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Interactions Between Water Chemistry and Waterborne Lead Exposure to Freshwater Organisms

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UNIVERSITY OF MIAMI

INTERACTIONS BETWEEN WATER CHEMISTRY AND WATERBORNE LEAD EXPOSURE TO FRESHWATER ORGANISMS

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Interactions Between Water Chemistry and Waterborne Lead Exposure to Freshwater Organisms (August 2010)

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This dissertation characterizes the influences of water chemistry on the acute toxicity of lead (Pb) to two of the long-standing sentinel test organisms commonly employed by the United States Environmental Protection Agency (USEPA), the fathead minnow (*Pimephales promelas*) and daphnid (*Ceriodaphnia dubia*), for parameterization of an acute Pb Biotic Ligand Model (BLM). In addition, a toxicogenomic approach was employed to identify genes that might serve as molecular markers of Pb exposure and long-term effects, as well as provide new insights as to the underlying toxic mechanisms of chronic Pb exposure in *P. promelas*. The endpoints of growth, reproduction, Pb accumulation, prey capture ability, and swimming performance of *P. promelas* were examined to assess the influences of water chemistry during chronic Pb exposures and to potentially link microarray-identified genes to outcomes of ecological significance.

Importantly, this work revealed that calcium does not protect against acute toxicity to *C. dubia* or chronic Pb accumulation by *P. promelas*, indicating that current hardness-based regulations are inappropriate and provide further support for the need for alternative approaches to setting environmental regulations for Pb. The findings reported herein should facilitate the arrival of such an approach in the form of a new acute Pb BLM. However, different responses with respect to the influences of water chemistry on

the acute toxicity of Pb were exhibited by these species suggesting that development of separate BLMs for *P. promelas* and *C. dubia* should be considered to ensure adequate protection for both species. Furthermore, the influences of water chemistry were found to be inconsistent during acute and chronic Pb exposures to *P. promelas* and thus caution against inferring chronic effects from acute exposures. A number of Pb-responsive genes were identified that exhibited a strong potential for serving as robust indicators of Pb exposure and accumulation in *P. promelas*. While these genes also provided insight as to the likely toxic mechanisms of Pb, additional work will be necessary to firmly link these genes to chronic outcomes of ecological relevance in the context of ambient water chemistry.

I dedicate this dissertation
to my parents, Edward and Patricia,
to my aunt and uncle, Carol and Bill,
to my brother and sister, Doug and Cindy,
to my cousin, Linda,
and to the love of my life, Carmen

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CHAPTER 1

INTRODUCTION

1.1. Lead in the environment.

Lead (Pb) is a class B, post-transition heavy metal (atomic number 82) which exists predominantly in its divalent oxidative state. The most prevalent and economically important mineral of Pb is galena (PbS), with cerussite ($PbCO_3$) and anglesite ($PbSO_4$) among the most substantial of the other Pb deposits. Lead can enter receiving waters from a variety of aquatic, atmospheric and terrestrial routes. Both natural and anthropogenic sources contribute, but the atmospheric dispersal of Pb, primarily from its use as a fuel additive, has made it a pervasive and persistent pollutant worldwide. In fact, estimates indicate that atmospheric Pb deposition has increased up to 1000-fold since prehistoric times (Renberg et al., 2000). Much of this is attributed to the >7 million metric tons of Pb burned as a gasoline additive between 1926 and 1985 in the US alone (Nriagu, 1990). In response to the growing awareness of the hazards of Pb pollution, the US phased out the use of leaded gasoline beginning in the 1970s under the Clean Air Act. This effort has been regarded as one of the great successes in environmental legislation having dramatically improved air quality with respect to Pb (Figure 1.1.). Naturally, the benefits have also translated into reduced atmospheric contributions of Pb to the aquatic environment, as evident by analysis of sediment cores from various lakes across the US (Mahler et al., 2006). However, the use of leaded gasoline persists in many developing countries today primarily in Africa and Asia.

Although diffuse in nature, the contribution of Pb to the aquatic environment via atmospheric fallout (to which both natural and anthropogenic sources contribute) cannot be overstated. Natural sources of Pb to the atmosphere include volcanoes, wild forest fires and seasalt spray, while anthropogenic input arises largely from mobile sources (e.g. leaded fuel, break and engine wear, battery leaks), combustion of coal, oil and wood, and various processes in metal production and manufacturing. Nriagu and Pacyna (1988) estimated the contributions for each of the primary atmospheric sources of Pb in 1983 (Table 1.1.). Total anthropogenic emissions were estimated at nearly 300,000 metric tons annually, far exceeding the 12,000 metric tons from natural sources. However, it should be noted that, owing to the decline in the use of leaded fuels, more recent estimates of mobile sources were about 30% of the 1983 amounts (OECD, 1993). Nevertheless, having accounted for this difference, anthropogenic contributions to atmospheric Pb would still greatly exceed those from natural sources.

Aquatic sources of Pb include natural processes, such as the weathering of Pb ores, although of greater significance to toxicity are typically those due to anthropogenic discharge. While estimates have placed the total river input of Pb to the world's oceans at approximately 150,000 metric tons annually (Demayo et al., 1982), natural weathering contributes relatively minor amounts of Pb on a local scale except in areas of high Pb mineralization. Total anthropogenic input of Pb to aquatic ecosystems has been estimated at 138,000 metric tons per year, although most of this (around 100,000) was attributed to atmospheric fallout (Nriagu, 1989; Table 1.2.). Today, most concern for Pb entering aquatic environments is from point-source discharges related to mining and smelting of Pb ores, largely for use in the production of storage batteries, as well as from

sewage sludge and domestic wastewater (Table 1.2.) (Nriagu and Pacyna, 1988; World Health Organization, 1995). Such localized, and likely concentrated, sources can lead to elevated levels of Pb in receiving waters with potentially toxic effects on the resident biota. The extent of this toxicity will depend largely on the influence of water chemistry on Pb speciation and bioavailability.

1.2. Chemical speciation and bioavailability of lead in freshwater.

In natural aquatic environments at or above neutral pH, Pb is readily complexed and most inorganic salts of Pb are poorly soluble with the exception of nitrate, chlorate and chloride salts. In contrast, Pb salts tend to be quite soluble under acidic conditions. Lead can also form stable organic compounds such as tetraethyl Pb, once a common antiknock additive in gasoline. Such influences of water chemistry not only impact the chemical form (speciation) of Pb, but also its bioavailability and toxicity. In general, the most toxic form of a metal is assumed to be the free ionic form (Pb^{2+}), although it remains to be seen whether other species (e.g. hydroxides and/or carbonates) contribute to the toxicity of Pb in freshwater organisms.

Under typical freshwater conditions, pH, alkalinity and the amount and quality of natural organic matter (NOM) will represent the parameters of greatest importance for Pb speciation. As shown in Table 1.3., changes in pH and alkalinity have a profound effect on the relative contributions of the free Pb^{2+} ion, Pb monohydroxide ($PbOH^{+}$) and the Pb carbonate ($PbCO_3^{2}$) species. This occurs in large part because, as for other divalent metals, Pb complexes strongly with CO_3^{2-} and OH^{-} ions. Accordingly, in waters of high pH/alkalinity the Pb carbonato and hydroxo species will dominate while in waters of low

pH/alkalinity a far greater percentage of ionic Pb^{2+} will prevail. The significance of pH and alkalinity in this regard has been recognized for decades and several studies have investigated their influences on Pb toxicity to freshwater organisms. One of the first studies examining the toxicity of Pb to fish in the context of speciation revealed that differences in carbonate concentration among two different natural waters contributed to large differences in Pb solubility, and therefore toxicity of Pb to rainbow trout (*Oncorhynchus mykiss*; Davies et al., 1976). Others have examined the effects of pH on Pb toxicity to the fathead minnow, *Pimephales promelas*, the amphipod, *Hyalella azteca*, and the daphnid, *Ceriodaphnia dubia* (Schubauer-Berigan et al., 1993), as well as the common carp, *Cyprinus carpio* (Stouthart et al., 1994), demonstrating that acute toxicity of Pb increases as pH decreases from neutral pH. Lead accumulation was also found to increase in the blood of rainbow trout (Hodson et al., 1978b) and various tissues of the sunfish, *Lepomis gibbosus* (Merlini and Pozzi, 1977) when exposed at pH 6.0 versus pH 10 and pH 7.5, respectively, indicating greater bioavailability of Pb at acidic pH.

Broadly characterized as a poorly-defined complex mixture of particulate, colloidal and dissolved organic matter (DOM; e.g. humic substances), natural organic matter (NOM) exhibits great potential for influencing Pb bioavailability, putatively owing to an abundance of various high affinity ligands. Indeed, NOM has been estimated to complex the vast majority of Pb (>98%) at environmentally relevant concentrations within the meromictic lake, Paul Lake, MI (Taillefert et al., 2000). It is important to note, however, that the protective nature of DOM is dependent on its quality, with darker allochthonous sources imparting greater protection than lighter autochthonous DOM, a measure of which can be reasonably estimated by a simple spectrophotometric analysis of

aromaticity (Richards et al., 2001). By adhering to this approach, Macdonald et al. (2002) estimated that the binding affinity of Pb for DOM is approximately 250 times greater than that for the gill of rainbow trout. Thus, there is clear evidence that DOM is likely to significantly limit the bioavailability of Pb to fish in natural waters.

Another important parameter to consider is the effect of water hardness (i.e. Ca and Mg). Changes in hardness can influence Pb speciation through cation competition for binding both inorganic and organic ligands. Most important for toxicity, however, is likely the competition between Ca and Pb for binding at the biotic ligand (e.g. gill). Lead gill accumulation has been shown to decrease with increasing Ca concentrations and, conversely, Pb has been shown to competitively inhibit Ca influx (Macdonald et al., 2002; Rogers and Wood, 2004). Additionally, Pb uptake was inhibited by known voltage-independent Ca channel competitors (La, Cd, and Zn) but not by the voltage-dependent Ca channel blockers, nifedipine and verapamil (Rogers and Wood, 2004). These findings clearly illustrate a competitive Pb-Ca interaction at shared binding sites on the gill and strongly indicate that Pb uptake occurs via a voltage-independent Ca channel. Interestingly, there is evidence of a second low affinity/high capacity population of branchial Pb binding sites demonstrating an apparent non-competitive interaction with Ca (Birceanu et al., 2008; Rogers and Wood, 2004), although the Pb concentrations needed for binding to these sites appear to exceed the range of environmental relevance in most cases (e.g. mg Pb L^{-1} range).

