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BIOAVALABILITY AND TOXICOLOGICAL ASSESSMENT OF LEAD-CONTAMINATED SEDIMENTS IN AN URBAN RIVERINE MARSH

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BIOAVALABILITY AND TOXICOLOGICAL ASSESSMENT OF LEAD-CONTAMINATED SEDIMENTS IN AN URBAN RIVERINE MARSH

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We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science.

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ABSTRACT

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The La Crosse, Wisconsin Gun Club maintained a trapshooting range at the site of the La Crosse River Marsh (LRM) for three decades (1929-1963) resulting in lead (Pb)-contaminated sediments. Because Pb causes physical deformities, behavioral changes, and reproductive dysfunctions in humans and wildlife, it is essential that the potential Pb bioavailability and risk of these contaminated sediments to wildlife and humans be determined. Several fish species were sampled from a variety of trophic levels at reference sites (0-200 mg/kg) and within areas that contained low (400-1000 mg/kg), medium (2,000 - 4,000 mg/kg) and high (4,000 – 8,000 mg/kg) Pb-contaminated surface sediments. Pb from contaminated sites was bioavailable for fish with no correlations between Pb levels among fish and Pb levels from contaminated surface sediments. Also, Pb levels among fish did not differ between species. Additional, laboratory toxicity assays were conducted with zebrafish larvae to determine whether Pb-contaminated surface sediments from the LRM pose toxicological risk to fish. Lab results indicate exposure to LRM surface sediments induced mortality, impaired development, and caused neurotoxicity; however, toxicity was minimal with no distinct dose-response curve. Further research would help determine if remediation is needed or the LRM needs to be listed as a contaminated waters.

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CHAPTER I

OVERVIEW OF LEAD TOXICITY AND HISTORY OF THE LA CROSSE RIVER MARSH

The La Crosse River Marsh

The La Crosse Wisconsin Gun Club maintained a trapshooting range at the site of the La Crosse River Marsh (LRM) for over three decades (1929-1963). During this period, ammunitions containing Pb accumulated in the marsh sediments. This resulted in a Pb-contaminated wetland with an unknown biotic impact. Pb from spent ammunition can degrade, becoming bioavailable to plants, animals and humans. This process can take as long as 100 to 300 years, depending on environmental conditions (Jorgensen and Willems, 1987). Given the known toxicity of Pb to both wildlife and humans, determining the bioavailability of Pb from sediments within the marsh can indicate whether remediation or long term monitoring is required to maintain a healthy ecosystem.

The LRM is an urban wetland within the floodplain of the La Crosse and Mississippi rivers, acting as drainage to the La Crosse River, distributing water during flooding, and providing water during periods of drought (Sha et al. 2011). Several animal species (e.g., invertebrates, fish, birds and mammals) are dependent upon the LRM's unique environment. The LRM also acts as a recreational park for people within the La Crosse community, providing walking, running and biking paths (Moyer 1989). The LRM also acts as an outdoor laboratory for students at University of Wisconsin – La

Crosse (UW-L) and other regional schools, where they are able to take classes that involve hands on experience with the marsh (Moyer 1989).

Recent work by Perroy et al. (2014) quantified the extent of Pb contamination, indicating which areas pose the greatest risk to the environment. Their work indicated that high levels of Pb remain in surface and core sediments, with surface sediments reaching as high as 42,854 mg/kg and core sediment samples reaching 26,709 mg/kg Pb dry weight (Perroy et al. 2014). Surface sediments from the aquatic portion of the 15 ha study site were highly contaminated, with 3.8 ha of the area being above the Environmental Protection Agency's (EPA) soil contamination threshold of 400 mg/kg Pb (US EPA 2001; Perroy et al. 2014). Further, 8.9 ha of surface sediments exceed the 130 mg/kg Pb probable effect concentration (PEC), above which adverse biological effects can be frequent within freshwater ecosystems (MacDonald et al. 2000; Perroy et al. 2014). Sediment containing Pb shot was typically buried 10-30 cm below surface sediments, with surface sediment Pb no longer containing Pb shot, but secondary Pb minerals likely bound to near-surface flocculent sediments (Perroy et al. 2014).

In an attempt to better understand the extent to which Pb has mobilized within the system, and whether the sediments pose a toxicological risk to animals present, a collaborative approach to survey the marsh was taken. Herein, I describe a series of field and laboratory experiments as a part of this project. A fish survey was done to determine whether Pb from contaminated sediments were bioavailable to fish species from the LRM, and laboratory sediment toxicity assays were used to determine LRM sediment toxicity.

Pb Toxicity and its Dangers

Contaminated sediments are concerning because heavy metals such as Pb pose significant environmental health risks (Firat et al. 2011). Heavy metals are defined as metallic chemical elements that have high specific gravity and are toxic or poisonous in low concentrations (Aktar et al. 2011). Pb (and its oxidative state Pb^{+2}) is a heavy metal of special concern because it is the most abundant of the heavy metals in the earth's crust, making it widely distributed and mobilized in the environment, resulting in toxicity to humans and wildlife (Tong et al. 2000). In developed countries, high concentrations of Pb are still present in reservoirs (e.g., dam impoundments) and in soils, even after considerable efforts have been made to control it (Tong et al. 2000). Some of the most common ways Pb enters the environment is through human actions such as mining, erosion of Pb-based paint from homes, and the discharging of Pb ammunition (e.g., firing ranges and hunting; Johnson 1998). Further, the prior burning of Pb-based fossil fuels have resulted in Pb entering water and soils via atmospheric deposition, thus becoming available to organisms through food webs (Jarup 2003). Once in the food web, most organisms do not possess efficient elimination mechanisms, making Pb a serious concern to environmental health, especially among aquatic systems (Osman et al. 2007; Rocha and Souza 2012).

Aquatic organisms are greatly affected by heavy metal pollutants, such as Pb, because unlike organic pollutants, heavy metal persistence does not allow them to be removed via biodegradation (Ashraf et al. 2012). Understanding how Pb becomes taken up into the water from sediments is important when determining whether it is bioavailable to organisms, such as fish. The process by which heavy metals become

available to the water column involves physical, chemical and biochemical weathering, and oxidative processes that alter the amount of Pb that becomes available to the environment (Velimirovic et al. 2011). This is dependent upon geochemical and geotechnical properties of the surrounding soil, climate, and site geology (Dermatas et al. 2004). Ultimately, the chemical form in which the metal is present in the sediments will affect its bioavailability to organisms (Velimirovic et al. 2011). Chemical form and availability can be altered by the pH of the sediment causing drastic effects on Pb mobility, becoming more mobile under acidic conditions (Dermatas et al. 2004; Adhikari el al. 2006). Therefore, it is important to consider the pH of the media (e.g., water or soil) when determining the movement of heavy metals through the environment (Ponce and Bloom 1991). Not only do the physiochemical factors of the environment influence the amount of available Pb to the environment, the type of sediment the contaminant is in also affects uptake into the water column (Luoma and Bryan 1982). One way Pb can become mobilized from its solid form is through the process of particle weathering (Heier et al. 2009). As Pb ages it becomes more mobile, resulting in it being taken up into the water column as a dissolved or soluble form (Velimirovic et al. 2011). Once Pb becomes available in the water column, it can be taken up by the aquatic organisms in the form of waterborne Pb (Schmitt et al. 2005).

Pb enters aquatic biota either through absorption or ingestion (Stauber et al. 1994). Once Pb is present in the lower trophic levels (e.g., phytoplankton) it is able to move up the food chain through predation (Soto-Jimenez et al. 2011). Lower trophic invertebrates can also acquire Pb through direct consumption of Pb-contaminated sediments (Hare et al. 1991). Though Pb does not tend to biomagnify across trophic

levels, it does tend to bioaccumulate when the ingestion rate exceeds the excretion rate (Rubio-Franchini and Rico-Martinez 2011). In vertebrates, Pb tends to become trapped in bone tissue as the result of being metabolically mistaken for calcium (Milton et al. 2003). Pb can then leach out of the bones and transfer to other parts of the body via the blood (e.g. kidney, liver and brain), resulting in toxicity from altered biochemical function and oxidative stress (Adonaylo and Oteiza 1999; Kumar and Sabhlok 2011).

General Pb Toxicity

Pb is toxic because it replaces essential metals and minerals involved in many biochemical functions. Certain heavy metals within earth's crust (i.e., zinc and iron) are important in redox activity; however, are inhibited when trace metals (i.e., Pb) become unnaturally abundant (Clemens 2006). Pb binds to thiol and phosphate groups of nucleic acids, proteins and cell membranes within the host organism, causing many adverse effects (Shimoni-Livny et al. 1998). This cellular binding process causes cellular damage, resulting in behavioral abnormalities, learning impairment, and impaired cognitive function in both humans and animals (Pokras and Kneeland 2009). Poisoning due to Pb can happen rapidly, resulting in acute and sublethal problems for a variety of different organisms (Pokras and Kneeland 2009).

Exposure of Pb under varying concentrations can result in both acute and sublethal toxicity, depending upon route of exposure, concentration, and the length of time the organism is exposed (Campana et al. 2003). Measures of acute toxicity include determining its concentration that results in 50% lethality (i.e., LC50; Scholz et al. 2008) and characterizing gross developmental effects. Acute toxic responses in fish to Pb include physical deformities such as changes in body color, descaling, fin erosion,

hemorrhaging, and death (Kumar and Sabhlok 2011). However, chronic exposure to sublethal concentrations of a contaminant can also cause subtle developmental malformations that impact the health and sustainability of the population. Sublethal toxicity can result in physiological changes that manifest in poor survival or reproductive success, which can be difficult to assess. For example, exposure to metals often results in oxidative stress by forming free radicals that alter cell activity, and lead to cardiovascular, renal, and neurodegenerative diseases (Campana et al. 2003; Ahamed and Siddiqui 2007). Heavy metals, such as Pb, can act as a catalyst in oxidative deterioration of cells, thus resulting in dysfunction (Adonaylo and Oteiza 1999). Oxidative stress is measured through antioxidant systems. Certain enzymes, such as superoxide dismutases, glutathione peroxidases, catalase and glutathione S-transferases, which are located within different cellular compartments, can be used as bioindicators of oxidative stress (Lopes et al. 2001). The effects of acute and sublethal toxicity from Pb can be seen in most animals, particularly aquatic species such as fish.

