Radio

Physiological consequences of bioaccumulated heavy metals in the freshwater mussel *Actinonaias ligamentia* (common mucket) along a pollution gradient

Nicholas M. Radio

Introduction:

Researchers have historically analyzed heavy metal concentrations in freshwater mussels to prevent consumption of a contaminated food source and for biomonitoring purposes (Manly & George 1977, Boyden 1977, Granley Jr. et al. 1984, Hinch and Stephenson 1987, Metcalfe-Smith et al. 1996). To date, the majority of such studies have been performed on marine mussels (Hugget et al. 1973, Granley Jr. et al. 1984). As Metcalfe-Smith et al. point out (1996) most freshwater mussel studies have been restricted to analyzing mussels upstream and downstream from a potential metal discharging industry.

Early bivalve studies analyzed marine oyster fitness affected by a variety of waterborne pollutants (Scott and Middaugh 1978). These preliminary studies depicted mollusk growth rates negatively affected by aquatic pollutants. These observations catalyzed intensive research into the use of oysters as biomonitors for aquatic pollutants. Since that time, few studies have analyzed possible physiological effects of heavy metals on freshwater mussel fitness. Furthermore, research is needed to determine the minimal heavy metal concentrations in the aquatic environment that have detrimental physiological effects to freshwater mussels.

Background Information:

According to Bryan (1976), concentrations of heavy metals are very low in natural aquatic environments unless they are contaminated via industrial discharges.

Heavy metals can anthropogenically enter aquatic environments indirectly via atmospheric pollution from sources miles away, or directly via industrial waste discharge into the aquatic environment. Analysis of DEP permit records for industries along the French Creek and Allegheny River depict six industries with heavy metals permits along the surveyed section of the Allegheny River, and four industries with heavy metal permits along the surveyed section of the French Creek. At high metal concentrations, both essential and non-essential metals act as enzyme inhibitors, allowing for further failure of metal regulation effects of pollutants (Bryan 1976). Heavy metal toxicity varies for each metal. According to Metcalfe-Smith et al. (1996), cadmium is more toxic than copper, zinc, lead, or nickel. Cadmium is a nonessential metal that does not serve any beneficial biological functions within the organism. Additionally, Metcalfe-Smith (1996) et al. found that the freshwater mussel *Elliptio complanata* was more effective in regulating concentrations of copper within the water column than concentrations of zinc or cadmium.

Various studies have linked cadmium bioaccumulation to reduced physiological fitness in freshwater mussels. In Couillard et al's 1993 study, condition indices for *A. grandis* were negatively correlated to whole organism cadmium concentrations, indicative of periods of stress. Couillard et al. speculated that the metal influx exceeded the mussel's regulating capacity, resulting in the onset of detectable adverse effects. In Wang et al's 1999 study, reproductive output, gill dry weight, and larval survival rate decreased as cadmium concentrations within their tissue increased. To minimize such adverse effects, freshwater mussels have been shown to reduce both the amount of time their valves are open and their filtration rate in waters polluted with heavy metals

(Stewart 1999). Toxicological studies on humans have shown that cadmium exposure may cause pulmonary and renal diseases, testicular and lung cancers (Heinrich 1988).

Freshwater mussels are becoming an increasingly popular biomonitoring species because they are a very hardy, long-lived, immobile organism that effectively retains heavy metals found in their aquatic habitat and are generally considered to be poor metal regulators (Huggett et al. 1973, Boyden 1977, Couillard et al. 1993, Metcalfe-Smith et al. 1996). Additionally, since freshwater mussels can live up to 40 years old, scientists can monitor heavy metal bioaccumulation over extended periods of time (Metcalfe-Smith and Green 1992). The test organism in my experiment will be the common mucket (*Actinonaium ligamentia*) because it is the prevalent mussel in both the Allegheny and French Creek rivers. It is doubtful whether another species type could be collected in the abundance required for this study. The common mucket is a common inhabitant of a wide range of habitats, from stony riffles to soft-bottomed pools. (Strayer and Jirka 1997).