Finally, speciation effects attributed to changes in one parameter are often complicated by concurrent changes in other parameters. For example, hardness often co-varies with alkalinity (as $CaCO_3$) leading to an inherent difficulty in elucidating the

relative protective contributions of each. Additionally, while a low pH will correspond with greater free Pb^{2+} concentrations, the bioavailability and toxicity of Pb may be offset somewhat by the competition provided by increased H^{+} concentrations. Of course, at some point the acidity itself will contribute to toxicity. Nevertheless, any such competing effects of protons, or other cations for that matter, with Pb^{2+} may not be completely offsetting due to differences in the binding affinities of each chemical species for various inorganic, organic and biotic ligands.

1.3. Environmental regulation of lead within the United States.

Under the Clean Water Act, the United States Environmental Protection Agency (USEPA) is charged with establishing national guidelines known as ambient water quality criteria (WQC) that are intended to protect 95% of aquatic taxa at all times from the release of metals and other toxicants to the environment. To efficiently address such a daunting task, the collection of toxicological data spanning a minimum of 8 different taxa are required as a means of extrapolating sufficient protection against wider ecological impacts. Two of the long-standing environmental sentinels commonly employed by the USEPA for these purposes, and the organisms chosen for study within this dissertation, are the fathead minnow, *Pimephales promelas*, and the water flea, or daphnid, *Ceriodaphnia dubia*.

The USEPA is also mandated to periodically reevaluate WQC and adopt new criteria as scientific advances warrant. Establishment of WQC for Pb continues to rely principally on the hardness (i.e. Ca^{2+}) of the receiving water despite growing evidence that other chemical parameters (e.g. pH and DOM as discussed previously), which may

vary greatly on a local basis, also strongly influence the toxicity of Pb (Grosell et al., 2006a; Macdonald et al., 2002). Several examples of current acute and chronic Pb criteria values corresponding to various levels of water hardness are listed in Table 1.4. Efforts to improve WQC for metals have given rise to several toxicity models designed to encompass the influences of all major water chemistry parameters that may influence a metal's toxicity. The most widely accepted of these, the Biotic Ligand Model (BLM), is currently used by the Agency to set WQC for copper (USEPA, 2007). In essence, the BLM accounts for site-specific water conditions by considering both the mitigating effects of competition with other cations (e.g. Ca^{2+}) for binding to the biotic ligand, and speciation effects due to pH or complexation with free anionic species (e.g. HCO_3^-) and DOM that prevent the metal from interacting with the site of toxic action (Paquin et al., 2002). Currently, utility of the BLM is limited to adjustment of acute WQC; however, it is hoped that through additional efforts, such as those reported herein, more sensitive, chronic BLMs will ultimately reach fruition.

Another factor lending uncertainty to the regulatory decision-making process is that metals and other toxicants are commonly present as mixtures in the environment. Genomic approaches are well suited to address such problems, filling in where more conventional methods prove insufficient to pinpoint key environmental stressors or elucidate the contributions and additive effects from multiple toxicants. Furthermore, microarrays provide opportunities not only for establishing the molecular basis of toxicity, but potential for gaining insights into modes of action and higher order effects. Thus, defining toxicant-specific mechanisms that link signature gene expression profiles to chronic effects would greatly aid in monitoring and diagnosing water quality and also

prioritizing higher-tier tests in ecological risk assessment. The significance of genomics in this regard was recently addressed by the USEPA as outlined in the Interim Genomics Policy (Dix et al., 2006).

Finding novel biomarkers of effects capable of predicting chronic outcomes of ecological importance, ideally in a site-specific manner, remains a highly prized, yet still elusive goal for regulators and industry alike. In practical terms, the implementation of genomics within established regulatory and risk assessment frameworks faces many challenges. Regulatory applications will require the demonstration of strong links between molecular responses and effects at multiple higher orders of biological organization (i.e. "phenotypic anchoring") including those endpoints typically measured from traditional toxicity tests (USEPA, 2004). Furthermore, validation with samples from the field will ultimately be required before adoption of microarray data for regulatory applications will gain widespread acceptance.

Ideally, chronic toxicity tests span an organism's full life cycle in order to examine potential reproductive effects. For many species, however, it may take many months to years to reach reproductive maturity obviating considerable time and cost demands for carrying out truly, full-term chronic exposures. To facilitate the process, the USEPA instead allows shorter tests for fish, typically 7d, early-life stage exposures. Although such tests may represent a good compromise by targeting the most sensitive time period of development, subtle effects on reproduction (fecundity) and/or overall fitness (physiology) may go unnoticed. It is possible that fundamental molecular responses to a given toxicant will be widely shared across different taxa. Thus, organisms of lower complexity may potentially offer a more efficient and cost-effective

means for extrapolating chronic adverse effects in other sensitive wildlife species, thereby serving as a powerful tool for quickly and more accurately assessing chronic toxicity (USEPA, 2004). However, for such a tool to become realized will demand the strong correlation of toxic effects observed across various vertebrate and non-vertebrate taxa.

1.4. Scope of research.

This dissertation characterizes the key water chemistry parameters mediating acute toxicity of waterborne Pb to the fathead minnow and *C. dubia*, as well as the chronic toxicity of waterborne Pb to the fathead minnow. Additionally, attempts are made to link molecular responses of sublethal Pb exposure during the early life stages of fathead minnows to chronic outcomes of ecological significance. Chapter 1 (Mager et al., 2010c) addresses the influences of hardness (as $CaSO_4$), DOM (as Aldrich humic acid (HA)) and alkalinity (as $NaHCO_3$) on the acute toxicity of Pb to the fathead minnow and *C. dubia*. Additionally, the influence of pH (5.5-8.3) on the acute and chronic toxicity of Pb to *P. promelas* was evaluated using a novel approach employing an automated titration system to adjust pH while minimizing concurrent changes in the carbonate buffering system. The main goals of Chapter 2 (Mager et al., 2008) were fourfold: (1) examine the influence of Ca^{2+} and DOC on the accumulation and chronic toxicity (as spinal curvature) of waterborne Pb during chronic (150 d) exposures (2) to identify transcriptional responses to chronic Pb exposure that might provide further insight as to the underlying toxic mechanisms of Pb, (3) determine whether such genes could serve as early markers of Pb exposure by assessing whether the transcriptional responses reflect

the influences of water chemistry on Pb accumulation, and (4) examine their potential for serving as indicators of long-term Pb effects as evaluated by spinal curvature. In Chapter 3 (Mager et al., 2010b), chronic (>300 d) Pb exposures examining the influences of alkalinity (as $NaHCO_3$) and DOM (as HA) on the reproductive and behavioral toxicity of Pb to fathead minnows are described. Finally, Chapter 5 (Mager and Grosell, 2010) addresses the ecological relevance of the potential hematological and neurological effects suggested by results obtained in chapters 3 and 4 by examining whether such effects translate to an impairment of fathead minnow swimming performance.

Table 1.1. Worldwide atmospheric Pb emissions in 1983 (metric tons yr^{-1}).

Source	Range (Median Value)
Natural:	
Wind-borne soil particles	300-7,500
Volcanoes	540-6,000
Wild forest fires	60-3,800
Seasalt spray	20-2,800
Biogenic, continental	30-2,500
Biogenic, marine	20-450
Total natural emissions	**970-23,000 (12,000)**
Anthropogenic:	
Mobile sources	248,030
Pb production	11,700-31,200
Cu-Ni production	11,050-22,100
Cement production	18-14,240
Steel & iron manufacturing	1,065-14,200
Zn-Cd production	5,520-11,500
Coal combustion, industry & domestic	990-9,900
Coal combustion, electric utilities	775-4,650
Pb mining	1,700-3,400
Wood combustion	1,200-3,000
Refuse incineration, municipal	1,400-2,800
Oil combustion, industry & domestic	716-2,150
Oil combustion, electric utilities	232-1,740
Secondary non-ferrous metal production	90-1,440
Refuse incineration, sewage sludge	240-300
Phosphate fertilizers	55-274
Miscellaneous	3,900-5,100
Total anthropogenic emissions	**288,700-376,000 (332,350)**
Total Pb emissions	***290,000-399,000 (344,000)***

Data from Nriagu (1989).

Table 1.2. Anthropogenic Pb input to aquatic ecosystems in 1983 (metric tons yr^{-1}).

Source	Range (Median Value)
Atmospheric fallout	87,000-113,000
Metal manufacturing	2,500-22,000
Dumping of sewage sludge	2,900-16,000
Domestic wastewater, central	900-7,200
Smelting & refining, non-ferrous metals	1,000-6,000
Domestic wastewater, non-central	600-4,800
Chemical manufacturing	400-3,000
Smelting & refining, iron & steel	1,400-2,800
Base metal mining & dressing	250-2,500
Steam electric	240-1,200
Pulp & paper manufacturing	10-900
Petroleum product manufacturing	0-120
Total anthropogenic input	**97,000-180,000 (138,000)**

Data from Nriagu and Pacyna (1988).

Table 1.3. Estimated inorganic speciation of Pb in conditions representative of typical natural waters at 25 °C. Two examples are provided for fresh water to illlustrate the influence of pH and alkalinity.