Given the dangers of Pb, and the potential for Pb to mobilize through aquatic systems, it was important to determine if this was taking place in the LRM. The goals of my work were to (1) survey a variety of fish species to see whether they contain significant concentrations of Pb and (2) evaluate the toxicity of surface sediments utilizing zebrafish as a model organism.

CHAPTER II

IS LEAD FROM LA CROSSE RIVER MARSH SEDIMENTS BIOAVAILABLE TO FISH?

Introduction

High levels of Pb found within LRM sediments are troubling; however, to determine if there are any potential risks to fish living in the marsh, Pb bioavailability must first be determined. Pb can enter fish from the environment through a variety of methods, the two most common being the gills and diet (Rubio-Franchini and Rico-Martinez 2011). Gills are the primary site of accumulation for waterborne Pb (Dai et al. 2009). Upon entering the gills from waterborne Pb (Hodson et al. 1982), Pb is manipulated as calcium (Ca^{+2}), resulting in Pb being taken up by the high-affinity Ca^{+2} uptake mechanism. This can also be referred to as a metal-gill interaction approach (Playle 1998), which results in the dissolved metals being taken up through biological membranes in the gills and being dispersed throughout the body via the circulatory system (Macdonald et al. 2002). The process of Pb entering fish tissue through the gills often causes lesions, resulting in gill damage (Vinodhini and Narayanan 2008). Once Pb from the environment enters through the gills it is distributed to the organs such as bones, scales, gills, kidneys and the liver via blood (Spry and Wiener 1991). The accumulation rate is dependent on the concentration of Pb in the environment, which dictates the absorption rate and severity of chronic effects on the organism (Spry and Wiener 1991; Rocha and Souza 2012). Fish size may also play a role in Pb accumulation, as larger size

fish tend to accumulate Pb more slowly from waterborne Pb to the blood than smaller sized fish, especially with regards to the opercular bone (Hodson et al. 1982).

Diet is another major route of Pb exposure for fish. Once in the environment, Pb becomes available to lower trophic organisms (e.g., zooplankton) via ingestion and absorption. Fish then consume zooplankton or higher trophic organisms (e.g., crustaceans and macroinvertebrates), resulting in Pb transfer through food web dynamics (Soto-Jimenez et al 2011). In fish, most Pb from dietary exposure accumulates in the digestive system and liver, which can cause severe tissue damage if Pb concentrations are high enough (Vinodhini and Narayanan 2008; Dai et al. 2009). After passing through the liver, Pb tends to accumulate in the same tissues following gill uptake. The effects of Pb from this uptake can be quantified by the presence of ALA-D (δ-aminolevulinic acid dehydratase) protein, a Pb toxicity biomarker indicating acute and sub-lethal effects (Hodson et al. 1982; Heier et al. 2009). In certain fish species, as little as 10 µg/L of waterborne Pb has shown to inhibit the activity of the enzyme ALA-D (Hodson et al. 1977), which can lead to oxidative stress (Heier et al. 2009). Therefore, ALA-D activity is often used as a biomarker to indicate the extent of Pb toxicity (Heier et al. 2009).

Fish are often used as indicators of aquatic ecosystem health because their entire life cycle takes place within the water column (Vinodhini and Narayanan 2008). Given the legacy contamination within the LRM, this study sought to conduct field evaluations measuring Pb levels within fish of different fish species and trophic level, thus determining if Pb was becoming bioavailable to fish. Fish were sampled and assessed from areas from varying Pb-contaminated sediments to determine potential bioaccumulation, and determine which areas of the marsh are greater or lesser concerns

with respect to Pb exposure. Fish likely moved throughout the different sites; however, this indicated if Pb in the sediments were bioavailable and posed a potential toxicological concern. The objective of this study was not simply to determine if Pb is bioavailable, but to determine if Pb levels within fish tissue warrant a concern to fish health and to humans if consumed. Human consumption advisories for Pb have been set forth by a number of global commissions. The European Commission (EC) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) have set consumption advisories for Pb within fish at 0.2 mg/kg and 0.25 mg/kg Pb (weight weight.), respectively (EC 2005; JECFA 2010; Ngelinkoto et al. 2014; Ahmed et al. 2015). This study should provide the necessary information to determine if this area of the LRM should be listed as an impaired water body or whether continued monitoring would be sufficient to protect this unique urban ecosystem.

Materials and Methods

Survey sites were separated into four areas based on concentration of Pb within the surface sediments, and accessibility for fish collection: reference (0-200 mg/kg), low (400-1,000 mg/kg), medium (2,000-4,000 mg/kg) and high (4,000-8,000 mg/kg) Pb contamination (Figure 1). All animal handling and euthanizing followed the University of Wisconsin – La Crosse's animal care protocols (7-12). Fish were collected under a State of Wisconsin Department of Natural Resources (WI DNR) Scientific Collection Permit (form 9400-379). At each site there were at least three sampling areas where fish were captured with mini-fyke nets or a backpack electroshocker. Mini-fyke nets were placed at random locations within each survey site and set at a depth that covered most of the net (Figure 1). Depth (m), location (global positioning system coordinates), and time

fished for each net was recorded. An electrofishing backpack-shocker (direct current) was used in order to sample minnow species from the different survey sites.

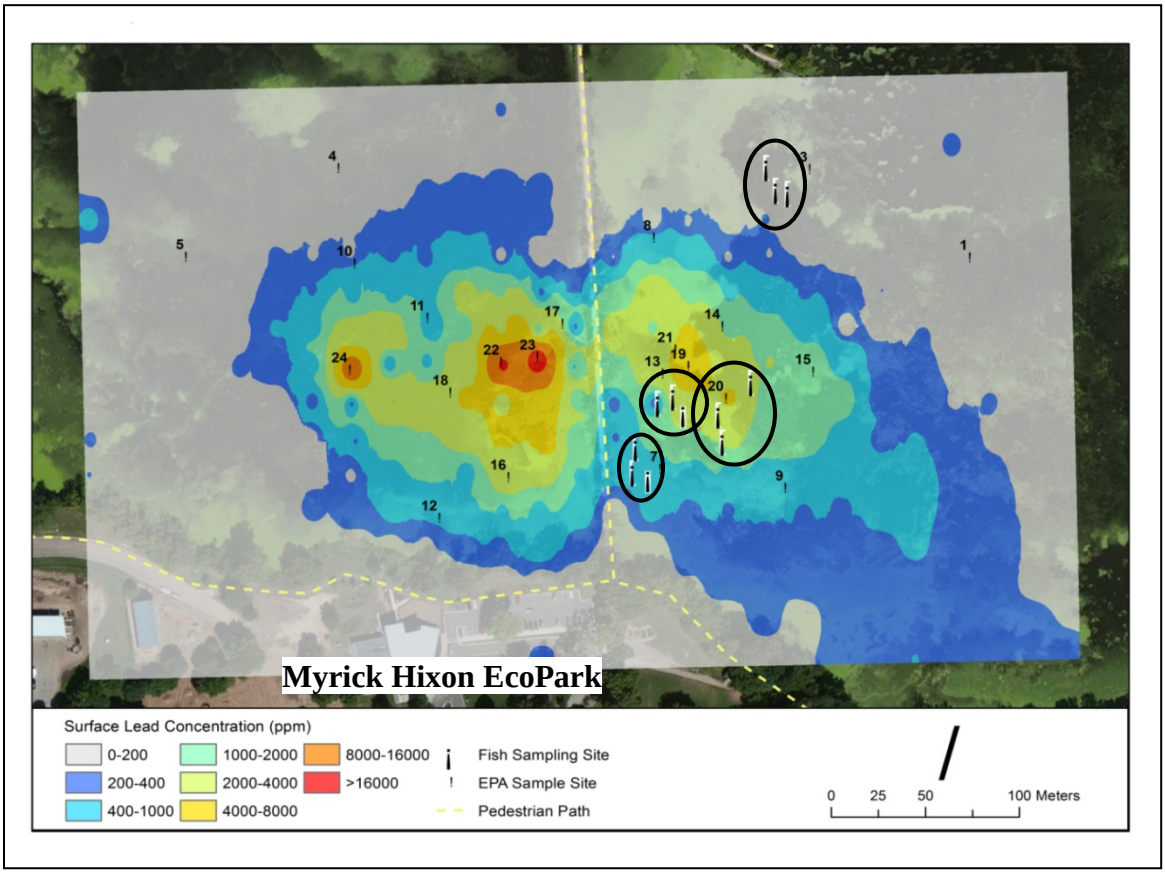

Figure 1. Mini-Fyke Sampling Map. Location of collecting sites within the La Crosse River Marsh based on sediment contamination levels from summer, 2012. Survey sites are indicated by circles, and placement of individual fyke nets within each site are noted.

For Pb analyses, we selected fish that were representative of those within different trophic levels and life histories found within the LRM, and that were caught consistently within each survey site, including: planktivores/zooplanktivores (golden shiners, *Notemigonus crysoleucas*), insectivores (bluegill, *Lepomis macrochirus*) omnivores (black bullhead, *Ameiurus melas*) and piscivores (northern pike, *Esox lucius*). A minimum of four fish from each species were processed for Pb content from each survey site (Table 1). Length and weight data were recorded for each fish.

Table 1. Summary of Fish Survey. Number of different fish species analyzed for Pb content from different survey sites within the La Crosse River Marsh in summer 2012.

		Number analyzed per site			
Species	Number analyzed	reference	low	medium	high
Golden Shiner	7	4	0	1	2
Bluegill	16	4	4	4	4
Black Bullhead	13	4	1	4	4
Northern Pike	8	0	4	0	4

Fish were euthanized according to the University of Wisconsin – La Crosse's animal care protocols and processed for Pb content. Smaller fish were euthanized with tricaine methanesulfonate (MS-222), while larger fish were stunned with a blunt blow to the head and euthanized via cervical transection. Fish tissues were stored frozen and processed to determine Pb content in whole fish. Samples were analyzed by the Wisconsin State Lab of Hygiene to maintain consistency and appropriate quality control. Fish were shipped as whole specimens because metal types do not accumulate equally in muscle tissues compared to other body parts (e.g., liver, other vital organs and bones), resulting in a full specimen homogenization being the most accurate measure of Pb content (Wagner and Bowman 2003). Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was used to measure Pb levels, with results given as µg/g (mg/kg) Pb, wet weight.

