentrations of these metals near the coal ash ponds suggest probable chronic loading of the sediments due to the coal ash effluents. More importantly, these results show that multiple toxic metals are present in coal ash and are likely having a synergistic effect on toxicity, complicating meaningful interpretation of SQGs. *In situ* deployment of *C. fluminea* allowed us to overcome some of the experimental limitations of using SQGs alone by transplanting animals into the ecosystem where they could integrate conditions over a one-month duration, and subsequently be assessed for bioaccumulation and physiological stress, thereby telling us more about the actual consequences to biota. When bioindicator and sediment chemistry

monitoring approaches are integrated, SQGs can be more useful by determining which metals are likely the principal drivers of toxicity.

Tissue metal concentrations of deployed clams indicated that higher sediment concentrations do not necessarily dictate increased bioaccumulation, at least not for the metals analyzed here over this deployment duration. Some differences in sediment and tissue metal trends could be a result of differences in bioavailability at each site due to factors such as primary productivity and metal speciation. Although pH measurements were not drastically different between treatments in this study, it was observed that the Outflow site had slightly higher pH for all exposures. This change in pH is an important consideration in regards to bioavailability because dissolved H^+ concentrations can have profound effects on metal speciation, particularly in freshwater systems (Luoma and Rainbow 2008). For most metals, higher pH will favor complexation with organic and inorganic ligands in the sediment, ultimately increasing sediment concentrations and lowering bioavailability (Burton 2010). This possibly explains some of the disparity observed between sediment and tissue metal concentrations in this study.

Tissue metal concentrations are also affected by route of uptake and clam ventilatory behavior. Historically, the primary route of metal contaminant uptake for aquatic animals was believed to be dissolved metal contact with the gills; however a number of studies with bivalves have shown that dietary uptake is a significant route for metal uptake and bioaccumulation (Luoma et al. 1992; Reinfelder 1997; Wang et al. 1996). Tran et al. (2002) found that cadmium bioaccumulation in *C. fluminea* was strongly correlated with ventilatory flow rate, and that ventilatory flow rate can be affected by temperature and phytoplankton density as well as pollutants. Therefore, it is

possible that clams deployed at the Outflow site simply filtered less water during deployment due to toxicant narcosis or toxicity effects on filtration rates. The high levels of lysosomal damage and DNA damage indicate a variety of types of cellular damage and toxicity. Furthermore, clams from the Outflow site were observed to have abnormally dark gills when dissected. While reduced filtration rates could serve to limit their exposure to toxicants, it can also be detrimental because it limits their food intake, and therefore their energetic resources needed to maintain normal biological functions. Clams from the Latta site were the healthiest group based on biomarker responses measured in this study, a condition that likely makes them better suited to maintain metal homeostasis through clearance and detoxification mechanisms (Mason and Jenkins 1995).

In addition to the mechanisms discussed above, the relationship between Se and Hg must also be considered when assessing Hg bioaccumulation and toxicity. It has been well documented that Se exhibits protective effects against bioaccumulation and toxicity of Hg (Bjerregaard et al. 1999; Dang and Wang 2011; de Freitas et al. 2009; Hamilton 2004; Luoma and Rainbow 2008). Although the mechanisms are still poorly understood, proposed mechanisms include Hg-induced oxidative damage prevention, competition for binding sites, and the formation of Hg-Se complexes (Dang and Wang 2011). It has also been suggested that formation of Hg-Se complexes can increase the excretion rates of Hg in some animals (de Freitas et al. 2009; Yang et al. 2008). Neufeld (2010) observed that *C. fluminea* has a much faster Hg turnover rate than most animals, and that Hg accumulation in *C. fluminea* shows seasonal variation. These processes may explain the decrease in Hg concentrations observed in sites near the coal ash effluents over the course of deployment in this and other studies (Bjerregaard et al. 1999; Peltier et al. 2009).

Although tissue metal concentrations did not exceed FDA shellfish contaminant advisory levels for human consumption (As: 86 ppm (FDA 1993); Cr: 13 ppm (FDA 1993); Hg: 1 ppm (FDA 1996)) over the course of this study, it is important to consider that we did observe significant bioaccumulation of Cr and moderately higher As concentrations downstream of the ash ponds after a one-month deployment. It is possible that a longer deployment duration or sampling of resident clams in this study system could result in contaminant levels above the advisory level. When considering tissue bioaccumulation in this context it is also critical to remember that these advisory levels are based on what is considered safe for human consumption, and that many invertebrates are sensitive to much lower levels of contamination than humans.