Major Pb Species	FW (pH 6.0)	FW (pH 9.0)	SW (pH 8.2)
	%		
Free	86	< 1	3
CO_3	7	95	41
Cl	1	< 1	47
OH	2	5	9
SO_4	4	< 1	1

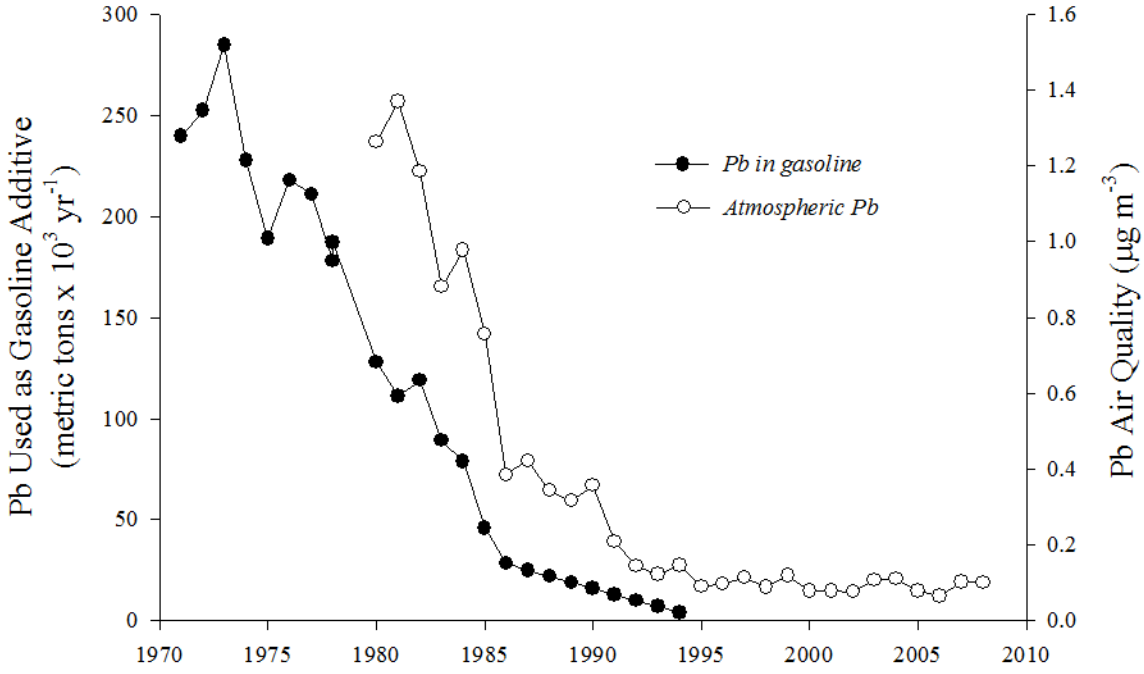
Data from Turner, D.E. et al. (1981).

Abbreviations: FW=freshwater; SW=seawater

Table 1.4. USEPA recommended ambient water quality criteria for lead calculated for various levels of water hardness.

Hardness (mg L^{-1})	Acute (µg L^{-1})	Chronic (µg L^{-1})
20	10.8	0.4
50	30.1	1.2
200	136	5.3

Figure 1.1. Parallel trends in improved air quality within the United States and the use of leaded gasoline following enactment of the Clean Air Act in 1970. *Source*: USEPA (1986 and 2010).



CHAPTER 2.

INFLUENCES OF WATER CHEMISTRY ON THE ACUTE TOXICITY OF LEAD TO *PIMEPHALES PROMELAS* AND *CERIODAPHNIA DUBIA*

2.1. Summary.

The acute toxicity of lead (Pb) was examined for fathead minnows (*Pimephales promelas*; 96-h) and daphnids (*Ceriodaphnia dubia*; 48-h) in waters modified for hardness (as $CaSO_4$), dissolved organic carbon (DOC; as Aldrich humic acid) and alkalinity (as $NaHCO_3$) for parameterization of an acute freshwater biotic ligand model (BLM). Additionally, acute (96-h) and chronic (30-d) bioassays were performed for *P. promelas* to more clearly define the influence of pH (5.5-8.3) on Pb toxicity as modified by addition of HCl or NaOH using an automated titration system. Results indicate that Ca^{2+} is protective against acute Pb toxicity to *P. promelas* but not *C. dubia*. Strong protection was afforded by DOC and $NaHCO_3$ against acute Pb toxicity to *P. promelas* whereas milder protection was observed for *C. dubia* with both parameters. Dissolved Pb LC50s from the *P. promelas* pH bioassays revealed a complex effect of pH on Pb toxicity that can likely be explained by Pb speciation and the competitive interaction of H^+ with ionic Pb^{2+}. Chronic pH bioassays also demonstrated that 30-d growth is not impaired in fathead minnows at relevant Pb concentrations. The findings reported herein suggest that development of separate BLMs for *P. promelas* and *C. dubia* should be considered.

2.2. Background.

Lead (Pb) is a nonessential metal that has gained much notoriety from its past use as an additive in gasoline and from Pb-based paints. Although such applications were phased out beginning in the 1970s in the United States, Pb remains an environmental concern today primarily as a consequence of anthropogenic sources such as those related to Pb mining and industrial processing (World Health Organization, 1995). Establishing safe environmental regulations for Pb, as with other metals, is a challenging endeavor due to natural variability in receiving water chemistry that can differentially impact its chemical speciation, and therefore bioavailability and toxicity. Factors important in determining metal toxicity include pH, concentration and quality of dissolved organic carbon (DOC), water hardness (Ca^{2+}, Mg^{2+}) and inorganic complexing agents (e.g. carbonates, sulfides, and chlorides) (Pagenkopf, 1983; Richards et al., 2001). Until recently, however, the USEPA has only recommended site-specific adjustments to water quality criteria (WQC) based on water hardness for several trace metals including Pb.

The biotic ligand model (BLM) represents a culmination of multiple efforts aimed at addressing all of the major water chemistry parameters that may influence a metal's toxicity. Under an assumption of chemical equilibrium, the BLM accounts for the effects of cations competing with a metal for binding at a biotic ligand and complexation with DOC or other inorganic species that render it unavailable for uptake (Paquin et al., 2002). Because of its flexibility and strong potential for application to many metals and a variety of test organisms, a BLM-based approach to establishing environmental standards for metals is being pursued in many countries. At present, a freshwater BLM has been implemented only for copper (Cu) within the United States (USEPA, 2007) and regional

risk assessments in Europe have employed BLMs for metals such as Cu, nickel and zinc (Bodar et al., 2005). Additional BLMs for other high priority metals such as aluminum, cobalt, iron, silver and Pb are likely to follow in the coming years as each are currently in various stages of development.

At a minimum, data sets for BLM development should include multiple measurements of the same toxicological endpoint (e.g. LC50) and well characterized water chemistry over a range of relevant conditions. Furthermore, sensitive test organisms should be used for toxicity testing if the results are to have ecological relevance. The fathead minnow (*Pimephales promelas*) and daphnid (*Ceriodaphnia dubia*) are two of the most commonly used test organisms for establishing WQC and both have documented sensitivity to Pb (Grosell et al., 2006a; Mager et al., 2010a; Mager et al., 2010b; Mager et al., 2008; Schubauer-Berigan et al., 1993; Spehar and Fiandt, 1986). Specifically, our lab recently investigated the influences of water chemistry on chronic Pb toxicity to fathead minnows during 30, 150 and 300-d flow-through exposures (Grosell et al., 2006a; Mager et al., 2010b; Mager et al., 2008) and to *C. dubia* during standard 7-d reproductive tests (Mager et al., 2010a). The 30-d fathead minnow studies allowed for derivation of 96-h LC50s, but obtaining acute effect levels in this manner is in contrast to the established test protocol for acute toxicity studies (Stephan et al., 1985) as the fish were fed daily throughout. Additionally, Schubaer-Berigan and colleagues (1993) investigated the importance of pH to acute Pb toxicity in very hard water to several freshwater organisms including fathead minnows and *C. dubia*. From these studies, as well as others using rainbow trout and common carp (Davies et al., 1976; Hodson et al., 1978b; Macdonald et al., 2002; Stouthart et al., 1994), it has become

evident that Ca^{2+}, DOC, pH and alkalinity are the primary parameters influencing Pb toxicity. Still, a comprehensive analysis of acute Pb toxicity sufficient for BLM parameterization has yet to reach fruition for either of these organisms.

We therefore undertook the present study with the specific goal of obtaining a data set for parameterization of an acute freshwater BLM for Pb. To this end, we evaluated acute Pb toxicity to fathead minnows and *C. dubia* using standard bioassays adjusted for parameters known to influence Pb toxicity (Ca^{2+}, DOC, pH and alkalinity). As part of our recent studies (Grosell et al., 2006a; Mager et al., 2010a) it was shown that pH manipulations using organic buffers (MOPS) are potentially problematic as the buffers themselves may alter the physiology of the test organisms thereby rendering them more sensitive to Pb regardless of the pH. These included 30-d fathead minnow tests performed at pH 6.3 and 8.3 (Grosell et al., 2006a). Thus, to more effectively assess the influence of pH on Pb toxicity to fathead minnows we conducted both acute and chronic (30-d) pH bioassays using an automated titration system. Finally, to evaluate whether 96-h LC50s estimated from our earlier 30-d chronic studies where fish were fed could be used in acute BLM parameterization, an additional bioassay was performed in which fathead minnows were fed daily throughout acute (96 h) Pb exposure.

2.3. Materials and methods.

2.3.1. Experimental design.

Acute Pb toxicity was evaluated for larval fathead minnows and *C. dubia* neonates using 96-h flow-through and 48-h static renewal survival tests, respectively, performed in accordance with standard USEPA guidelines (USEPA, 2002). To examine

the influence of feeding during fathead minnow acute toxicity tests, two bioassays were first performed in the base water, one in which fish were fed daily and another in which fish were not fed. As feeding was shown to potentially influence Pb toxicity (see *Section 2.4.1*), all subsequent acute tests were conducted without feeding. For both organisms, water chemistry was manipulated to investigate the influence of Ca^{2+} (as $CaSO_4$), DOC (as Aldrich humic acid (HA)), and alkalinity (as $NaHCO_3$) on acute Pb toxicity (as $PbNO_3$). A NaCl test was also performed for each organism to distinguish any potential protective contribution from the Na^+ component of the $NaHCO_3$ salt. Additionally, acute and chronic (30-d) pH tests were performed for fathead minnows as described below. The complete lists of tests are provided in Tables 2.1. and 2.2. All tests included a treatment-matched control without Pb. Both test organisms were purchased from Aquatic BioSystems, Inc. (Fort Collins, CO). Fathead minnows were <24 h post-hatch on arrival, and an in-house culture of *C. dubia* was maintained to provide <24 h old neonates for testing. All chemicals used for modifying test waters were obtained from Sigma–Aldrich (St. Louis, MO).