Data Analysis

Statistical analysis was performed with IBM SPSS Statistics (22.0). All data were analyzed for normality. Fish samples were summarized by average Pb levels among whole body fishes, collection site and species using a two-way ANOVA, with a Tukey post-hoc test to indicate differences between site, species and site vs. species with regards

to Pb among fish. Each species was analyzed for a comparison between Pb levels among whole body fishes, survey site and total length (mm) via ANCOVA.

Results

All fish analyzed contained detectable concentrations of Pb; however, the extent of bioaccumulation did not vary between fish species and collection site. Across all species concentrations of Pb within whole fish were variable, indicating no significance between Pb among fish tissue and collection site (Figure 2, P = 0.384). Results indicate there was no difference in fish Pb concentration between species (Figure 2, P = 0.478). Incorporating both site and species to fish Pb concentration indicated no statistical difference (Figure 2, P = 0.644).

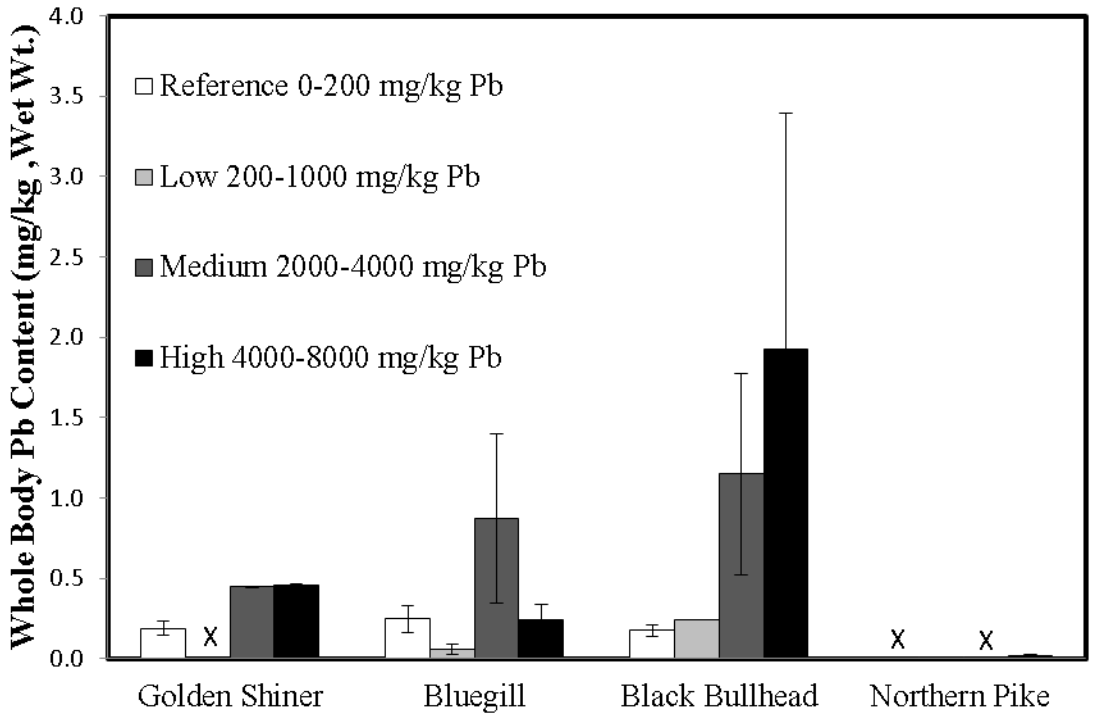

Figure 2. Concentrations of Pb within Fish Collected at Different Survey Sites. Pb concentration within whole fish samples **(**mean ± SE) based on species and collection sites from the LRM, summer 2012. X denotes unavailable samples.

Total length of fish was taken to see whether Pb contamination increased with fish size. Incorporating total length did not demonstrate a relationship between size and Pb contamination (Figure 3). Golden shiners were the only exception, with total length correlating with Pb-accumulation, suggesting a negative relationship. As size increased in golden shiners, Pb-contamination decreased (Figure 3). In the case of bluegill, as size increased Pb-contamination among tissues decreased, despite being captured from areas that had relatively high contamination (Figure 3). Total length of black bullhead, and northern pike compared to tissue-Pb indicated no correlation (Figure 3). This result suggests there is a lack of linear relationship between fish size and Pb contamination among tissues for these three fish species.

According to human consumption advisories by the EC and JECFA Pb levels among fish for should not exceed 0.2 and 0.25 mg/kg Pb (wet weight.) for generalized fish species (EC 2005; Ngelinkoto et al. 2014; JECFA 2010; Ahmed et al. 2015). Overall, 57 and 41% of fish samples from the LRM were considered unhealthy for consumption. Looking specifically at game fish from the LRM (e.g., most likely consumed by humans; bluegill, black bullhead, and northern pike) 56 and 50% of bluegill and 77 and 56% of black bullhead were over the allotted consumption limit set forth by the EC and JECFA, while none of the sampled northern pike contained levels of Pb that are of an advisory concern (Table 2).

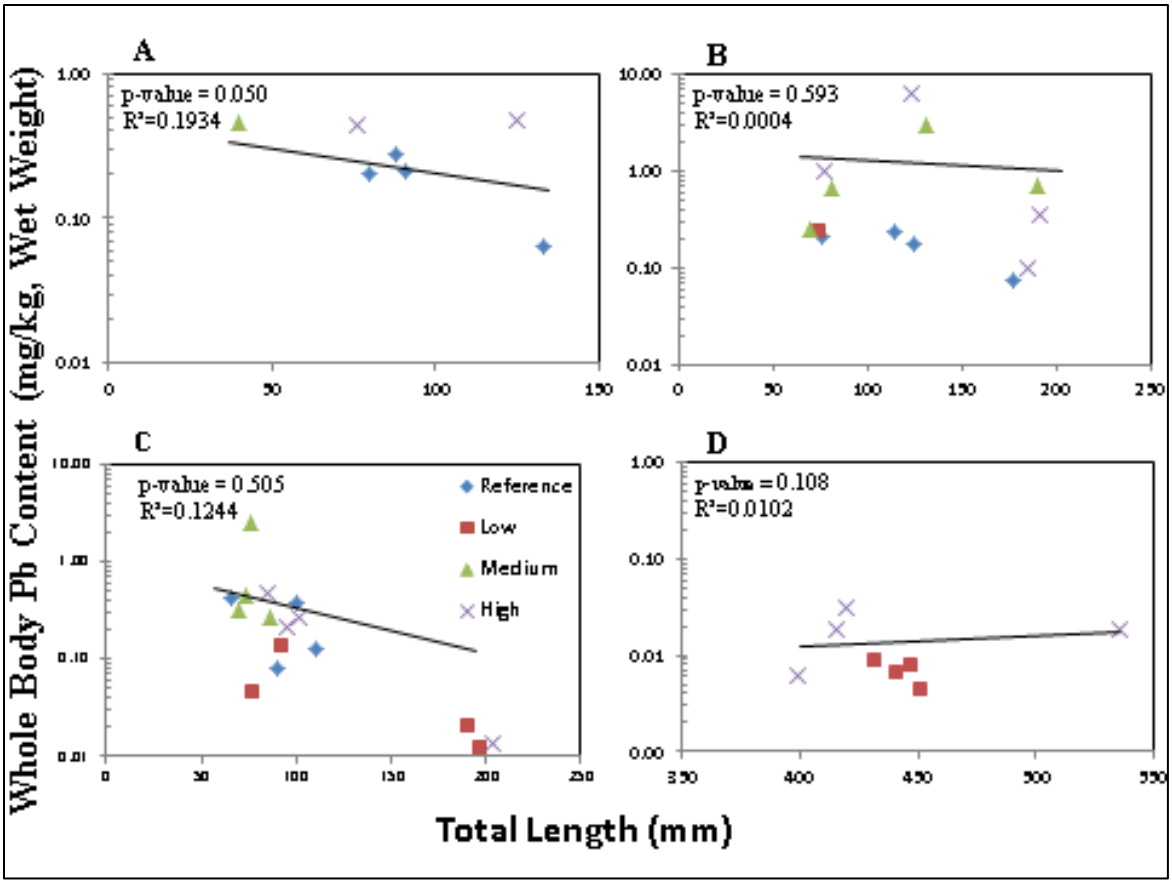

Figure 3. Effects of Length on Accumulation of Pb. Relation between total length and concentration of Pb in whole fish compounded with collection sites from the LRM, summer 2012. Graphs are denoted for the following fish species: A) golden shiner, B) black bullhead, C) bluegill, and D) northern pike.

Table 2. Consumption Advisory. Percentage of fish from the LRM that are over the advised human consumption limit of Pb (wet weight) for fish.

	Species				
Regulation	Golden shiner	Bluegill	Black bullhead	Northern pike	Total
0.20 mg/kg Pb[a]	86%	56%	77%	0%	57%
0.25 mg/kg Pb[b]	44%	50%	46%	0%	41%

[a] Human consumption limit set forth by the European Commission (EC).
[b] Human consumption limit set forth by the Joint FOA/WHO Expert Committee on Food Additives (JECFA).

Discussion

While the high concentrations of Pb found within surface sediments and core samples within the La Crosse River Marsh generated concern, full risks of the contaminated sediments was unclear since Pb bioavailability was not known. This survey was intended to establish whether Pb is moving through the ecosystem and into fish. The data suggests that Pb from contaminated sediments is bioavailable to various fish species living within the LRM. Results demonstrated that 57% of all fish from the LRM were over the EC's allotted consumption limit for Pb and 41% were over the JECFA's consumption limit (EC 2005; JECFA 2010; Ngelinkoto et al. 2014; Ahmed et al. 2015). Based on these levels, it is evident that there may be fish consumption issues for anglers within the Pb-contaminated areas of the LRM. Given the variability in total Pb content within the different species of fish, additional sampling is warranted. Further, assessing Pb content within fillets of fish tissue with respect to potential human consumption would also help to determine whether warnings should be posted within the LRM. Given subtle differences in Pb bioaccumulation across species, understanding the life history of fish species may indicate why Pb bioaccumulation took place.