Heavy metals within mussels:

Researchers have linked two variables, age and body weight to heavy metal concentrations within mussels. Most reports agree that a positive relationship exists between age and tissue metal concentrations (Hinch and Stephenson 1987, Metcalfe-Smith and Green 1992). Discrepancies exist within the relationship between body weight and bioaccumulated heavy metal concentrations. Williamson's 1980 study suggested that concentrations of cadmium within a gastropod increased with age, yet at any given age, larger animals had larger cadmium concentrations then smaller ones. Similarly Boyden (1977) determined that body size had a positive relationship with cadmium

bioaccumulation, presumably due to longer exposure times to the environmental metals. However, in an earlier study by Boyden (1974), suggests that heavy metal concentration is directly proportional to body weight. Boyden reasoned that larger mussels are better suited to remove cadmium from their body circulation. In Manly and George's 1977 study, no correlation between body weight and heavy metal concentration was found in areas of low metal concentration (0-0.4 $\mu g\ g^{-1}$). However, in areas of higher heavy metal contamination (>1.0 $\mu g\ g^{-1}$), cadmium in mussels increased with body weight. Malley et al. (1989) found that smaller freshwater mussels (*A. cygnea*) accumulated proportionately more cadmium than larger sized mussels. Based on their results Malley et al. reasoned that cadmium bioaccumulation is weight-dependent, such that small mussels will accumulate cadmium faster than larger ones. To fully utilize freshwater mussel's biomonitoring capabilities, relationships such as body weight to metal concentration should be better understood.

Pollution status effect on metal content and body size:

Evidence exists within the literature that the concentration of heavy metals within the water column may dictate the amount of heavy metals bioaccumulated within the mussel (Manly and George 1977). No correlation existed between mussel body weight and the amount of bioaccumulated cadmium when cadmium pollution within the water was relatively low (0-0.4 $\mu g\ g^{-1}$). However, in areas of higher cadmium concentration (>1.0 $\mu g\ g^{-1}$), cadmium bioaccumulation within the mussels increased proportionately to body weight. Similar metal concentration to dry body weight trends were also found for zinc, lead, copper, and mercury in some or all of the samples of more highly contaminated individuals. Manly and George (1977) suggested that in waters relatively

unpolluted with heavy metals such as cadmium, bioaccumulation does not occur because the mussel is able to regulate the limited cadmium ions. As cadmium pollution increases, the excretion of cadmium from the mussel is superseded by accumulation and finally bioaccumulation of cadmium within the mussel. Similarly in Metcalfe-Smith et al.'s 1996 study, significant body to heavy metal concentrations was significant at heavily cadmium-polluted sites and insignificant at cadmium clean sites. They suggested that at relatively uncontaminated cadmium sites, bivalves are better able to regulate heavy metals such that body accumulation does not occur over time. These metal defense mechanisms are overwhelmed at polluted sites, and body burdens increase with size and age.

Research analyzing the detoxifying protein metallothionein (MT) seems to support Manly & Owens (1977) and Metcalfe-Smith & Green's (1996) findings. Metallothionens (MT) are low molecular weight, heat stable, soluble, intracellular proteins that are inducible by heavy metal exposure typically, Cd, Cu, Hg, Zn (Wang et al. 1999). Roesijadi proposed in her 1992 experiment that the detoxifying capability of metallothionein is dependent upon the heavy metal concentration within the water. Metallothionein can successfully detoxify low concentrations of heavy metals by binding to the heavy metals permitting their removal from the body via excretion. At higher heavy metal concentrations, toxic consequences are thought to occur as the MT binding capacity is exceeded, resulting in binding to other cytosolic molecules (Wang et al 1999). In Couillard et al.'s 1993 study, Cd^{+2} activity was suggested as being the key metal to stimulate metallothionein levels within freshwater mussels. The heavy metals copper and zinc were significantly less effective in triggering metallothionein synthesis within the