2.3.2. Toxicity tests – Pimephales promelas.

Acute (96-h) and chronic (30-d) bioassays were administered with a base water of 2:1 deionized water:dechlorinated Virginia Key tap water using a gravity flow-through approach as previously described (Grosell et al., 2006a). Except for pH, water chemistry and Pb concentrations were adjusted by a constant drip of concentrated stock solution delivered by Mariotte bottle or peristaltic pump (2.5 mM $CaSO_4$). Addition of Pb was initiated at least 24 h prior to the introduction of fish to allow for equilibration. To

modify pH, an automated titration system was used to more accurately assess the influence of pH and to minimize concurrent changes in the carbonate buffering system. In brief, a MasterFlex peristaltic pump automated through a pump controller (Cole-Parmer, Vernon Hills, IL) with feedback from a pH electrode (sc-100, Hach Co., Loveland, CO) in the primary mixing chamber was used to maintain constant pH values targeting 5.5, 6.4, and 8.3 by addition of HCl (pH ≤6.4) or NaOH (pH 8.3). The pH of the exposure chambers was assessed independently using a combination glass electrode coupled to a PHM201 pH meter (Radiometer, Copenhagen, Denmark) which was calibrated daily using IUPAC standards (Radiometer, Copenhagen, Denmark). The entire system was allowed to operate for at least 3 days prior to the introduction of animals to ensure pH stabilization. An additional base water control test of pH 7.5, unmodified by acid/base addition, was performed alongside the first of these tests (pH 6.4).

Fish were fed *Artemia* nauplii daily (*ad libitum*) and gradually acclimated to water chemistry for 7 d prior to Pb exposures and were thus 8 days old at the onset of toxicity testing. Acclimation to test waters is important as it has been demonstrated to affect gill-metal binding in fathead minnows (Bielmyer et al., 2008). For the acute bioassays, food was withheld 24 h before and during Pb exposures except for the test examining the effect of feeding in which case fish were fed daily. Chronic bioassays to examine the effect of pH on Pb toxicity were performed as for the acute tests except that in all cases fish were fed daily and on the final day of exposure all surviving fish were collected and weighed to analyze potential growth effects. Initial sample sizes comprised a minimum of 10 larvae per each of 3, 1 L plastic beakers per Pb concentration. Mortality was

recorded and uneaten food and fecal matter was siphoned from beakers daily. All procedures were approved by the University of Miami Animal Care and Use Committee.

2.3.3. Toxicity tests – Ceriodaphnia dubia.

Test waters for 48-h *C. dubia* bioassays were supplemented with 1 µg/L selenium and initially prepared with 2:1 dechlorinated Virginia Key tap water:deionized water. However, as it became difficult to maintain cultures under these low ionic strength conditions later tests were prepared with full strength dechlorinated tap water. Control tests were performed for both ionic strength base waters. Additionally, because the switch in base water occurred in the middle of the HA series, duplicate tests were performed for the 8 mg/L HA treatment using each of the different base waters. Cultures were allowed to acclimate to test waters for 5-7 days prior to test initiation with <24 h old neonates. Lead was added to aliquots of test water the day before testing to allow for equilibration. For each bioassay, 5 replicate 30 ml polypropylene cups each containing 20 ml of test solution and 5 *C. dubia* neonates were used for each Pb treatment. All mass cultures and bioassays were maintained in a controlled environmental chamber at 25°C.

2.3.4. Water chemistry.

For the acute fathead minnow tests, dissolved Pb was measured daily and general water chemistry was measured at least twice during the 96-h exposure period. For the chronic tests, dissolved Pb was measured daily for the first 4-5 days and then at least twice per week thereafter and general water chemistry was typically measured once per week (n=3-4). For the *C. dubia* bioassays, Pb concentrations and general water chemistry

were measured on d 0 and day 2. A Varian 200Z graphite furnace (Australia) was used to measure dissolved Pb concentrations by atomic absorption spectroscopy. Samples were first filtered through a 0.45 μm syringe filter (Pall LifeSciences, MI) and acidified to 1% HNO_3 (trace metal grade, Fisher Scientific, PA). Concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+} were determined by flame atomic absorption spectroscopy (Varian 220FS, Australia) and Cl^- and SO_4^{2-} concentrations were measured by anion chromatography (DIONEX DX120, CA). Total CO_2 (dissolved inorganic carbon) was measured using a Corning 962 carbon dioxide analyzer (UK) except for pH and $NaHCO_3$ treatments which were measured by manual double endpoint titrations using a 2 mL Gilmont micrometer burette (Cole-Parmer, Vernon Hills, IL). The fine-scale manual fluid dispense (±0.5% accuracy) by the latter allowed for higher resolution measurements over those obtained by the CO_2 analyzer. High temperature catalytic oxidations using a Shimadzu total organic carbon-VCSH analyzer (Kyoto, Japan) were used to measure dissolved organic carbon (DOC) concentrations (Hansell and Carlson, 2001).

2.3.5. Calculations and statistical analyses.

All statistical analyses were performed according to USEPA guidelines using ToxCalc v. 5.0 (Tidepool Scientific Software). LC50s were estimated using Maximum Likelihood-Probit (preferred), Trimmed Spearman-Karber or Maximum Likelihood-Logit analyses. The LC20s and LC10s for all chronic tests were estimated using Linear Interpolation (survival was more sensitive than growth in all tests). See Tables 2.3.-2.5. for the exact statistical method used for each test. Calculations of Pb speciation were performed using the Biotic Ligand Model v. 2.3.3 (HydroQual, Inc.;

http://www.hydroqual.com/wr_blm.html). The BLM input for %HA was set at 10% except for tests in which HA was added. For the HA tests, the %HA values were estimated proportionally relative to the base water control by assuming all additional DOC was attributable to 100% HA. Measured values from the respective tests were used for all other parameters, except when unavailable, in which cases measured control values were used.

2.4. Results and discussion.

2.4.1. Acute lead toxicity to P. promelas – influence of feeding.

A comparison of LC50s from the base water tests during which fish were either fed or unfed initially suggested that providing food during Pb exposure affords significant (2.5 fold) protection against acute Pb toxicity (Table 2.3.). This result indicated that 96-h LC50s estimated from our previous 30-d chronic studies (Grosell et al., 2006a) during which fish were fed may be inappropriate for acute BLM parameterization. However, it should be noted that the present acute fed and unfed bioassays were conducted at different times and that the water chemistry varied between tests. Specifically, the base water in the fed bioassay had proportionately higher concentrations (typically 10-20%) in all water chemistry parameters measured except K^+ and pH than in the unfed bioassay (Table 2.1.). Thus, some of the protection during the feeding tests may have been due to other factors (e.g. Ca^{2+} and DOC) particularly in light of results from the other acute bioassays described below. Nonetheless, to eliminate any potential influence of feeding and to ensure adherence to USEPA standards food was withheld during all subsequent bioassays.

Further insight into the effect of feeding was gained upon completion of the acute and chronic pH bioassays, as fish were fed during the latter but not the former, and 96-h LC50s were calculated for each. It should be noted that these were the last of all tests performed, and therefore were unavailable for our initial assessment of a feeding effect. Comparing the LC50s from these two data sets suggested that there were no consistent trends supporting an influence of feeding on acute Pb toxicity (Tables 2.3. and 2.4.). These findings further indicate that the apparent feeding effect observed from the initial bioassays was likely due to differences in water chemistry as opposed to feeding. However, as mentioned previously, the fact that fish were not fed during the subsequent acute bioassays evaluating the influences of water chemistry eliminates any concerns for future regulatory adoption of a Pb BLM based on the results reported herein.

2.4.2. Acute lead toxicity to P. promelas and C. dubia – influences of calcium, DOC and alkalinity.

Water chemistry measurements and Pb toxicity results for *P. promelas* and *C. dubia* bioassays are summarized in Tables 2.1.-2.5. For the fathead minnow tests, measured Ca^{2+} concentrations were consistently lower than targeted nominal concentrations (including two attempts at 1.5 mM) due to the inherent difficulty associated with the solubility limit of $CaSO_4$ in water and therefore large stock solution volumes needed to adjust concentrations during flow-through exposures. An exception was the 2.5 mM $CaSO_4$ test for which delivery by peristaltic pump rather than Mariotte bottle helped address this issue. In any event, a range of concentrations useful for evaluation of a Ca^{2+} effect was still achieved. Ambient Ca^{2+} demonstrated a clear

protective effect against acute Pb toxicity to fathead minnows exhibiting an apparent saturation pattern as would be expected for a competitive inhibitor of Pb uptake (Figure 2.1.). Water samples from the 0.5 and 1.0 mM nominal $CaSO_4$ tests for *C. dubia* were lost prior to completion of water chemistry analysis, although Ca^{2+} was one of the parameters measured prior to loss. Nonetheless, it is clear that unlike for fathead minnows there was no appreciable effect of ambient Ca^{2+} on acute Pb toxicity to *C. dubia* (Figure 2.1.).

The results for fathead minnows and *C. dubia* with respect to Ca^{2+} suggest different and potentially complex interactions between water chemistry and the organisms. It has been shown in rainbow trout that at relatively high Pb concentrations (as used in acute fathead minnow tests) Pb enters via a Ca^{2+} channel and that competition between Ca^{2+} and Pb^{2+} for uptake at this site results in reduced Pb bioavailability (Rogers et al., 2004). This mechanism is supported by the Pb speciation data as ionic Pb^{2+} concentrations at the dissolved Pb LC50 increase with increasing Ca^{2+} concentrations suggesting that Pb^{2+} and Ca^{2+} are indeed competing for binding to the biotic ligand (Table 2.6.). Conversely, this trend was not well supported for *C. dubia* (Table 2.7.). Thus, at the lower Pb concentrations used in the *C. dubia* tests, Pb^{2+} may be entering by another mechanism, for example through a high affinity divalent metal transporter (DMT1) that has low affinity for Ca^{2+} (Bury and Grosell, 2003; Gunshin et al., 1997). As a result, competitive interactions between Ca^{2+} and Pb^{2+} are possibly minimized under these conditions and Ca^{2+} does therefore not reduce Pb toxicity. Interestingly, Komjarova and Blust (Komjarova and Blust, 2009a, b) used a stable Pb isotope to show that Pb uptake rates in *Danio rerio* and *Daphnia magna* were both inhibited in water with

2.5 mM Ca^{2+}, but not in water with 0.5 mM Ca^{2+}. These experiments were performed at a low Pb concentration (5 µg/L dissolved Pb), comparable to exposure levels in the *C. dubia* toxicity tests, and thus suggest that the lack of Ca^{2+} protection at low Pb concentrations may not apply uniformly or that Pb accumulation and acute toxicity are not strictly linked. Additional study of this issue is clearly needed.