Golden shiners and bluegills exhibited similar levels of Pb contamination. This is most likely attributed to their feeding habits. Golden shiners are a minnow species that are omnivorous, with nearly half of their diet coming from vegetation and the other half coming from insects and crustaceans (i.e., zooplankton; Johannes et al. 1989; Browman and O'Brien 1992). Collaborative research indicated that Pb is bioavailable to *Lemna* sp. residing in areas containing Pb-contaminated sediments. If *Lemna* sp. contain Pb, there is a good chance other vegetation, including algae, contain Pb as well. If golden shiners are

consuming any vegetation (i.e., algae) and invertebrates from contaminated sites, this may explain Pb bioavailability for this species. Bluegill Pb contamination could be explained in a similar fashion. Bluegill typically feed on invertebrates within the water column (Taguchi et al. 2014). Preliminary results regarding invertebrate collection from the LRM for Pb analysis revealed that caddisfly tissue Pb concentrations correlate with sediment Pb concentrations (Ryan et al. in prep). Presence of Pb in caddisfly larvae and other invertebrates would allow for a possible vector for Pb contamination to move into fish species that primarily consume invertebrates.

Fish species that feed on detritus and interact more with contaminated sediments could see a higher bioaccumulation rate of Pb. Black bullheads had the highest levels of Pb among fish, though contamination levels were highly variable. This is likely due to omnivorous benthic feeding habits (Leunda et al. 2008). Feeding on detritus, invertebrates and small fish species directly with contaminated sediments would result in higher Pb contaminated tissue. Being omnivorous, black bullheads are more likely to consume Pb from their food, because they are wide-range spectrum feeders. Additional sampling may reveal a correlation between concentrations of Pb within sediments and uptake in this species of fish.

Northern pike captured from the marsh exhibited the least Pb contamination. This is most likely due to feeding habits, with northern pike being mostly piscivorous and Pb levels not biomagnifying through aquatic systems (Rubio-Franchini and Rico-Martinez 2011). This process was evident in all other fish species collected from the marsh, which had lower tissue Pb concentrations than preliminary research involving duckweed and caddisfly larvae collected from similar sites. All fish sampled did have some level of Pb

bioaccumulation, however; these levels did not appear to be correlated with the contaminated sites they were collected from.

Life history traits such as diet, movement patterns and size are likely to influence how much time is spent at a particular site, and therefore the extent of Pb bioaccumulation. Though there was not a significant correlation that took place, black bullhead did have a slight indication of a dose-response curve with regards to increased Pb among tissues with increasing sediment Pb contamination (Figure 2). The most contaminated fish came from high and medium contaminated sites (in that order); however, the large standard error and high variability among these samples is why this correlation is not statistically significant. A larger sample size for black bullhead may have indicated a correlation between Pb tissue level and site Pb contamination. A possible reason why there was no clear correlation between the Pb in fish tissue and the contaminated areas they were collected from may be due to fish movement patterns. The area of contaminated sediments is rather small (especially the highest contamination), and does not cover the entire marsh habitat. Certain fish species have demonstrated movement throughout a wide range on any given day, especially with regards to feeding and spawning runs (Goclowski et al. 2013). Golden shiners and bluegills have demonstrated diel movements based on zooplankton movement (Baumann and Kitchell, 1974; Lague and Reebs 2000). Fish collected from sampling areas may not have been there for extended periods of time due to diel movement patterns. If fish were to stay in contaminated areas where waterborne Pb levels are high, Pb uptake into the blood may increase. If fish species were to stay in one place instead of moving throughout the marsh, Pb contamination may have been larger than what was observed. With fish

movement there is a chance that Pb-contaminated fish could be caught by the public in other areas of the marsh. Fish movement is perhaps the most pertinent aspect of this study and why Pb contamination and site contamination did not correlate to one another. Fish size also needs to be incorporated into the discussion of tissue Pb contamination correlated to site Pb contamination.

Fish size, growth rate, and age are other life history traits to consider with respect to Pb bioaccumulation. Fish size did not correlate with site Pb concentration and fish concentration between all fish species, indicating that size was not a major factor in determining the Pb contamination that was present in fish. For bluegill, the largest fish tended to have the lowest Pb concentration, despite being from the most contaminated areas. Research has shown that smaller fish tend to uptake Pb at a higher rate through their opercular bone when compared larger fish of the same species, which is inherent of Pb uptake by other tissues (Hodson et al 1982). Also, the Pb uptake process takes longer for larger sized fish, due to larger levels of blood to equilibrate (Hodson et al. 1982), which would explain why fish size and site Pb concentration were not correlated.

Concentrations of Pb within sampled fish indicate that accumulation of Pb could potentially be sufficient to cause sublethal toxicity, impacting fitness. A study by Heier et al. (2009) looked at the effects of heavy metals (including Pb) from drainage water associated with a shooting range. Brown trout (*Salmo trutta*) were exposed to drainage water containing 15-45 µg/L Pb, resulting in Pb accumulation as high as ~14 mg/kg Pb in gill tissue after 11 days of exposure and approximately 0.3 mg/kg of Pb in liver tissues after 23 days of exposure. These Pb levels were enough to decrease condition factor, increase oxidative stress and, inhibit ALA-D activity, causing a suite of adverse effects

(Heier et al. 2009). Total Pb from unfiltered water column samples collected from the LRM study by the WI DNR during summer 2012 averaged 13.1 µg/L ± 2.4 µg/L waterborne Pb, which is nearly enough to induce ALA-D inhibition and cause toxic effects in certain fish species (i.e., brown trout). Water samples taken by UW-L researchers indicated slightly lower values for summer 2013 and 2014 with 4.8 ± 2.2 and 3.1 ± 1.5 µg/L waterborne Pb from unfiltered surface water (unpublished data). This information, along with the Pb levels found within fish tissues from the LRM, would suggest that fish within contaminated areas may be experiencing adverse effects, or at the very least ALA-D inhibition. Fish sampled from the LRM indicate that Pb from contaminated sediments is bioavailable to all fish species; however, at this time it is uncertain these levels are not negatively impacting fish with regards to population dynamics.

CHAPTER III

TOXICITY OF Pb-CONTAMINATED SEDIMENTS

Introduction

Mobilization of Pb from its solid form (e.g., lead-shot within LRM sediments) takes place through the process of particle weathering and changes in pH (Heier et al. 2009); however, this process can take as long as 100-300 years, depending on environmental conditions (Jorgensen and Willems, 1987). Once mobilized, fish can be exposed to waterborne Pb causing severe physical deformities such as descaling, fin erosion and hemorrhaging, and even death (Heier et al 2009; Kumar and Sabhlok 2011). Sublethal concentrations of Pb can cause subtle physical deformities or alterations in general physiology that result in reduced survival and fitness. For example, Pb is also a potent neurotoxicant, which can cause behavioral changes (irregular movement), resulting in increased predation via easier means of capture or reductions in the efficiency by which they capture prey items (Schmitt et al. 2005), or even halting nesting behaviors leading to reproductive failure (Scott and Sloman, 2004). Exposure to Pb can lead to anemia and oxidative stress. Pb alters heme synthesis and iron metabolism by inhibiting enzyme activity, ultimately leading to porphyria, anemia, or both (Schmitt et al. 2005). The creation of free radicals from the exposure of trace levels of Pb results in damaging of proteins, enzyme inhibition, can impair cell signaling leading to various physiological alterations (Benedetti et al. 2007). Collectively, such sublethal effects can impact recruitment and sustainability of wild fish populations (Scott and Sloman 2004).

As with most toxicants, age at exposure influences the toxicity of Pb. Embryonic development is often considered the most sensitive stage within the fish lifecycle (Hallare et al. 2004; Dou and Zhang 2011), where even low levels of Pb can cause developmental toxicity (Boyle et al. 2010). Embryonic exposure to high concentrations of Pb (480-960 µg/L Pb) results in embryonic death, indicating that if Pb levels are high enough, new cohorts cannot be maintained (Goran and Xiu 1991). However, exposure to sublethal concentrations during early stages of development can also result in subtle growth abnormalities, resulting in death early on in development (Grosell et al. 2006) or reduced fitness. Exposure to Pb has been shown to impair fertilization and affect early cleavage patterns and hatching times of fertilized eggs, impacting recruitment (Osman et al. 2007).

While Pb levels within the sediments from the LRM are high (surface sediment up to 42,854 mg/kg; Perroy et al. 2014), it is not known whether these sediments pose a toxicological threat to biota within the marsh that come in contact with the sediments. Therefore, I used the zebrafish (*Danio rerio*) embryo model to evaluate both acute and sublethal developmental toxicity to evaluate the potential risks that marsh sediments may pose to wild fish populations. Zebrafish are good toxicological models because developmental abnormalities can be easily quantified during embryonic and larval growth (Linney et al. 2004), and findings can be used to predict potential risk to both wild fish populations and humans (Scott and Sloman 2004; Wang et al. 2005). Therefore, using standard sediment toxicity assays with zebrafish embryos and larvae should determine whether (1) sediments with different contaminant levels are toxic and (2) whether pH influences toxicity.

Materials and Methods

Standard sediment-toxicity assays were performed using sediment samples from the LRM. All experiments followed animal care protocols (7-13) sanctioned and accepted by the University of Wisconsin – La Crosse. Pilot studies identified that 0.25 g of sediment within 2 ml of zebrafish water allowed for limited observation of developing larvae, but did not induce toxicity to zebrafish from sediments alone. Toxicity was evaluated by separating sediments into reference sites (10 mg/kg Pb), low (269 mg/kg Pb), medium (4,463 mg/kg Pb) and high (12,520 mg/kg Pb) sediments, which were represented by one sample of marsh surface sediment. Sediments were dried, analyzed for Pb concentration using X-ray fluorescence (XRF) spectrometry (Perroy et al. 2014) and stored until use. LRM sediments were compared to artificial sediments that mimic organic sediments and no-sediment negative controls (water). Two established lines of AB zebrafish were used for sediment toxicity assays: a transgenic EPRE AB line, and a non-transgenic line of AB. EPRE zebrafish were initially selected as a means to measure whether fish were under oxidative stress; however, after a prolonged period of time that they were not spawned, they were not producing viable eggs for experiments. Therefore, I switched to using the standard AB line of zebrafish. Survival between EPRE and AB strains were determined to be the same via one-way ANOVA, and so data across experiments were combined.

Sediments from the LRM were homogenized with zebrafish water (60 mg/L Instant Ocean and distilled water) and placed into 24 cell-well plates with one egg per well. Three or four replicate experiments of 24 cell-well plates were performed (total replicates: n=72-96, as each well represented n=1; Table 3). Embryos were exposed to

the varying sediments (control sediments: water and artificial; LRM sediments: reference, low, medium and high) for 120 hours and maintained at 28°C with a 14hr light, 10hr dark photoperiod (Table 3). At 120 hours post fertilization (hpf), surviving fish were moved to clean 24-cell well plates for viewing purposes and mortality was recorded to assess acute toxicity. Once assessed for mortality, zebrafish larvae were allowed to acclimate in clean 24-cell well plates at least five minutes before neurotoxicity analysis.