mussel. In Wang et al.'s 1999 study, internal MT not only strongly correlated with internal cadmium, but also closely reflected the changes in free Cd^{+2} within the water column. As free Cd^{+2} within the water column increased above ~1 nM, the amount of cytosolic cadmium bound to cytosolic ligands increased significantly, possibly because the MT binding capacity had been exceeded. Wang et al. then analyzed fitness of those mussels with cytosolic cadmium bound to cytosolic ligands. The shape of the gill appeared necrotic and its dry weight decreased proportional to the bioabsorbed cadmium concentration. Additionally, larval stages of mussels exhibited reduced fitness with increasing cadmium concentrations by a marked imbalance in tissue Cd:MT ratios. Thus, Wang et al's 1999 experiment strongly supports the theory that freshwater mussels cannot regulate heavy metals within the water column at high concentrations as efficiently as regulating heavy metals at lower concentrations.

Influences that affect heavy metal bioaccumulation:

Detecting a definite trend may be difficult, since so many biological influences may affect heavy metal bioaccumulation in freshwater mussels. Perhaps the largest disadvantage in using mussels as bioindicators of water quality is their strong correlation with biological conditions. As a result, a number of biological and physiological variables must be considered while utilizing mussels as bioindicators. Several potential biological factors that may influence the rate at which mussels bioaccumulate contaminants were analyzed in Metcalfe-Smith et al's 1996 study. Their results indicated that mussel species was the most influential biological variable, followed by size, age, growth rate, and sex. The authors suggested that experiments using freshwater mussels as heavy metal biomonitors should standardize for these biological influences on metal

bioaccumulation to improve precision. In Graney Jr. et al.'s 1984 study, substrate composition was shown to influence both filtering rate and metal solubility. Marine mussels were shown to reduce their filtering rate when placed in sandy or muddy substrates in comparison to rocky substrates. Metal solubility was also reduced in sandy and/or muddy sediment by altering the metal species. Acidic water conditions ($pH \leq 5.0$) were shown to significantly reduce accumulation of cadmium in *C. fluminea*. Additional soluble cadmium is available for bioaccumulation to mussels as additional free hydrogen ions are added into the water. The authors hypothesized that mussels become stressed as water turns acidic. As a result, mussels reduce their filtering activity under acidic conditions, reducing the amount of soluble cadmium entering their bodies. Finally, an increase in temperature from 9° to 21°C resulted in a 2-3-fold increase in cadmium bioaccumulation. The authors speculated that mussels were forced to increase filtering rate to gain more food and oxygen, thus increasing their exposure to cadmium.

In Stewart's 1999 study, cadmium bioaccumulation in the freshwater mussel (P. *grandis*) was reduced when copper, zinc, lead, and/or nickel were present. In his experiment, proportionally less cadmium was bioaccumulated in mussels when mixed with other metals (Cu, Zn, Pb, and Ni) than when cadmium was present by itself. These results seem significant since there was a higher cadmium concentration within the metal mixture than within the cadmium alone. Stewart suggested that direct competition among the heavy metals for sites within mussel tissue might have reduced total cadmium uptake.

The rate at which cadmium is bioabsorbed may be influenced by the amount of calcium within the water column. According to Wang and Evans 1993 study, cadmium

uptake by the freshwater mussel *E. complanata* has a negative linear relationship with calcium levels. They suggested that calcium depresses cadmium uptake within the mussel because cadmium and calcium enter organisms through the same type of binding site within the cell membrane. Therefore calcium inhibits cadmium bioaccumulation by clogging membrane-binding sites.

Methods:

Mussels will be collected from two river systems, the Allegheny River near Warren, PA and the French Creek, near Meadville, PA. Department of Environmental Protection reports suggest that both river systems are burdened by heavy metal pollution from local industries. The heavy metals copper, cadmium, nickel, lead, and zinc will be analyzed in both the water column and sediment via AAS along potential sites on the French Creek and Allegheny River. These heavy metals have been selected based upon preliminary results (Table 1) and DEP industrial permits for heavy metals discharged within the rivers (Table 2).

Table 1: AAS results of heavy metals detected in whole-tissue common muckets on 9-17-2000 from the French Creek and Allegheny River.