Dissolved organic carbon (added as HA) offered robust protection against acute Pb toxicity for both *C. dubia* and fathead minnows (Tables 2.3. and 2.5.). However, linear regressions of the LC50s indicate that HA affords stronger protection for fathead minnows than for *C. dubia* as evident by a higher slope for the former (Figure 2.2.). Interestingly, the response of *C. dubia* to increased DOC may actually be sigmoidal in contrast to the clearly linear response of fathead minnows. The underlying mechanism for these different response relationships is unclear at this time.

Recent studies have shown that the effect of DOC on metal toxicity is not strictly the result of complexation with bioavailable metal species. Additionally, DOC interacts directly with exposed respiratory surfaces leading to alterations of epithelial potentials in fish (Galvez et al., 2008). Specific to daphnids, it has been shown that DOC as HA stimulates Na^+ uptake and that this stimulation is dependent on both ambient Ca^{2+} and pH (Glover et al., 2005; Glover and Wood, 2005). This stimulatory action is believed to be the result of the same epithelial hyperpolarization that occurs in fish, as daphnids possibly take up Na^+ via an electrogenic $2Na^+$:H^+ exchanger (Bianchini and Wood, 2008). An interaction between Pb^{2+} and Ca^{2+} might be involved because Ca^{2+} can substitute for Na^+ at this exchanger (Ahearn et al., 2001). Hence, the interactions among DOC, Ca^{2+}, and Na^+ homeostasis in daphnids is likely to be quite different from that observed in fish

which lack this transporter. These differences may explain the observed discrepancies in DOC protection against Pb toxicity in fish versus daphnids, although the complexity of the interactions between these parameters remains to be fully characterized.

Alkalinity adjusted by $NaHCO_3$ addition also afforded protection against acute Pb toxicity, but as for the DOC effect, it appears that alkalinity adjustments are more potent in altering acute Pb toxicity for fathead minnows than for *C. dubia* (Tables 2.3. and 2.5.). Acute toxicity thresholds could not be determined for fathead minnows at total CO_2 concentrations above approximately 1 mM due to lack of mortality. This lack of Pb-induced mortality was a product of, on one hand the protective effect of alkalinity (as HCO_3^-), and on the other hand the influence of alkalinity on Pb solubility. At high total CO_2 concentrations, obtaining enough Pb in solution to cause mortality was not possible. It should be noted that alkalinity (as $NaHCO_3$) adjustments automatically result in pH changes and that alkalinity (total CO_2) and pH therefore co-vary. Furthermore, no effects of NaCl addition were observed confirming that effects from $NaHCO_3$ additions were due to HCO_3^- and associated pH changes rather than Na^+.

2.4.3. Acute and chronic lead toxicity to P. promelas – influence of pH.

2.4.3.1. 96-h mortality.

To more accurately assess the direct influence of pH on Pb toxicity to fathead minnows, acute and chronic toxicity tests were performed at four different pH values (5.5, 6.4, 7.5 and 8.3) using an automated titration system. Measured pH values were stable and within ~0.1 unit of our target nominal values in all cases (Table 2.1.). Comparing total CO_2 concentrations from the pH series with those from the $NaHCO_3$

series confirms this approach allowed for a more independent analysis of pH without concurrent alkalinity adjustments owing to changes in carbonate/bicarbonate concentrations. This is well illustrated by comparing the 2.0 mM nominal $NaHCO_3$ test with the pH 8.3 acute and chronic tests. Mean total CO_2 concentration during the $NaHCO_3$ test was 1662 μM compared to 667 μM and 798 μM for the acute and chronic pH tests, respectively, despite a pH range of only 8.22 – 8.30 across the 3 tests (Table 2.1.). Thus, while changes in alkalinity due to equilibration with atmospheric CO_2 could not be eliminated, the effect appeared to be minimized.

To the best of our knowledge, this is the first analysis of the influence of pH on Pb toxicity to fathead minnows using HCl and NaOH addition during a flow-through exposure. Schubauer-Berigan et al. (1993) used static exposures to fathead minnows performed by adjusting the pH of very hard water (300-320 mg/L $CaCO_3$) by addition of HCl and capping exposure chambers to control pH fluctuations. Their results demonstrated increased Pb toxicity at low pH (6.3), but distinct LC50s could not be obtained at pH 7.3 and 8.3 likely owing to the high alkalinity and hardness of the test waters, which can influence Pb solubility. Stouthart et al. (1994) observed a similar influence of low pH (5.6) on Pb toxicity to the egg and larval stages of common carp (*Cyprinus carpio*) using a pH-stat flow-through system. While our findings were consistent with an increase in Pb toxicity at low pH, a clear relationship between 96-h dissolved Pb LC50s and pH was not immediately apparent (Tables 2.3. and 2.4.). However, a closer analysis of the speciation data for all pH tests helps to explain the observed effects. Figure 2.3A. illustrates a plot of 96-h ionic Pb^{2+} LC50s as a function of H^+ concentration using data from both acute and chronic pH bioassays (Tables 2.6. and

2.8.). A strong Michaelis-Menten type (i.e., competitive) interaction suggests that the H^+ concentration is the likely reason why, despite a nearly 10-fold increase in Pb^{2+} concentration from pH 8.3 to pH 5.5, there is only an approximately 2 to 4-fold increase in dissolved Pb LC50s. By performing negative log transformations, the same data can be presented alternatively as a plot of the pPb^{2+} LC50 as a function of pH (Figure 2.3B.). From these analyses it appears that the influence of pH on Pb toxicity in the present study can be largely explained as a function of both Pb speciation and the competition between H^+ and Pb^{2+} for binding at the gill.

2.4.3.2. 30-d mortality and growth.

Detailed mortality profiles for each of the 30-d bioassays are shown in Figure 2.4. Chronic toxicity could not be assessed at pH 5.5 as complete mortality in the control treatment had occurred by day 9. This is likely because fathead minnows cannot survive low pH water for prolonged periods; an observation that is supported by a previous study which documented complete mortality in 15 d of older (and thus presumably less sensitive) fathead minnows subjected to pH 4.8 (Leino et al., 1987). As in previous studies (Grosell et al., 2006a), most of the mortality in the remaining three tests occurred within the first 10 d of Pb exposure with the exception of two concentrations in the pH 6.4 test. Body masses were measured for the surviving fish at the end of 30 d revealing no discernable Pb effects on growth in all but one of the chronic pH tests. In the lone case in which Pb appeared to reduce growth (pH 8.3 at 434 μg/L), only 2 surviving fish were available for comparison with the control (Table 2.9.). However, since the concentration at which this apparent growth inhibition occurred was well in excess of the

LC20, the evidence indicates that chronic Pb exposure has little effect on fish growth at concentrations sufficient to result in mortality.

2.5. Conclusions.

Based on the above, it seems likely that different BLMs will be required to ensure adequate environmental protection of both species. While differences in sensitivity to metals among organisms are common and have been dealt with by calibrating BLMs to toxicity data for metals such as Cu, such an approach will likely be insufficient for Pb. Rather, since it appears that water chemistry parameters which protect fathead minnows against Pb are either less effective or without effect on *C. dubia*, particularly with respect to the role of Ca^{2+}, different BLMs (or other model predictions) taking these differences into account will need to be considered. Finally, it is clear that the influences of pH and alkalinity on Pb toxicity to fathead minnows are complex and that controlling alkalinity and pH separately is not trivial, but fortunately these parameters tend to co-vary in nature. Nevertheless, the findings reported herein should help elucidate the respective significance of each, as well as those for other key parameters such as DOC and Ca^{2+}, for the purposes of BLM parameterization.

Table 2.1. Water quality data for acute and chronic *P. promelas* bioassays (mean±SEM). The ranges of dissolved Pb test concentrations (excluding no Pb controls) are provide in the last column.

	Concentration (in µM)							DOC	Hardness		Temp.	[Pb] Test Range
Test	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	Total CO_2	(µmol C/L)	(mg/L)	pH	(°C)	(µg/L)
Acute (96 h)												
Unfed Base H_2O	519±6	25±0	131±3	54±0	431±3	40±1	182±24	79±2	19±0	7.58±0.04	22.7±0.2	49-901
Fed Base H_2O	579±10	25±0	159±4	60±1	511±2	45±1	267±7	88±2	22±1	7.43±0.07	23.1±0.2	58-838
0.5 mM $CaSO_4$	624±8	26±0	258±3	62±1	567±6	153±1	236±24	97±2	33±1	7.46±0.05	24.4±0.2	103-661
1.0 mM $CaSO_4$	643±2	25±0	445±6	70±1	590±7	334±8	202±3	94±3	53±1	7.55±0.02	23.6±0.2	300-1993
1.5 mM $CaSO_4$ A	658±4	25±0	590±13	68±2	616±4	507±10	192±9	90±2	67±2	7.42±0.03	24.5±0.2	580-4154
1.5 mM $CaSO_4$ B	962±28	27±3	550±46	95±1	732±31	377±15	422±27	95±1	66±5	7.71±0.09	19.0±0	460-3438
2.5 mM $CaSO_4$	633±9	20±0	2950±132	62±2	462±10	2019±69	265±21	88±7	309±14	7.47±0.04	17.5±0.5	1019-8031
2 mg/L HA	600±2	26±0	137±2	59±1	598±4	54±1	167±27	115±1	20±0	7.61±0.03	23.8±0.1	177-1158
4 mg/L HA	605±9	25±0	154±10	60±2	606±4	52±1	185±15	138±5	22±1	7.59±0.04	24.0±0.2	109-1252
8 mg/L HA	616±3	26±0	152±4	63±1	609±4	52±1	176±29	216±6	22±1	7.51±0.09	24.9±0.1	231-3671
16 mg/L HA	630±7	26±0	148±2	61±1	618±2	55±1	248±6	420±23	21±0	7.61±0.02	24.6±0.2	884-6481
0.5 mM $NaHCO_3$	853±10	28.±1	157±9	66±3	736±7	63±1	428±14	88±1	23±1	7.52±0.05	22.2±0.2	145-1929
1.0 mM $NaHCO_3$	1089±15	28±0	160±5	66±1	722±3	63±1	744±19	91±1	23±1	7.87±0.01	22.1±0.4	173-1679
1.5 mM $NaHCO_3$	1353±8	33±0	149±5	70±1	592±57	42±1	1002±28	96±0	22±0	7.99±0.01	22.3±0.1	636-1859
2.0 mM $NaHCO_3$	2073±21	29±0	156±8	68±2	746±1	64±1	1662±44	93±1	23±1	8.30±0.03	21.5±0	665-1210
1.5 mM NaCl	1361±30	29±1	165±10	69±4	1956±9	62±1	258±25	92±3	24±1	7.50±0.02	22.5±0.1	58-1069
pH 5.5	716±19	12±0	218±10	60±1	1473±458	52±2	21±15	134±1	28±1	5.35±0.07	19.8±0.3	60-1109
pH 6.4	561±9	10±0	226±15	61±1	903±8	41±2	24±12	118±1	29±2	6.34±0.04	20.5±0	69-1207
pH 7.5	579±6	10±0	234±14	63±1	415±7	43±0	514±58	104±2	30±2	7.50±0.02	20.5±0	86-1723
pH 8.3	1050±114	10±0	186±5	60±2	577±16	48±1	667±25	123±1	25±1	8.26±0.06	20.0±0	231-1949
Chronic (30 d)												
pH 5.5	718±6	11±0	257±48	66±5	1443±135	55±1	19±13	136±13	21.7±5.3	5.64±0.11	20.5±0.5	30-447
pH 6.4	555±12	9±0	203±27	53±1	934±57	42±1	49±16	115±4	25.7±2.8	6.41±0.03	20.2±0.4	29-1348
pH 7.5	569±16	11±0	202±29	54±1	698±92	47±2	543±69	108±4	25.6±2.8	7.50±0.03	20.2±0.4	32-1828
pH 8.3	1215±133	13±0	180±12	58±2	648±35	60±3	798±66	123±5	23.8±1.1	8.22±0.03	18.3±0.3	25-1049