Table 3. Summary of Sediment Exposure Experiments. Various measures of acute and sublethal toxicity were examined at 120 hours post fertilization as described in the text.

	Sediment, pH=7	Replicates	Sediment, pH=6	Replicates
Controls	water control	4	water control	1
	artificial sediment	3		
Sediments	reference (10 mg/kg Pb)	4		
	low (269 mg/kg Pb)	4		
	medium (4,463 mg/kg Pb)	4	medium (4,462 mg/kg Pb)	3
	high (12,520 mg/kg Pb)	4	high (12,520 mg/kg Pb)	3

Because Pb is a known neurotoxicant (Adonaylo and Oteiza 1999), qualitative C-start touch-response-assays were done to evaluate potential neurotoxicity due to Pb (Hill et al. 2009). C-start touch-response-assays involve administering a stimulus (touch) to larval fish. The response the larvae made was scored on a scale from 0-3, with 0 being a normal C-start, 1 being a delayed C-start, 2 being an improper C-start (e.g., twitching) and 3 being no movement. Survival across treatment groups varied, therefore n=74-87.

Once C-start touch-response assays were completed, zebrafish were scored for toxicity using gross abnormality toxicological endpoints (i.e., developmental toxicity endpoints) as described by King-Heiden et al. (2007). Developmental toxicity endpoints

were assessed qualitatively by scoring individual fish for sublethal toxicity using a range from 0-3: 0 (healthy); 1 (mild toxicity, 1 endpoint), 2 (moderate toxicity, 2-3 endpoints) and 3 (severe toxicity, >3 endpoints). To quantify observed sublethal toxicity, a subset of larvae was used to quantify the presence of toxic endpoints. Eight representative fish from each sediment assay were immobilized in 2% methylcellulose, and lateral images were taken using a microscope-mounted camera. Lateral images were scored based on the number of visible gross abnormalities and used to evaluate overall toxicity (Figure 4).

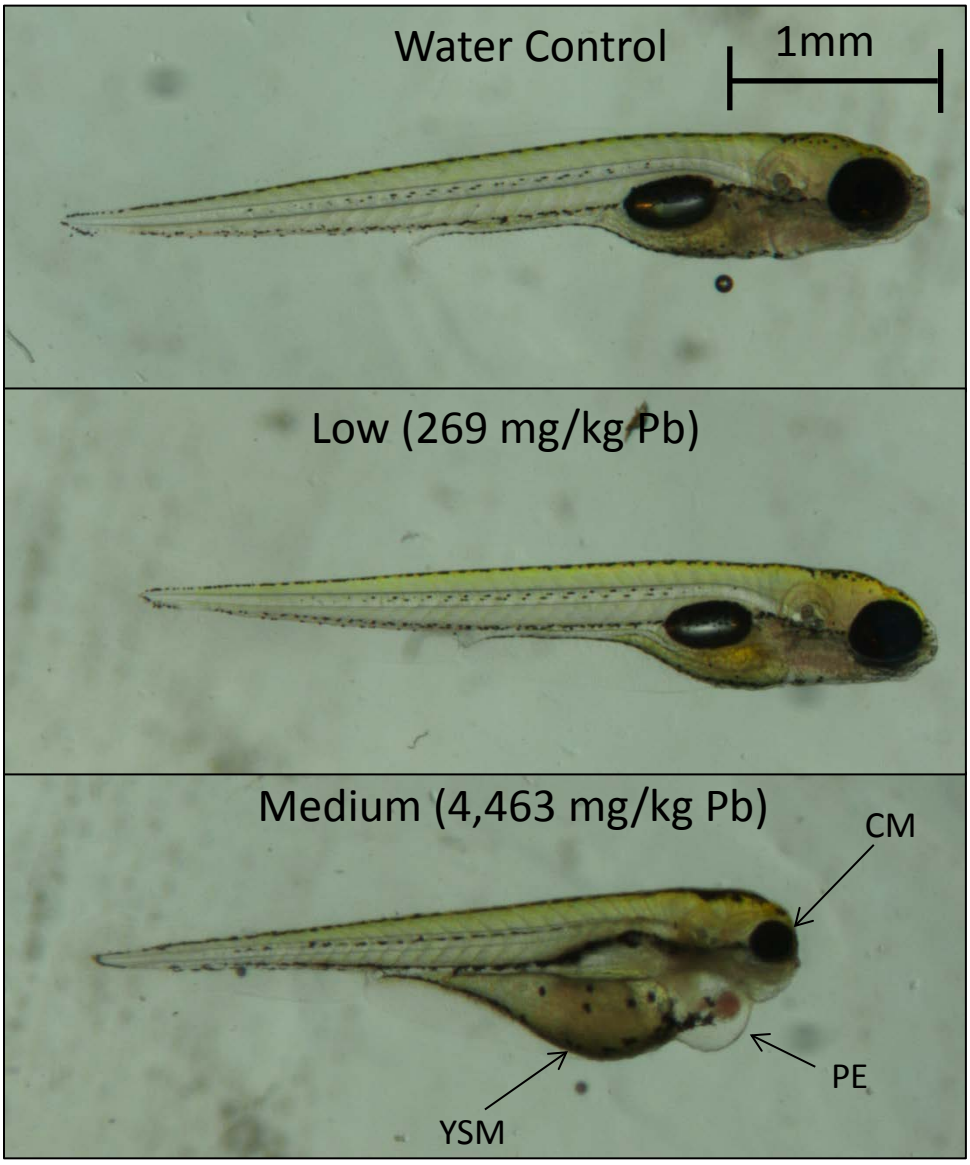

Figure 4. Representative Micrographs of Zebrafish Following Sediment Exposure. Abbreviations: YSM= yolk sac malformation (including yolk sac edema), PE = pericardial edema, CM = cranial malformations

Since low pH can influence the sorption of Pb to sediments and therefore bioavailability (Dermatas et al. 2004; Adhikari el al. 2006), we tested the toxicity of selected LRM sediments under slightly acidic pH conditions. Pb-contaminated sediments from high (12,520 mg/kg Pb) and medium (4463 mg/kg Pb) sites were homogenized in E3-media (NaCl: 17.4 mM, KCl: 0.21 mM, $MgSO_4$ ($7H_2O$): 0.12 mM,

$Ca(NO_3)2(4H_2O)$: 0.18 mM, and HEPES (pH 7.6): 0.15 mM), at a pH of 6. Exposures and data analysis was done as described above (Table 3). Upon completion of all toxicity tests, zebrafish larvae were anesthetized using tricaine methanesulfonate (MS-222).

Data Analysis

Data analyses were performed using IBM SPSS Statistics (22.0). All data were analyzed for normality. A one-way ANOVA with a posthoc Tukey test was used to compare zebrafish mortality, neurotoxicity and developmental toxicity scores across treatments. A random block ANOVA test was used to compare incidence of specific endpoints of toxicity with sediment types.

Results

While exposure to LRM sediments did not induce significant mortality (Figure 5, $P = 0.268$), approximately 67% of larvae exposed to contaminated sediments displayed at least one developmental anomaly (Figure 6). The most prevalent endpoints of toxicity were yolk sac malformations (ysm), pericardial edema (pe), and craniofacial malformations (cm); however, incidence of toxic endpoints did not differ across sediment types (Figure 7, $P = 0.874$).

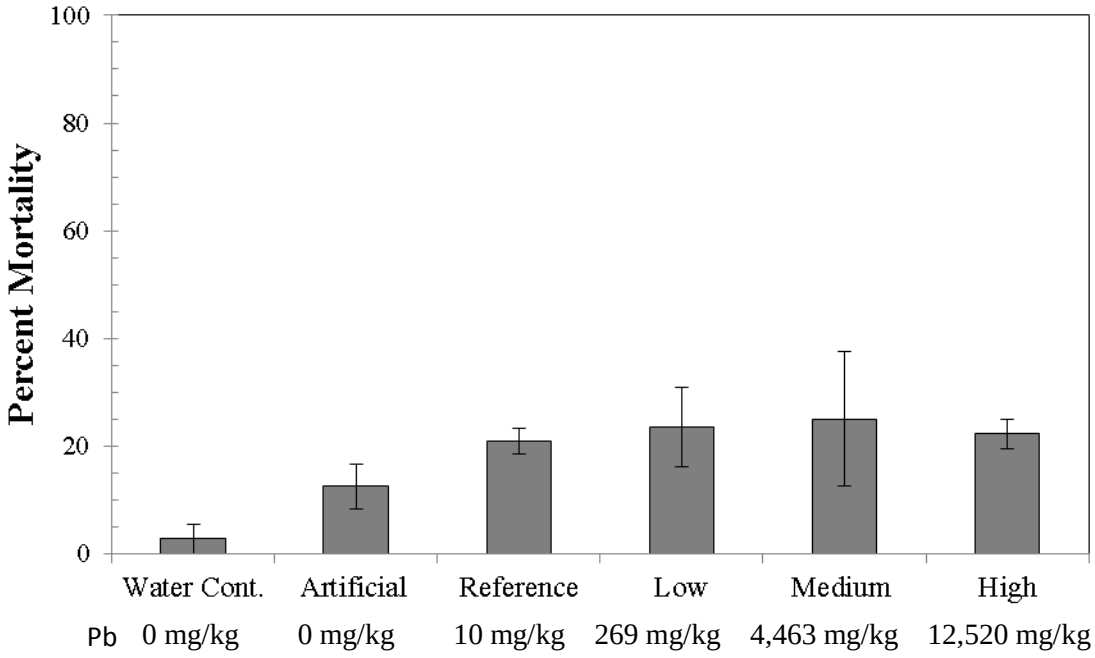

Figure 5. Mortality Following Sediment Exposure. Percent mortality of zebrafish at 120 hpf (mean ± SE) from sediment toxicity test involving control and Pb sediments from the LRM at a pH=7.