	Pb	Cu	Zn
French Creek			
1	2.5	20.6	9.3
2	2.1	13.6	10.4
Allegheny River			
1	3.8	7.6	10.9
2	1.9	15.6	10.5

Table 2: Heavy Metal Concentrations (ug/L) detected along industrial section of the Allegheny River. Information obtained from 1998 DEP permit application data.

Cadmium	0.2
Copper	983
Lead	43.7
Nickel	1300
Zinc	30

Since mussels are residents of the water-sediment interface, they can be affected by heavy metals within both the sediment and water column (Malley et al. 1989). Several prospective sites along both river systems will accordingly be screened for heavy metal pollution within the water column and sediment. These preliminary screenings will ultimately determine the five sites along both river systems from which mussels will be collected. The sites chosen will differ in metal pollution levels, such that a gradient in heavy metal pollution levels will exist from which mussels will be collected. To analyze the calcium concentration and heavy metal concentrations in the water, 400 ml of water will be collected from each site. The collected water sample will be returned to the lab, and preserved with 1 ml of sub-boiling distilled nitric acid. Two hundred ml of the water sample will be evaporated via hot water bath. 0.5 ml of nitric acid will then be added to the sample to digest most of the organic matter. The resulting ash will be taken up in 0.25 ml of nitric acid, whereupon the total volume will be adjusted to 5 ml with deionized water. The solution will be analyzed on the atomic absorption spectrum for calcium, cadmium, copper, zinc, and lead (adapted from Malley et al. 1988). Calcium measurements are necessary based upon previous reports (Wicklund and Runn 1988, Wang and Evans 1993) that calcium inhibits copper, zinc, lead, and cadmium uptake in freshwater mussels. Sediment cores will be collected from each site to analyze sediment

for heavy metal content. Sediment samples will be placed in 500-ml centrifugation bottles half filled with lake water during transport to the laboratory where they will be kept frozen until analysis. The sediments will be digested in sub-boiling solution (~96°C) of 0.04 M NH_2OH_3 HCl in 25%(vol/vol) acetic acid. The sediment solution will then be analyzed on the atomic absorption spectrum for cadmium, copper, zinc, and lead.

Collectors dressed in chest-waders will collect ten mussels from each of the ten total sites. The rather large sample size is in accordance with Boyden's previous studies (1974, 1977) stating the best method to counter the many biological influences that effect mussel growth is to analyze as many mussels from the same vicinity as possible. The collected mussels will be rinsed clean of any sediment with river water, and then immediately frozen via ice without being permitted to clear their digestive tracts (as suggested by Metcalfe-Smith et al. 1996). Once returned to Allegheny College's toxicology lab, mussel shells will be cleaned to remove the attached sediment and algae. The mussels will then be aged by counting the macroscopically visible external growth rings on their shells from the umbo outwards. Several studies have shown that counting these annuli rings is an accurate method of determining age if care is taken to not include the incomplete, lighter pseudoannuli growth rings (McCuaig and Green 1982, Hinch and Stephenson 1987, Metcalfe-Smith and Green 1992, Veinott and Cornett 1996). In accordance with Manly and George's 1977 study, mussels less than 4 years old will not be included in this study since previous works have shown that young mussels do not accumulate metals at a steady, constant rate.

To analyze metals within the mussel's soft tissue, mussels will be shucked from their shells, whereupon both the tissue and shells will be weighed. The soft tissue will be

dried to constant weight at 80° C for 24 hours and weighed to the nearest 0.01 g after a 5-10 minute cooling period (adapted from Lawrence and Scott 1982). Twenty ml of 70% nitric acid will then be added to each gram of dried mussel tissue. The flasks will then be placed in a water bath, covered with a watchglass, and heated at 80-90°C until the solution turns a yellowish color. The watchglass will then be removed from the Erlenmeyer flasks so that the remaining solution may evaporate. Ten ml of 2% nitric acid will be added to each solution, and then stirred to resuspend the solution. The solution will then be filtered to remove particles that could potentially clog the AAS. The digested solution can then be analyzed on the atomic absorption spectrophotometer for copper, cadmium, nickel and lead (adapted from Malley et al. 1989).