Table 2.2. Water quality data for acute *C. dubia* bioassays (mean±SEM). The ranges of dissolved Pb test concentrations (excluding no Pb controls) are provided in the last column.

			Concentration (in µM)					DOC	Hardness		Temp	[Pb] Test Range
Test	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	Total CO_2	(µmol C/L)	(mg/L)	pH	(°C)	(µg/L)
Base water A	1260±79	64±4	315±22	146±14	1063±78	85±5	506±23	213±27	47±4	7.58±0.06	25	180-907
Base water B	2369±103	109±5	828±77	309±18	2120±110	205±12	1085±59	334±16	116±10	8.01±0.01	25	107-544
0.5 mM CaSO4	735±20	50±3	614±39	130	1110	90	587	242	76	7.44	25	355-1851
1.0 mM CaSO4	792±30	51±9	1088±23	130	1110	90	587	242	125	7.44	25	261-1707
1.5 mM CaSO4	1272±11	60±1	1653±4	130±1	964±8	1037±1	489±12	282	183±1	7.44±0.01	25	164-523
2.0 mM CaSO4	1411±128	67±1	2302±9	142±1	1078±8	1528±18	555±23	321	250±1	7.43±0.02	25	157-440
2 mg/L Humic	1179±78	58±1	256±17	149±1	887±7	63±3	714±20	228	41±2	7.80±0.01	25	219-1303
4 mg/L Humic	1216±97	66±1	361±2	170±1	1012±26	75±11	793±23	266	54±1	7.80±0.01	25	491-1695
8 mg/L Humic A	1256±164	55±1	221±1	134±1	861±5	63±14	621±25	171	36±1	7.49±0.01	25	203-1569
8 mg/L Humic B	2477±250	110±10	701±46	297±23	2198±193	204±14	1125±99	415±21	102±7	7.89±0.01	25	101-802
32 mg/L Humic	2535±193	110±11	673±59	302±34	2152±209	215±29	1184±118	897±32	99±9	8.15±0.01	25	239-1439
64 mg/L Humic	4034±167	282±11	796±28	242±12	3391±268	368±29	1250±66	1298±41	106±4	8.15±0.02	25	271-5026
0.5 mM NaHCO3	2168±193	74±1	400±2	104±2	1385±5	133±1	857±13	214±12	51±1	7.96±0.11	25	279-1344
1.0 mM NaHCO3	2823±76	74±2	396±7	104±3	1485±52	96±2	1326±50	233±7	51±1	8.19±0.02	25	416-1547
1.5 mM NaHCO3	2937±209	69±6	295±20	142±11	1445±132	108±8	1715±137	228±33	44±3	8.06±0.04	25	389-1876
2.0 mM NaHCO3	2944±99	58±2	289±10	105±3	928±41	70±2	2175±87	199±13	40±1	8.23±0.04	25	355-1890
1.5 mM NaCl	2164±179	59±3	216±45	106±5	2066±183	119±19	428±51	216±30	33±5	7.48±0.03	25	345-1732

Notes: values in italics were not measured due to loss of samples but were estimated from averages of measurements on comparable test solutions for speciation calculations. All regressions were tested using Maximum Likelihood-Probit.

Table 2.3. 96-h LC50s (95% CI) for acute *P. promelas* bioassays.

Test	[µg/L Pb]	[nmol/L Pb]
Unfed Base H_2O	178 (151-209)	859 (729-1009)
Fed Base H_2O	439 (214-961)	2119 (1033-4638)
0.5 mM $CaSO_4$	744 (629-1031)	3591 (3036-4976)
1.0 mM $CaSO_4$	1015 (910-1111)	4899 (4392-5362)
1.5 mM $CaSO_4$ A	1068 (919-1227)	5154 (4435-5922)
1.5 mM $CaSO_4$ B	1148 (962-1342)	5541 (4643-6477)
2.5 mM $CaSO_4$	1719 (1487-1948)	8296 (7177-9402)
2 mg/L HA	608 (553-662)	2934 (2669-3195)
4 mg/L HA	1075 (973-1219)	5188 (4696-5883)
8 mg/L HA	1356 (1162-1534)	6544 (5608-7403)
16 mg/L HA	3249 (3004-3500)	15681 (14498-16892)
0.5 mM $NaHCO_3$	816 (713-927)	3938 (3441-4474)
1.0 mM $NaHCO_3$	996 (864-1155)	4807 (4170-5574)
1.5 mM $NaHCO_3$	698 (340-905)[a]	3369 (1641-4368)[a]
2.0 mM $NaHCO_3$	N/A[c]	N/A[c]
1.5 mM NaCl	370 (130-1067)	1786 (627-5150)
pH 5.5	162 (118-224)[b]	782 (569-1081)[b]
pH 6.4	265 (149-416)	1279 (719-2008)
pH 7.5	624 (444-891)	3012 (2143-4300)
pH 8.3	340 (282-410)[b]	1641 (1361-1979)[b]

[a-b]All LC50s were obtained using Maximum Likelihood-Probit unless otherwise indicated as follows: [a]Maximum Likelihood-Logit; [b]Trimmed Spearman-Karber.
[c]Sufficient mortality needed to obtain an LC50 was unachievable likely due to precipitation of Pb carbonates.

Table 2.4. Acute and chronic toxicity data for *P. promelas* from 30-d pH bioassays.

	96-h LC50 (95% CI)		30-d LC20 (95% CI)		30-d LC10 (95% CI)	
Test	[µg/L Pb]	[nmol/L Pb]	[µg/L Pb]	[nmol/L Pb]	[µg/L Pb]	[nmol/L Pb]
pH 5.5	188 (181-195)[a]	907 (874-941)[a]	N/A[b]	N/A[b]	N/A[b]	N/A[b]
pH 6.4	169 (149-192)[a]	816 (719-927)[a]	41 (37-48)	198 (179-232)	33 (29-38)	159 (140-184)
pH 7.5	293 (124-331)	1414 (598-1597)	74 (4.6-128)	357 (22-618)	44 (0-81)	212 (0-391)
pH 8.3	790 (651-1022)	3813 (3142-4932)	149 (8.5-243)	719 (41-1173)	99 (4.1-181)	478 (20-874)

[a]LC50 was obtained using Trimmed Spearman-Karber. All other LC50s were obtained using Maximum Likelihood-Probit; LC20s and LC10s were obtained using Linear Interpolation.
[b]All fish had perished prior to completion of 30-d test.

Table 2.5. 48-h LC50s (95% CI) for acute *C. dubia* bioassays.

Test	[µg/L Pb]	[nmol/L Pb]
Base water A	395 (368-416)	1906 (1776-2008)
Base water B	387 (336-418)	1868 (1622-2017)
0.5 mM $CaSO_4$	433 (375-498)	2090 (1810-2403)
1.0 mM $CaSO_4$	597 (503-682)	2881 (2428-3292)
1.5 mM $CaSO_4$	385 (348-432)	1858 (1680-2085)
2.0 mM $CaSO_4$	319 (285-358)	1540 (1375-1728)
2 mg/L Humic	425 (337-507)	2051 (1626-2447)
4 mg/L Humic	546 (441-631)	2635 (2128-3045)
8 mg/L Humic A	591 (461-682)	2852 (2225-3292)
8 mg/L Humic B	532 (473-604)	2568 (2283-2915)
32 mg/L Humic	964 (376-2606)	4653 (1815-12577)
64 mg/L Humic	3116 (2457-3611)	15039 (11858-17428)
0.5 mM $NaHCO_3$	384 (341-428)	1853 (1646-2066)
1.0 mM $NaHCO_3$	779 (697-840)	3760 (3364-4054)
1.5 mM $NaHCO_3$	571 (473-663)	2756 (2283-3200)
2.0 mM $NaHCO_3$	765 (665-856)	3692 (3209-4131)
1.5 mM NaCl	446 (377-515)	2153 (1819-2486)

Notes: all LC50s were obtained using Maximum Likelihood-Probit.

Table 2.6. BLM-predicted concentrations (in µg/L) for major Pb species at the 96-h LC50 for acute *P. promelas* bioassays. For $Pb(OH)_2$, $Pb(OH)_3^-$ and $PbCl_2$ values were <1 µg/L (<1%) in all cases. The relative abundances of each species expressed as the percentage of total dissolved Pb are shown in parentheses.