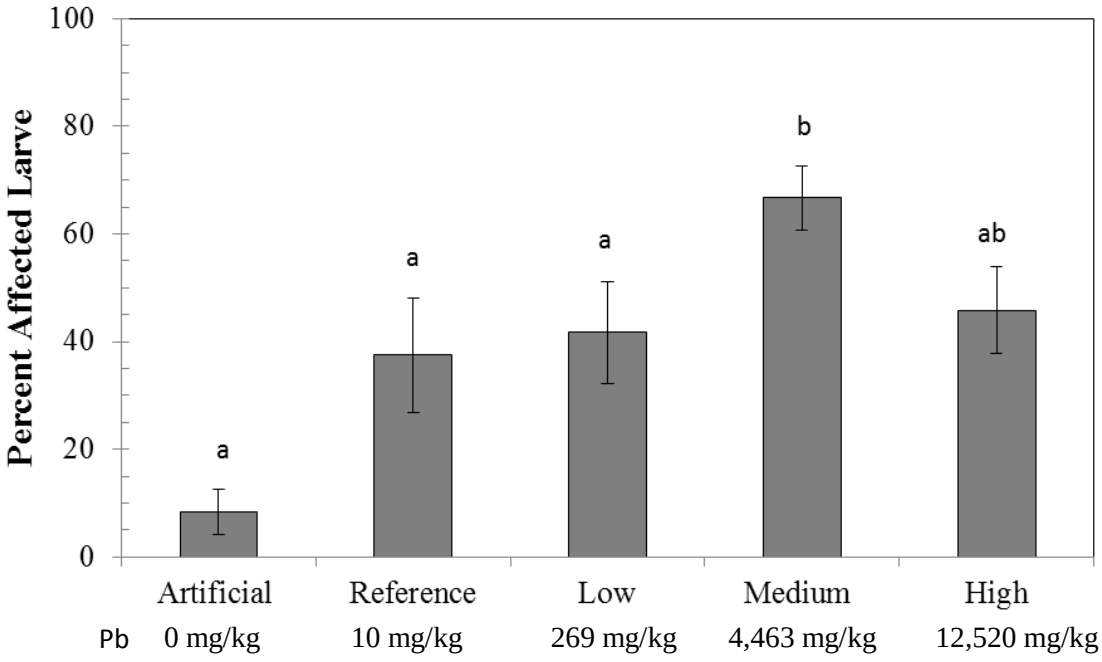

Figure 6. Incidence of Developmental Toxicity Following Sediment Exposure**.** Proportion of fish showing a toxicity score ≥ 1 following exposure to Pb-contaminated sediments from the LRM at pH=7. Letters denote significance ($p < 0.05$).

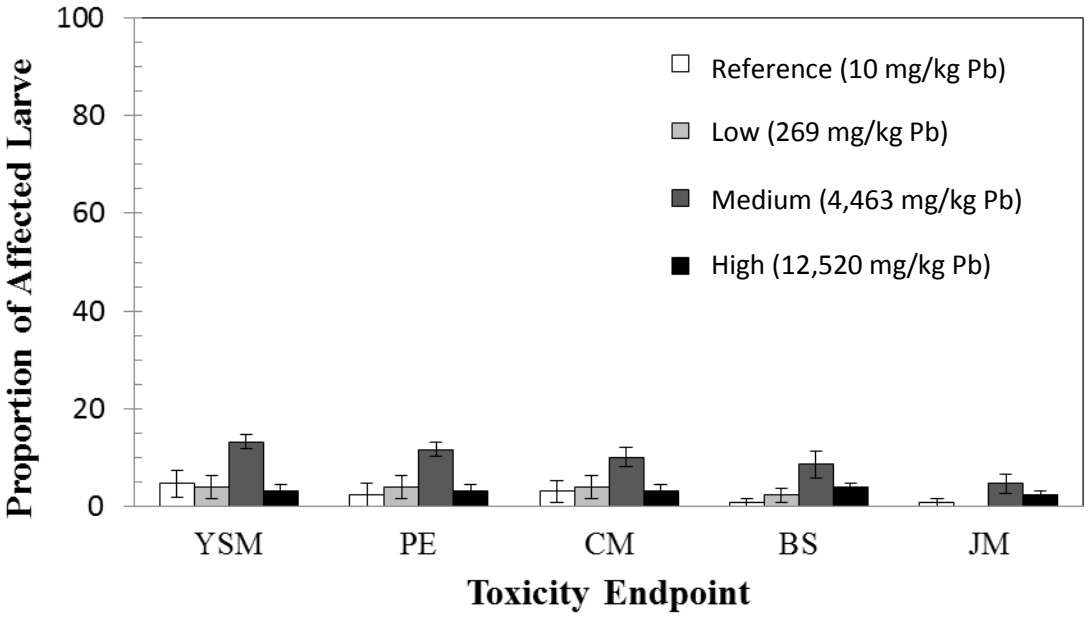

Figure 7. Incidence of Toxicity Endpoints Following Sediment Exposure. Exposure to Pb contaminated sediments resulted in various developmental abnormalities. Percent of affected fish were determined from for eight representative fish from sediment toxicity tests (N=4) involving control and Pb-contaminated sediments from the LRM at pH=7. Abbreviations: YSM= yolk sac malformation (including yolk sac edema), PE = pericardial edema, CM = cranial malformations, BS = bent spine and, JM = jaw malformation.

Zebrafish larvae exposed to sediments that contained 4,463 mg/kg Pb (medium) did show increased overall sublethal toxicity via developmental endpoints (Figure 8).

Zebrafish larvae exposed to all LRM sediment types demonstrated at least minimal signs of neurotoxicity; however it was largely variable with no visible dose response observed. Exposure to sediments containing low (269 mg/kg Pb) and medium (4,463 mg/kg Pb) levels of Pb resulted in a delayed response with regards to the C-start start response stimuli (Figure 9).

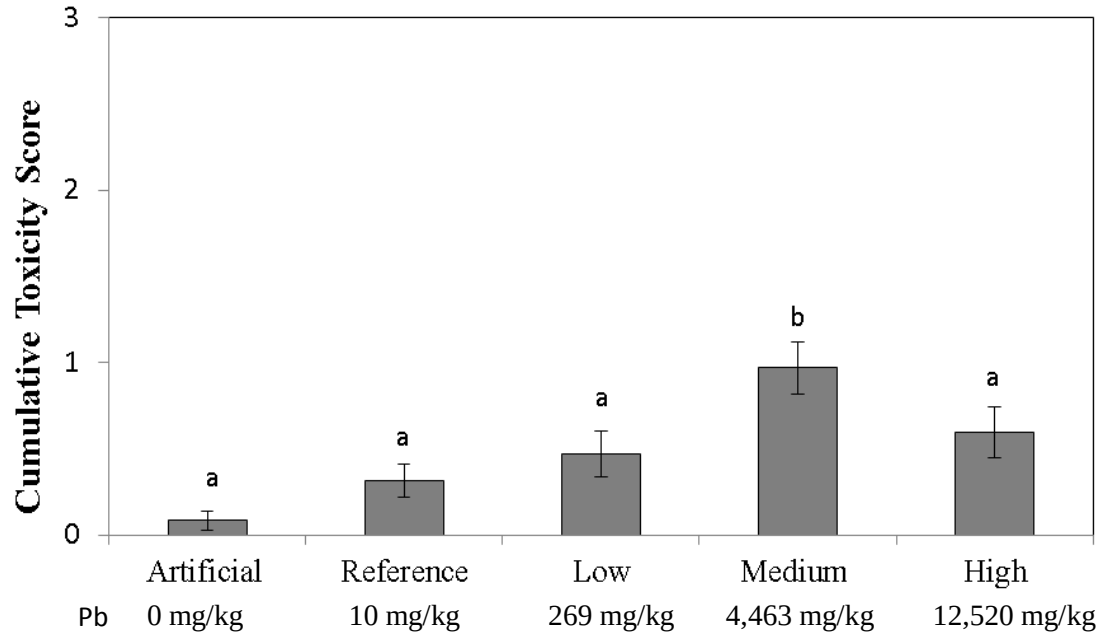

Figure 8. Sublethal Toxicity Following Sediment Exposure. Average cumulative toxicity score at 120 hpf (mean ± SE) for eight representative fish from sediment toxicity tests (N=4) involving control and Pb-contaminated sediments from the LRM at pH=7. Letters denote significance ($p < 0.05$).

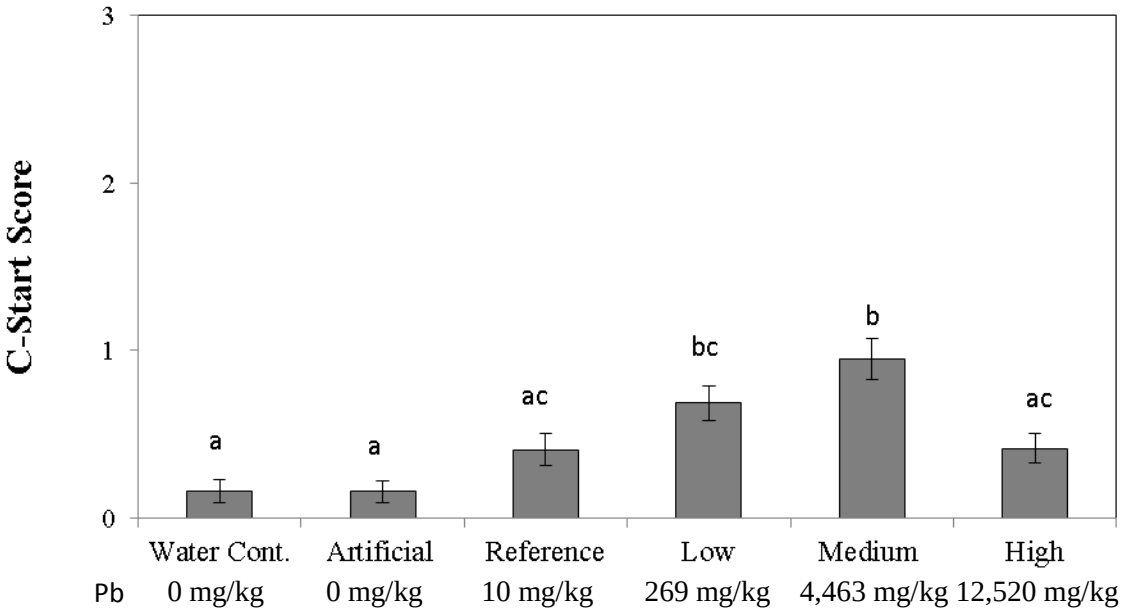

Figure 9. Neurotoxicity in Zebrafish Following Sediment Exposure. C-start score at 120 hpf (mean ± SE) for zebrafish from sediment toxicity tests involving control and Pb-contaminated sediments from the LRM at pH=7. Letters denote significance ($p < 0.05$).