In addition to bioaccumulation potential, it is important to assess the cellular and physiological impacts of metals. Lysosomal destabilization assays have been used in a variety of bivalve mollusks as a valuable indicator of pollutant exposure and adverse effects of organic contaminants as well as metals (Edge et al. 2012; Lowe et al. 1995; Moore 1982; Moore et al. 2006; Regoli and Giuliani 2014; Ringwood et al. 2002). This assay has proven to be particularly useful in regards to toxic metal exposures due to the role that lysosomes play in heavy metal sequestration and detoxification (Viarengo et al. 2000). Viarengo et. al. (2000) found that low dose mixtures of heavy metals have a more pronounced effect on lysosomal destabilization than exposure to a single metal alone. This synergistic effect that multiple metals have on lysosomal stability make this assay particularly attractive to coal ash studies due to the multiple metals and other compounds commonly associated with coal ash.

Other valuable biomarker assays that have been well established in marine bivalves are also valuable for freshwater systems, such as the MN assay for DNA damage. Micronuclei are typically formed when DNA suffers a double-strand break or some other damage that results in a piece of DNA without a functional chromatid (Bolognesi et al. 2004). During the next subsequent cell division, this chromatid deficient piece of DNA cannot properly segregate into the daughter cells and thus becomes a smaller, distinct MN inside the same cellular membrane. Because this process requires both DNA damage and subsequent division of the cell, the MN assay is regarded as a more effective indicator of chronic exposures to genotoxins than acute exposures (Bolognesi and Hayashi 2011). This likely explains the relatively lower incidence of micronuclei in the shorter laboratory exposures. Regardless, performing lysosomal destabilization and MN assays on sub-samples of the same cellular preparation allowed us to demonstrate a highly significant correlation between the lysosomal and MN biomarkers, and these results serve to further validate the efficacy of each assay for environmental monitoring.

While no significant differences were observed in lipid peroxidation, the observed trends can still be informative. Hepatopancreas tissues showed higher mean MDA concentrations inside the mixing zone, although the differences did not reach significance. Gill tissues, however, showed much more consistent mean MDA concentrations between treatments. This is important because it suggests that dietary exposure is likely a more significant route of exposure than dissolved metal contact with gills in regards to coal ash associated toxicity in *C. fluminea*. It is also interesting to consider the observed variation in this biomarker in hepatopancreas tissue, which could

reflect differential sensitivities between individuals. It is possible that the increased variation in oxidative stress near the coal ash effluent is indicative that the more sensitive individuals were showing negative effects of exposure and that other individuals were successfully compensating for metal toxicity and minimizing oxidative stress. Species and organisms that successfully upregulate antioxidant responses like glutathione and catalase, and stress proteins such as metallothioneins and heat shock proteins can at least temporarily avoid cellular damage from ROS (Kelly et al. 1998; Khan and Ringwood 2016; Regoli and Giuliani 2014). If this is the case, then perhaps with a longer deployment duration, the differences in lipid peroxidation would be more pronounced and reach significance. For future studies, antioxidant responses should be considered for investigation.

Hemocytes were valuable cells for the bivalve toxicity assays due to their ease of extraction and their variety of important physiological roles (Bolognesi and Fenech 2012). In bivalves, hemocytes are the circulating cells in an open circulatory system and play important roles in host defense, transport and detoxification of xenobiotics (Smith et al. 2016), and therefore have been shown to be sensitive to pollutant exposure. In addition to demonstrating the efficacy of these well-established assays with *Corbicula* hemocytes, these studies also demonstrate the potential use of these non-destructive techniques for characterizing the health of endangered freshwater bivalves.

The results of these studies provide evidence that regions of Mountain Island Lake, NC have been negatively impacted by the inputs of CCRs. Furthermore, this study elucidates some of the sublethal biological effects that conventional CCR storage and disposal techniques create for freshwater ecosystems in general, and provides a

framework upon which future studies can build. In addition to the biomarker assays used in this study, a variety of other biomarker assays have been proven to be effective indicators of stress in bivalves, and the particular biomarkers used for any given study can be catered to the pollutants and ecosystem of interest. Traditional environmental monitoring techniques based on periodic water sampling can be augmented with bivalve caging techniques, thereby integrating conditions over the length of deployment, and providing links from exposure to observed consequences on biological functions. While invasive Asian clams may not be regarded as a local resource worth protecting, these studies demonstrate that they are potentially a valuable species for assessing potential effects that can be calibrated for species and resources of concern. The integration of bioindicator studies into existing freshwater monitoring programs has the potential to facilitate a better understanding of the potential chronic effects of coal ash and other contaminated regions.

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