To perform a condition index analysis, internal cavity volume will determined via water displacement. A 2000-ml cylinder filled to a reference level of 1000 ml will be used. The intact mussel shell will be placed into water, and the final water level will be recorded. The cavity capacity will be determined by subtracting the two sets of measurements for each mussel (adapted from Lawrence and Scott 1982). The mussel shells will be air dried at room temperature for 24-30 hours. The mussel shells will then be weighed to the nearest 0.01gram. The A. E. Hopkins Condition Index analysis will be calculated for each measured mussel by: Condition Index = (dry meat weight in g * 100)/(internal volume in cm^3) (Lawrence and Scott 1982). The A. E. Hopkins condition index has been recommended as the optimal condition index by various scientists (Lawrence and Scott 1982, Couillard et al. 1993)

Project Statement:

The purpose of this study is to analyze freshwater mussel's ability to detoxify heavy metals along a heavy metal pollution gradient. Furthermore, I wish to analyze the effect bioaccumulated heavy metals have on the mussel's fitness. Finally, I wish to analyze possible effects various water parameters have on metal uptake. I will test the following three hypotheses to analyze these relationships:

1) Mussel's ability to regulate heavy metal uptake will become less efficient as the concentration of heavy metals within the water column and or sediment increase.
2) Mussel's physiological fitness will decrease (as depicted via Hopkins condition index) with increasing heavy metal concentrations in the sediments and/or water column.
3) Calcium levels within the water will negatively affect with heavy metal concentrations within the mussel tissues.

As previously mentioned, discrepancies exist within the literature as to the relationship between body size or age and metal concentrations. Various experiments have suggested that body size is negatively related to metal accumulation, possibly due to a greater ability to excrete accumulated metals as the mussel increases in size (Boyden 1974, Malley et al. 1989). However, several other studies have shown that metal bioaccumulation within mussels is positively related to body size, possibly due to a longer exposure time to the polluted site (Boyden 1977, Williamson 1980). The confusion may be due to altering heavy metal pollution levels within the water table. As Manly and George (1977) and Metcalfe-Smith et al's (1996) experiments suggest, heavy metal bioaccumulation within the freshwater mussels may be related to pollution status. Mussels may be able to efficiently regulate metals at relatively uncontaminated sites, but

metal bioaccumulation occurs at contaminated sites as metal regulatory systems fail to eliminate the polluted heavy metals from bioaccumulating into the mussel's body. I will then attempt to correlate the bioaccumulated heavy metals to mussel fitness levels via A. E. Hopkins Condition Index. As previously stated, research has neglected to analyze the effect accumulated heavy metals have on freshwater mussel's fitness. Finally, I will propose acceptable heavy metal levels that do not inflict physiological damages to the freshwater mussel.

Materials and Budget:

Cadmium lamp (approximately $500-700)
Insulated container to store frozen mussels from site to lab (Allegheny College)
Concentrated Nitric Acid (Allegheny College)
Twenty 500 ml centrifugation bottles (Allegheny College)
0.04 M NH_2OH_3HCl (Allegheny College)
Copper, Nickel, Zinc, and Calcium Lamps (Allegheny College)
Atomic Absorption Spectrum (Allegheny College)
Water Bath (Allegheny College)
Heating oven (Allegheny College)

Timeline:

Late September: Analyze potential sites along French Creek and Allegheny River for Cd, Cu, Zn, Ni, Pb, Cr in water and sediment samples via AAS. Results of analysis will ultimately determine site selection. Select sites so that pollution gradient obtained for each river.
Early October: Collect mussels from selected sites along the French Creek and Allegheny River.
Late October-November: Digest mussel tissue, analyze mentioned heavy metal concentrations within whole-tissues of mussels from each site via AAS.
Late November-December: age mussels, and perform condition index
Remaining of 1st and hopefully early segment of 2nd semester: Begin data analysis and complete writing

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