Interestingly, zebrafish mortality was significantly lower when exposure to LRM sediments under slightly acidic pH (Figure 10). The addition of slightly acidic pH to sediment exposures did not result in any statistical differences for sublethal toxicity, incidence of sublethal toxicity, and neurotoxicity, indicating that these parameters were not altered by pH (Figures 11-13).

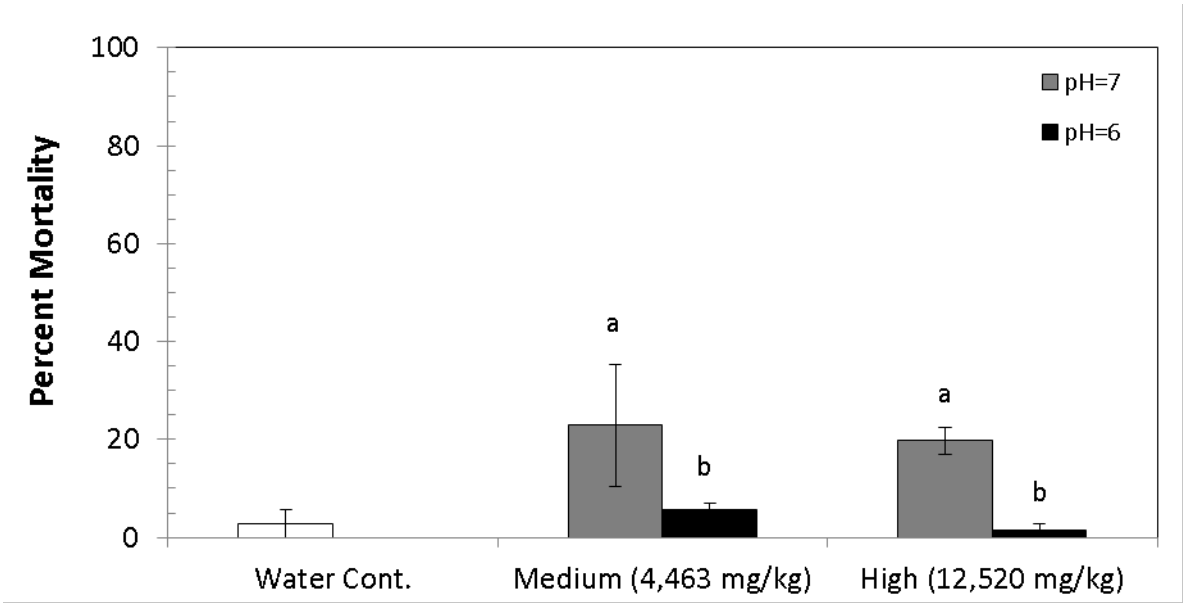

Figure 10. Impacts of pH on Mortality. Percent mortality of zebrafish at 120 hpf (mean ± SE) from sediment toxicity test involving control, medium, and high Pb-contaminated sediments from the LRM at a pH=7 and pH=6. Letters denote differences ($p < 0.05$).

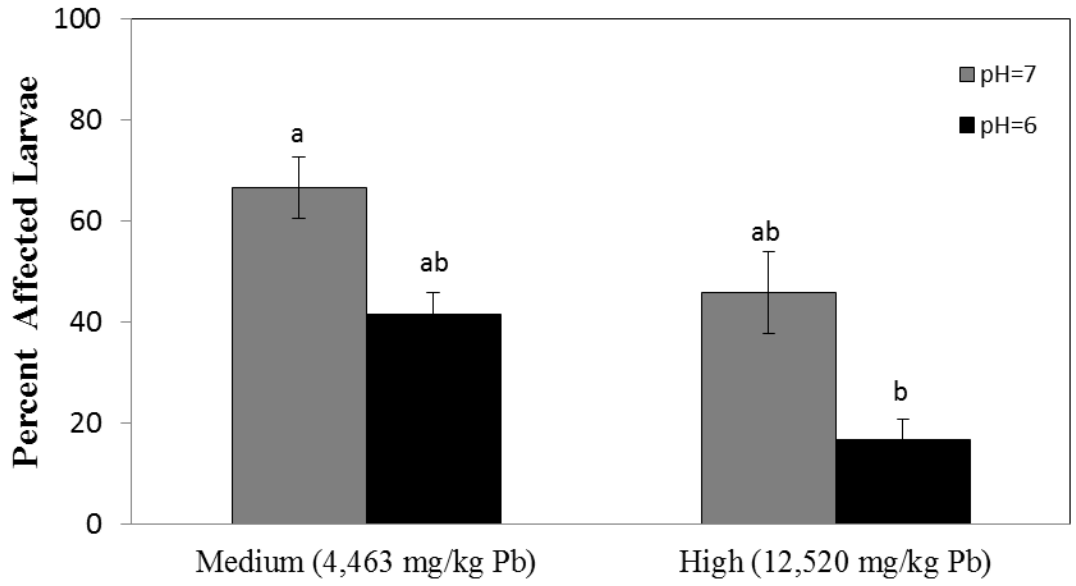

Figure 11. Impacts of pH on Incidence of Toxicity Endpoints. Proportion of fish showing a toxicity score ≥ 1 at 120 hpf (mean ± SE) following exposure medium and high Pb-contaminated sediments from the LRM at pH=7 (N=4) and pH=6 (N=3). Letters denote significance ($p < 0.05$).

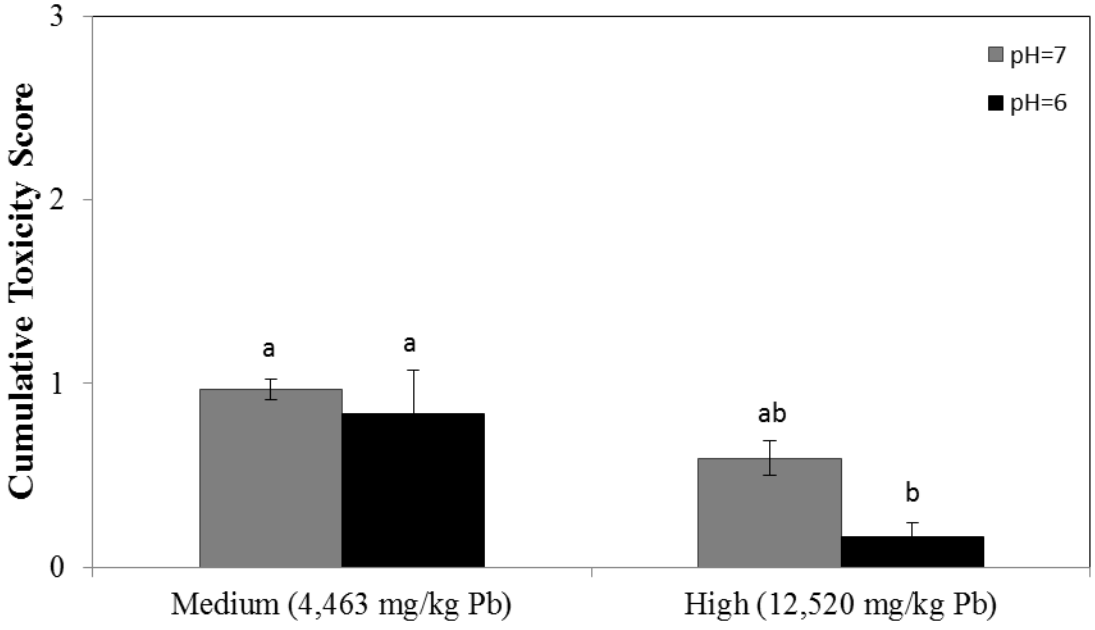

Figure 12. Impacts of pH on Sublethal Toxicity. Average cumulative toxicity score at 120 hpf (mean ± SE) for eight representative fish from sediment toxicity tests involving medium and high Pb-contaminated sediments from the LRM at pH=7 (N=4) and pH=6 (N=3). Letters denote significance ($p < 0.05$).

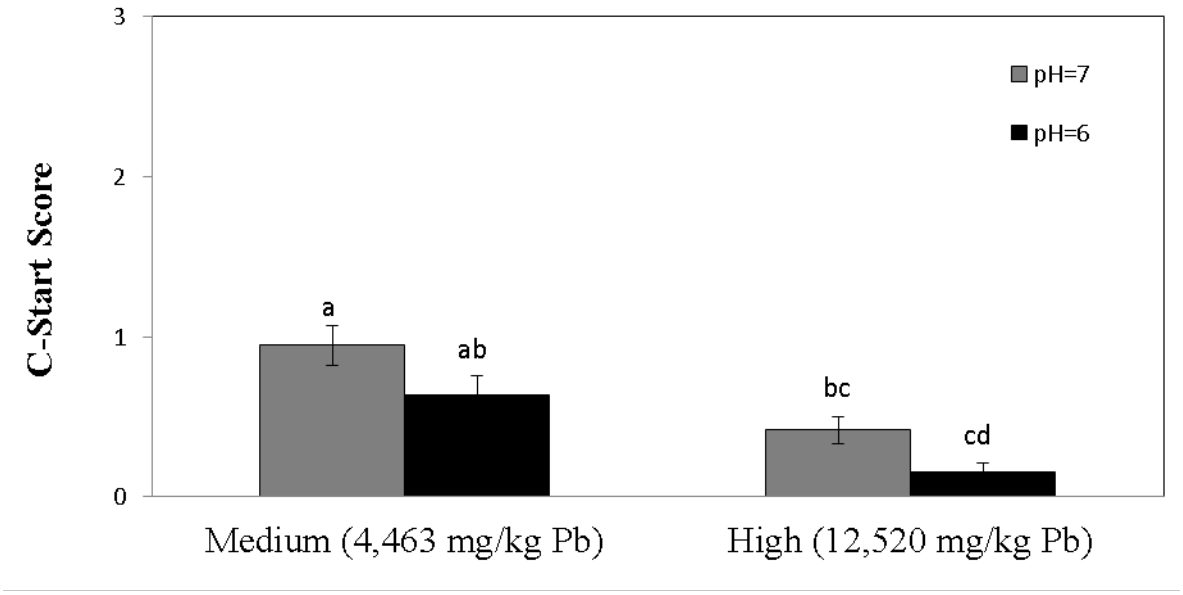

Figure 13. Impacts of pH on Neurotoxicity. C-start score at 120 hpf (mean ± SE) for zebrafish from sediment toxicity tests involving medium and high Pb-contaminated sediments from the LRM at pH=7 and pH=6. Letters denote significance ($p < 0.05$).