Test	Pb^{2+}	$PbOH^+$	$PbCO_3$	$(PbCO_3)_2^{2-}$	$PbSO_4$	$PbCl^+$	Total Organic Pb
Unfed Base H_2O	4.0 (2.3)	2.2 (1.3)	16 (8.8)	0.01 (0.010)	0.067 (0.038)	0.056 (0.031)	156 (88)
Fed Base H_2O	32 (7.4)	13 (3.0)	126 (29)	0.084 (0.019)	0.59 (0.13)	0.53 (0.12)	267 (61)
0.5 mM $CaSO_4$	75 (10)	35 (4.7)	278 (37)	0.18 (0.025)	4.4 (0.59)	1.4 (0.18)	350 (47)
1.0 mM $CaSO_4$	121 (12)	64 (6.3)	444 (44)	0.31 (0.031)	14 (1.4)	2.2 (0.21)	370 (36)
1.5 mM $CaSO_4$ A	173 (16)	71 (6.7)	435 (41)	0.22 (0.020)	29 (2.7)	3.2 (0.30)	356 (33)
1.5 mM $CaSO_4$ B	70 (6.1)	37 (3.2)	697 (61)	1.39 (0.12)	8.7 (0.76)	1.4 (0.12)	333 (29)
2.5 mM $CaSO_4$	312 (18)	75 (4.4)	861 (50)	0.63 (0.037)	137 (7.9)	3.2 (0.19)	330 (19)
2 mg/L HA	28 (4.7)	18 (3.0)	109 (18)	0.071 (0.012)	0.62 (0.10)	0.55 (0.090)	452 (74)
4 mg/L HA	66 (6.2)	41 (3.8)	268 (25)	0.19 (0.017)	1.4 (0.13)	1.3 (0.12)	697 (65)
8 mg/L HA	49 (3.6)	27 (2.0)	159 (12)	0.088 (0.010)	1.0 (0.076)	1.0 (0.073)	1119 (83)
16 mg/L HA	74 (2.3)	50 (1.6)	425 (13)	0.42 (0.013)	1.6 (0.05)	1.5 (0.046)	2698 (83)
0.5 mM $NaHCO_3$	56 (6.9)	25 (3.1)	418 (51)	0.55 (0.067)	1.4 (0.17)	1.3 (0.16)	313 (38)
1.0 mM $NaHCO_3$	22 (2.2)	22 (2.2)	651 (65)	3.5 (0.35)	0.53 (0.053)	0.48 (0.048)	296 (30)
1.5 mM $NaHCO_3$	8.0 (1.1)	11 (1.5)	417 (59)	4.0 (0.57)	0.125 (0.018)	0.14 (0.020)	264 (38)
2.0 mM $NaHCO_3$	No LC50	No LC50	No LC50	No LC50	No LC50	No LC50	No LC50
1.5 mM NaCl	21 (5.7)	9.2 (2.5)	87 (24)	0.068 (0.018)	0.48 (0.13)	1.2 (0.33)	250 (68)
pH 5.5	55 (34)	0.26 (0.16)	0.016 (0.010)	$8.1x10^{-10}$ (<0.01)	1.1 (0.67)	2.8 (1.7)	103 (63)
pH 6.4	41 (15)	2.0 (0.76)	0.72 (0.27)	$2.2x10^{-6}$ (<0.01)	0.65 (0.25)	1.3 (0.50)	219 (83)
pH 7.5	27 (4.3)	21 (3.3)	271 (43)	0.48 (0.076)	0.45 (0.072)	0.41 (0.066)	304 (49)
pH 8.3	1.1 (0.33)	3.4 (0.99)	78 (23)	0.98 (0.29)	0.021 (0.010)	0.021 (0.010)	256 (75)

Table 2.7. BLM-predicted concentrations (in µg/L) for major Pb species at the 48-h LC50 for acute *C. dubia* bioassays. For $Pb(OH)_2$, $Pb(OH)_3^-$ and $PbCl_2$ values were <1 µg/L (<1%) in all cases. The relative abundances of each species expressed as the percentage of total dissolved Pb are shown in parentheses.

Test	Pb^{2+}	$PbOH^+$	$PbCO_3$	$(PbCO_3)_2^{2-}$	$PbSO_4$	$PbCl^+$	Total Organic Pb
Base water A	3.3 (0.84)	2.1 (0.53)	34 (8.6)	0.067 (0.017)	0.099 (0.025)	0.11 (0.028)	355 (90)
Base water B	0.57 (0.15)	0.90 (0.23)	31 (7.9)	0.38 (0.098)	0.032 (0.010)	0.035 (0.010)	354 (92)
0.5 mM $CaSO_4$	3.1 (0.71)	1.3 (0.30)	21 (4.8)	0.032 (0.010)	1.2 (0.27)	0.091 (0.021)	406 (94)
1.0 mM $CaSO_4$	8.1 (1.3)	3.4 (0.57)	54 (9.0)	0.082 (0.014)	2.8 (0.47)	0.23 (0.039)	529 (89)
1.5 mM $CaSO_4$	3.5 (0.92)	1.5 (0.38)	21 (5.5)	0.031 (0.010)	0.92 (0.24)	0.095 (0.025)	358 (93)
2.0 mM $CaSO_4$	2.1 (0.66)	0.83 (0.26)	13 (4.2)	0.022 (0.010)	0.72 (0.23)	0.061 (0.019)	302 (95)
2 mg/L Humic	1.5 (0.36)	1.6 (0.38)	38 (9.0)	0.18 (0.042)	0.035 (0.010)	0.043 (0.010)	383 (90)
4 mg/L Humic	1.6 (0.30)	1.7 (0.31)	43 (8.0)	0.23 (0.042)	0.041 (0.010)	0.051 (0.010)	500 (91)
8 mg/L Humic A	15 (2.5)	7.7 (1.3)	153 (26)	0.30 (0.050)	0.34 (0.057)	0.41 (0.069)	414 (70)
8 mg/L Humic B	0.61 (0.11)	0.73 (0.14)	26 (4.9)	0.25 (0.047)	0.035 (0.010)	0.038 (0.010)	504 (95)
32 mg/L Humic	0.14 (0.014)	0.30 (0.031)	11 (1.2)	0.21 (0.022)	0.010 (<0.010)	0.010 (<0.010)	952 (99)
64 mg/L Humic	0.69 (0.022)	1.5 (0.047)	57 (1.8)	1.2 (0.037)	0.066 (<0.010)	0.065 (<0.010)	3056 (98)
0.5 mM $NaHCO_3$	1.2 (0.31)	1.7 (0.45)	49 (13)	0.41 (0.11)	0.051 (0.013)	0.050 (0.013)	332 (86)
1.0 mM $NaHCO_3$	2.4 (0.30)	5.8 (0.74)	250 (32)	5.6 (0.72)	0.071 (0.010)	0.10 (0.013)	516 (66)
1.5 mM $NaHCO_3$	1.5 (0.26)	2.7 (0.48)	152 (27)	3.3 (0.58)	0.051 (0.010)	0.064 (0.011)	412 (72)
2.0 mM $NaHCO_3$	1.7 (0.22)	4.6 (0.60)	322 (42)	13 (1.7)	0.038 (0.010)	0.047 (0.010)	423 (55)
1.5 mM NaCl	5.7 (1.3)	2.8 (0.62)	37 (8.4)	0.050 (0.011)	0.23 (0.051)	0.36 (0.080)	400 (90)

Table 2.8. BLM-predicted concentrations (in µg/L) for major Pb species at the 96-h LC50s and 30-d LC20s for chronic *P. promelas* bioassays. For $Pb(OH)_2$, $Pb(OH)_3^-$ and $PbCl_2$ values were <1 µg/L (<1%) in all cases. The relative abundances of each species expressed as the percentage of total dissolved Pb are shown in parentheses.

Test	Pb^{2+}	$PbOH^+$	$PbCO_3$	$(PbCO_3)_2^{2-}$	$PbSO_4$	$PbCl^+$	Total Organic Pb
96 h LC50							
pH 5.5	48 (25)	0.27 (0.14)	0.035 (0.018)	$4.8x10^{-9}$ (<0.01)	0.98 (0.52)	2.0 (1.1)	137 (73)
pH 6.4	15 (8.9)	0.69 (0.41)	0.63 (0.37)	$4.6x10^{-6}$ (<0.01)	0.25 (0.15)	0.47 (0.28)	152 (90)
pH 7.5	6.3 (2.2)	3.6 (1.2)	61 (21)	0.11 (0.036)	0.12 (0.039)	0.15 (0.050)	221 (76)
pH 8.3	5.6 (0.71)	14 (1.8)	412 (52)	5.6 (0.70)	0.13 (0.016)	0.11 (0.014)	352 (45)
30 d LC20							
pH 5.5	No LC20	No LC20	No LC20	No LC20	No LC20	No LC20	No LC20
pH 6.4	1.4 (3.4)	0.065 (0.16)	0.059 (0.14)	$4.3x10^{-7}$ (<0.01)	0.023 (0.057)	0.044 (0.11)	39 (96)
pH 7.5	0.33 (0.45)	0.19 (0.25)	3.2 (4.4)	0.010 (0.010)	0.010 (0.010)	0.010 (0.010)	71 (95)
pH 8.3	0.20 (0.13)	0.50 (0.34)	14 (9.7)	0.20 (0.13)	<0.010 (<0.010)	<0.010 (<0.010)	134 (90)

Table 2.9. Whole body mass data (wet weight in mg) for surviving *P. promelas* from chronic Pb bioassays (mean±SEM).

Test	Pb (µg/L)	Mass (n)
pH 6.4	Control	75.2±6.0 (35)
	29.3±2.7	98.2±4.3 (34)
	89.1±8.2	94.5±5.2 (34)
	148±13	86.5±13 (7)
	297±27	57.2±20 (3)
pH 7.5	Control	70.2±5.8 (34)
	31.7±4.2	86.1±4.6 (34)
	156±19	93.5±4.8 (32)
	333±22	104±7 (18)
	453±64	134.1 (1)
pH 8.3	Control	94.0±3.7 (30)
	25.4±1.9	83.3±5.0 (30)
	82.2±3.8	86.5±4.5 (27)
	250±20	88.4±6.0 (17)
	434±14	58.2±16[a] (2)

[a]Significantly different from corresponding control as determined by Bonferroni *t*-test ($P \leq 0.05$).

Figure 2.1. Influence of calcium on acute Pb-induced mortality (LC50±95% CI) from 96-h larval fathead minnow (filled circles; y=2060.5927*x/(517.5896+x); r^2=0.9426; *P*=0.0013) or 48-h *C. dubia* (open circles) bioassays.