Discussion

Given the high levels of Pb contamination within surface sediment of the LRM, there were concerns that Pb could potentially be affecting fish recruitment in the marsh. For this reason, sediment toxicity tests using zebrafish and Pb-contaminated sediments from the LRM were done. Despite the high concentration of Pb that embryos were exposed to, we did not observe a significant difference in mortality across control and Pb-contaminated sites following 120 hour exposure. Though mortality was not significant, exposure to all sediment types from the LRM resulted in signs of either developmental abnormalities or neurotoxicity in zebrafish, suggesting that sediments from the marsh are marginally toxic to fish embryos or larvae. While LRM sediments were not overtly toxic, zebrafish larvae did display signs of Pb- toxicity given the developmental endpoints that were observed. Prior to LRM sediment toxicity assays preliminary trials were done using

Pb-nitrate ($Pb(NO_3)_2$) to determine developmental endpoints for Pb, clarifying if developmental endpoints from LRM sediments were due to Pb. Preliminary results indicated that pericardial edema (pe), yolk sac malformations (ysm), cranial malformations (cm), and jaw malformations (jm) were the most evident developmental abnormalities for zebrafish exposed to Pb-nitrate (Schneider et al. 2013). Sediment toxicity assays involving LRM sediments demonstrated similar developmental abnormalities that would suggest that sublethal toxicity was due to Pb from LRM sediments (Figure 8). The use of C-start touch-response assays to analyze neurotoxicity can also be used to determine if Pb from LRM sediments are minimally toxic to larval fish.

Subtle neurotoxicity was present in all Pb sediment types; however, only sediments with a concentration of 269 mg/kg Pb (low) and 4,463 mg/kg Pb (medium) yielded any statistical significance from other sediment types. Neurotoxicity for zebrafish embryos followed a similar pattern to that of sublethal toxicity (i.e., developmental abnormalities), where there was an evident dose-response curve in terms of C-start scores until a decrease in toxicity from sediments containing 12,520 mg/kg Pb (high). This would make sense, because neurotoxicity is often linked to sublethal toxicity (Beauvais et al. 2001). C-start touch-response-assays are a good representation for neurotoxicity, because movement from stimuli is a direct indicator of the stability of the neurological system (Stehr et al. 2006). Since Pb is a known neurotoxicant (Adonaylo and Oteiza 1999), the C-start assay may be a good indicator of Pb toxicity taking place in zebrafish embryos from contaminated sediments. Observations from neurotoxicity and developmental endpoints suggests that toxicity observed in zebrafish larvae exposed to

LRM sediments was, at least in part, due to Pb. Developmental abnormalities and neurotoxicity that was observed may have been due to Pb toxicity, however; there may be other reasons as to why we do not see a distinct dose-response curve from LRM sediments. These reasons include the presence of other toxicants and a lack of Pb leaching from LRM sediment.

Sediment assays indicated that toxicity was taking place at varying degrees throughout sediment types; however, it is not clear how much toxicity was due solely to Pb, which is partially attributed to a lacking dose-response curve. Marsh-like habitats and their sediments have been shown to contain a variety of toxicants (Serafim et al. 2013), meaning that not all toxicity in this study could have been due to Pb. A number of other possible toxicants (e.g., heavy metals, PCBs, organic matter, etc.) could be present in the LRM sediments, much like sediments from other marshes (Kim et al. 2012).

Developmental toxicity endpoint score and neurotoxicity was greater in medium than high sediments which would imply a true dose response curve is not taking place. If Pb were the main toxicant, high sediments (12,520 mg/kg Pb) should have been more toxic, considering high sediments contain almost three times more Pb than medium sediments (4,463 mg/kg Pb). For this reason there may be other toxicants present besides Pb in the sediments, especially from medium sediments. The composition of medium sediments (e.g., physio-chemical composition) may favor the release of Pb into water over other LRM sediments.

Another possible explanation as to why we did not observe a clear dose-response curve to Pb-contaminated sediments may be due to leaching. Toxicity was present in all studies; however, leaching from LRM sediments may not be happening fast enough to

cause noticeable signs within the short time period of the study. Pb from sediments used may not have been in their most readily bioavailable form, affecting the overall toxicity (Jorgensen and Willems, 1987). Preliminary work in our lab indicated that even after sonication, LRM sediments did not cause significant toxicity, which would suggest that Pb may be more bound to LRM sediments than expected. In order to determine if sediment toxicity is solely due to Pb, a biomarker (i.e., ALA-D) for Pb should be utilized in sediment toxicity assays.

With the addition of slightly acidic pH to sediment toxicity assays, toxicity scores were lower in studies with a pH=6 compared to a pH=7. In fact, with the addition of slightly acidic pH mortality decreased significantly. There was no evidence that developmental toxicity and neurotoxicity increased with the addition of slightly acidic pH. Since Pb tends to increase in toxicity under slightly acidic condition (Dermatas et al. 2004; Adhikari el al. 2006), it was surprising to find that toxicity was not increased under slightly acidic conditions. Given the limited breadth of pH analysis, we cannot be sure that sediments would not be more toxic under acidic conditions. The main point that can be taken away from the pH comparison is that slightly acidic pH did not result in more Pb toxicity than a neutral pH.

Sediment toxicity assays involving Pb-contaminated LRM sediments suggests that at the very least Pb may be minimally toxic to embryos or larvae, which is evident through developmental abnormalities and neurotoxicity. The lack of a dose-response cannot be fully explained, only hypothesized, which is why further studies should be done to ensure that Pb-contaminated sediments from the LRM are not having a biological impact on native fishes.

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

Bioavailability of Fish from the La Crosse River Marsh

This initial fish survey of the LRM indicated that Pb from the LRM is bioavailable to native fish. Based on fish consumption advisory limits (i.e., EC and JECFA), consumption of fish from areas of Pb-contaminated sediments is not recommended based on observed fish tissue Pb levels. Due to potential harm of the fish population and possible human consumption, further sampling of fish is warranted. Further experiments could be done to focus on consumption of fish caught by local anglers. This would involve spending more time targeting game species of interest (e.g., bluegill, yellow perch, black bullhead, largemouth bass and northern pike). Fish species could be analyzed for Pb within fillets, which could be more closely linked to angler consumption. This would allow us to determine if the Pb-contaminated area in the LRM requires a fish consumption advisory.

Variability of Pb content in the fish tissues was high, which could be correlated to fish movement throughout the LRM. Caging fish within contaminated areas could provide a means of understanding Pb uptake between the different Pb-contaminated sites. This would involve creating containment nets that could keep fish within a certain area while exposed to the environmental conditions (Fredriksson et al. 2014). This way Pb exposure, movement, and the amount of time fish are exposed to Pb-contaminated sediments can be controlled. This experiment could also involve the use of laboratory

fish (i.e., fathead minnows) that would then be able to be analyzed for Pb accumulation and sublethal toxicity, much like the experiments involving zebrafish. This would provide us better insight into the potential risks these contaminated sediments pose to fish living in the LRM.

Age data was one aspect that was not incorporated into this project. Adding different age structures for different species would allow for a better correlation between age and Pb contamination. Taking the most appropriate age structures for species would allow the most accurate aging (Kowalewski et al. 2012). For example, otoliths should be taken for bluegill (Kowalewski et al. 2012), cleithra for northern pike (Faust et al. 2013), and spines for black bullhead (Pedicillo et al. 2008). This would allow for a clear and concise understanding of age vs. Pb contamination. Though these suggestions would help to polish the project, there are conclusions that can be made from the wild fish samples from the LRM.

Toxicity of Pb-Contaminated Sediments

Acute and sublethal toxicity tests can be a good indicator of what is taking place in an environment with regards to contaminates (Kusik et al. 2008); however, a natural environment may have multiple toxicants and/or stressors present. Therefore, it is important to know that Pb is the main toxicant causing problems. This can be done by analyzing toxicity with the presence of known Pb-biomarkers (i.e., ALA-D). Knowing that Pb is the main toxicant causing problems from the marsh sediments will be an excellent addition; however, there are a number of things that could have been altered to ensure sound results. While laboratory assays do provide us with some information regarding the toxicity of the LRM sediments, they are not a perfect representation of what

is taking place in the LRM with regards to native fish recruitment. Using a native species or at least a non-tropical fish (i.e., fathead minnow) with microhabitats and running reproduction studies (e.g., recruitment) would be a good addition.

Management Implications

There are a number of conclusions that can be determined based on the results of the wild fish survey and sediment toxicity assays regarding Pb contamination from the LRM. With regards to the wild caught fish, Pb was being made bioavailable into all fish species that were sampled. Fish Pb contamination was to the extent that certain organizations (i.e., EC; JECFA) would advise against consumption. Waterborne Pb levels would suggest that ALA-D inhibition and toxicity is possible based on whole fish samples (Heier et al. 2009). With regards to what was seen in northern pike we can conclude that Pb is not increasing as it moves up the food chain, at least with regards to fish trophic levels. Because the level of Pb in these top tier fish species is so low, we would not expect that level to affect higher trophic levels above it (e.g., birds, mammals), because Pb levels decline as trophic levels increase (Chen et al. 2000). Based on fish results, continued monitored should be done to determine the full extent of Pb-contamination on the fish population from the LRM.

Sediment toxicity tests from Pb-contaminated sediments from the LRM indicate that there is evident toxicity taking place from all sediment types that were used, even from control sediments (reference). Developmental endpoints and neurotoxicity suggests that there may be some toxicity taking place due to Pb; however, because there was an absence of a dose response curve from the different toxicity tests, it would suggest that Pb may not be the only toxicant present. There is the possibility that the Pb from LRM

sediments did not leach out fully over the 120 hour toxicity trial. Toxicity assays involving a Pb biomarker should be utilized to insure toxicity is due to Pb. Minimally, this study should be done, along with fillet samples of sports fish to determine the full extent of Pb toxicity on fish from the LRM. From this initial survey and future studies, a remediation or monitoring plan can be created to insure the sanctity of the LRM fish population.

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