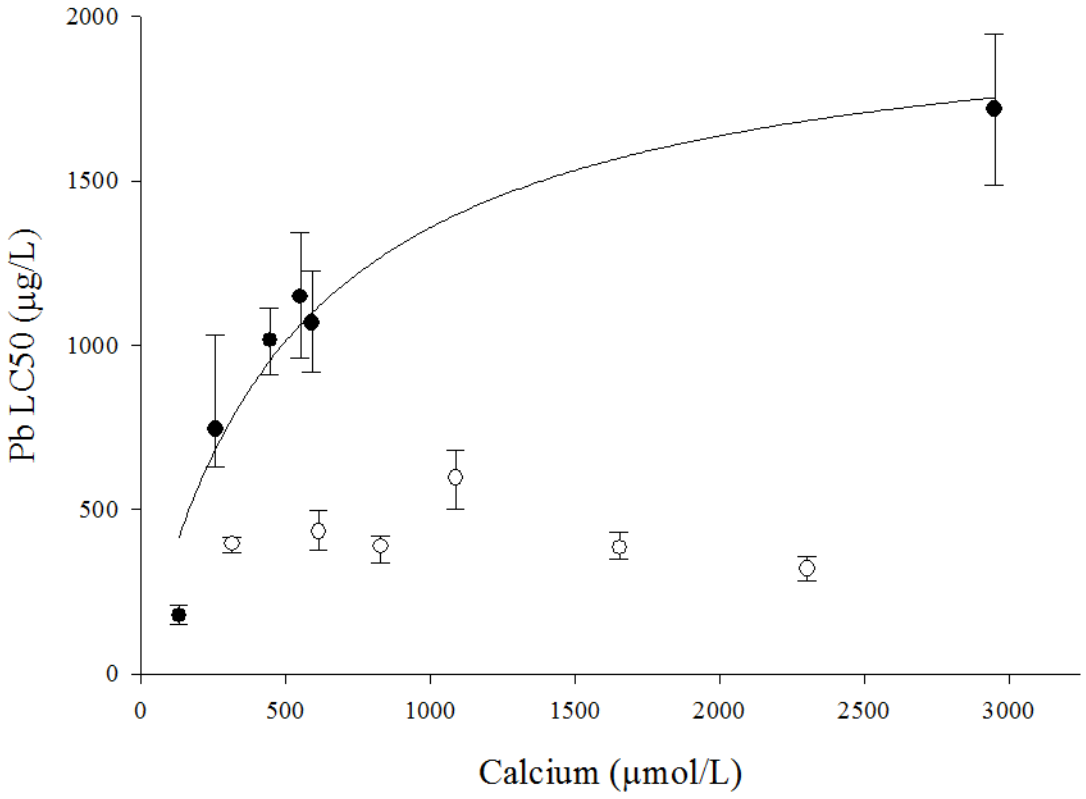


Figure 2.2. Influence of DOC (as Aldrich humic acid) on acute Pb-induced mortality (LC50±95% CI) from 96-h larval fathead minnow (filled circles; y=8.6026*x-372.2428); r^2=0.9819; *P*=0.0010) or 48-h *C. dubia* (open circles; y=2.0645*x-116.5652); r^2=0.8122; *P*=0.0022) bioassays.

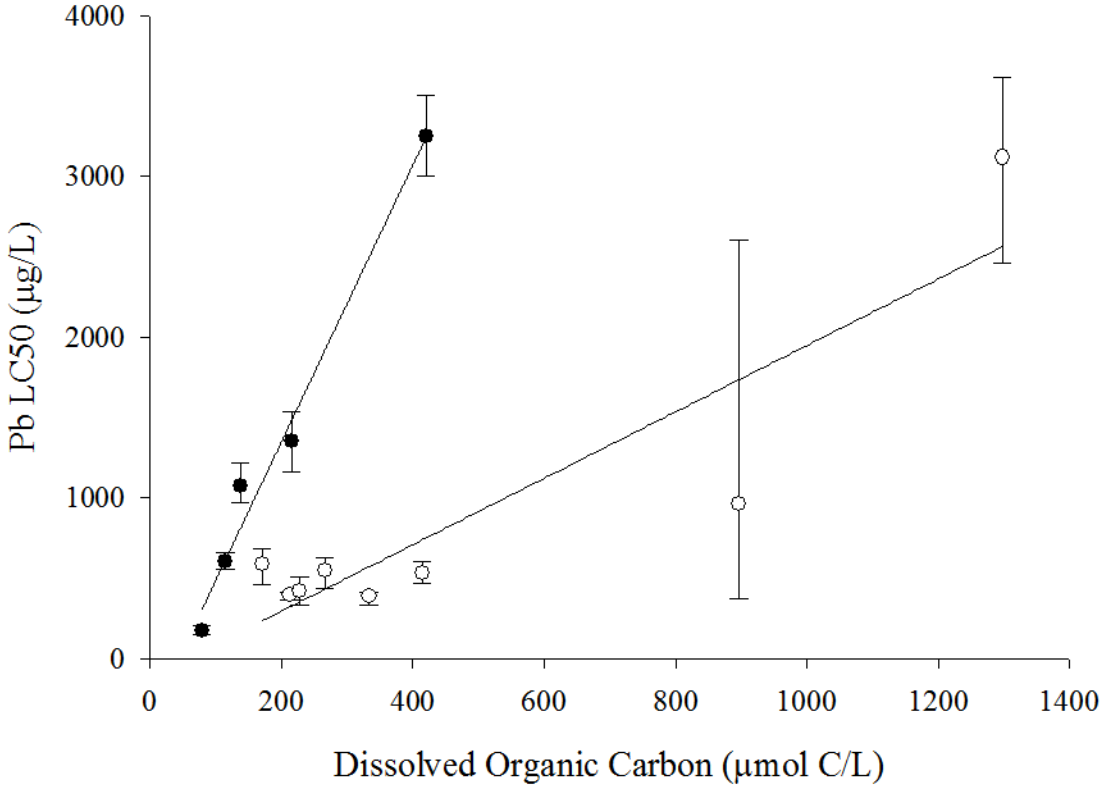
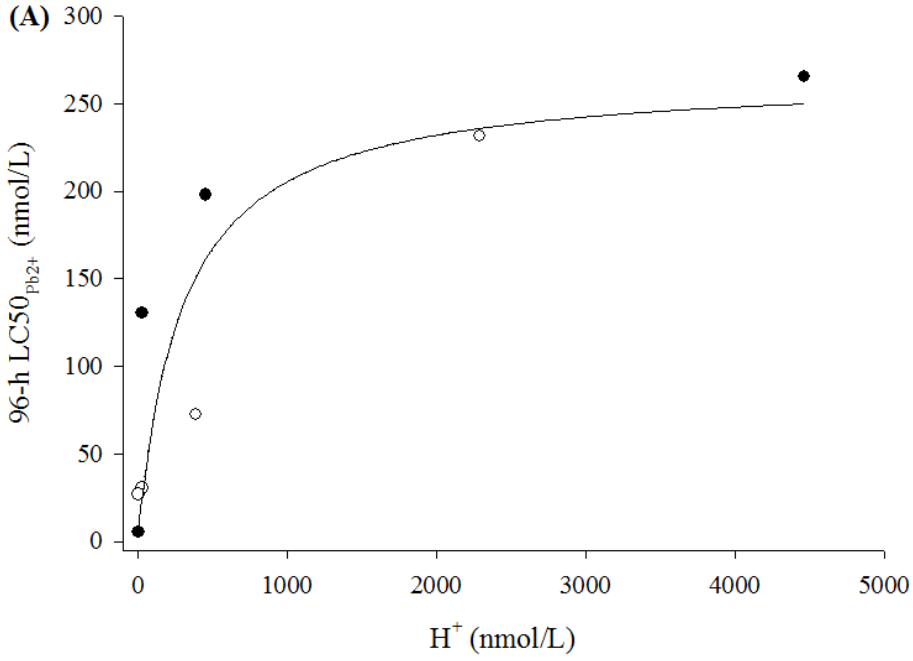


Figure 2.3. Influence of pH on acute (96-h) Pb toxicity to fathead minnows. A plot of ionic Pb^{2+} LC50s as a function of H^+ concentration reveals a Michaelis-Menten type relationship (y=266.5813*x/(299.5607+x); r^2=0.7325; P=0.0067) in (A). A linear regression of 96-h LC50s (expressed as pPb^{2+}) as a function of pH yields an r^2=0.7176 (y=0.4445*x+4.0946; P=0.0079). Filled circles indicate data from acute (unfed) bioassays and open circles indicate data from chronic (fed) bioassays.



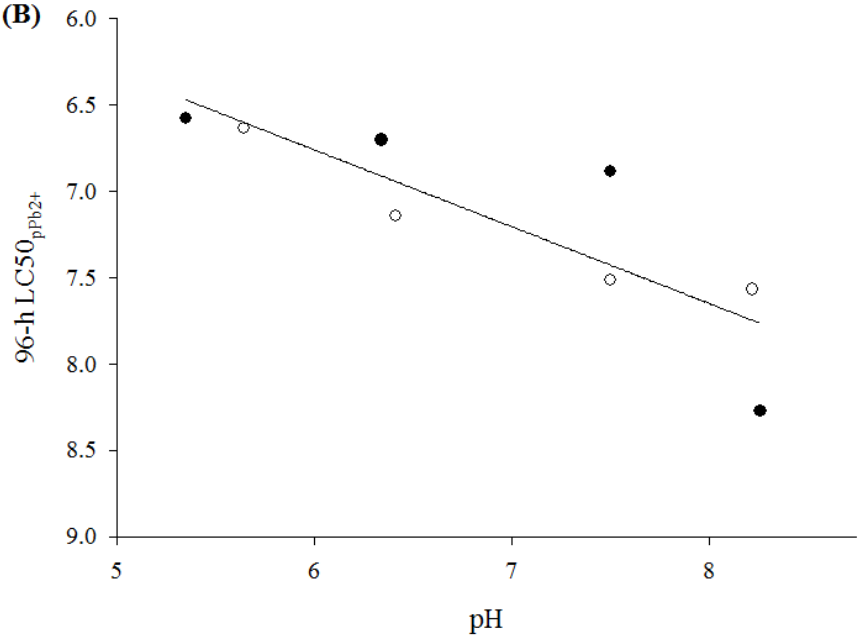
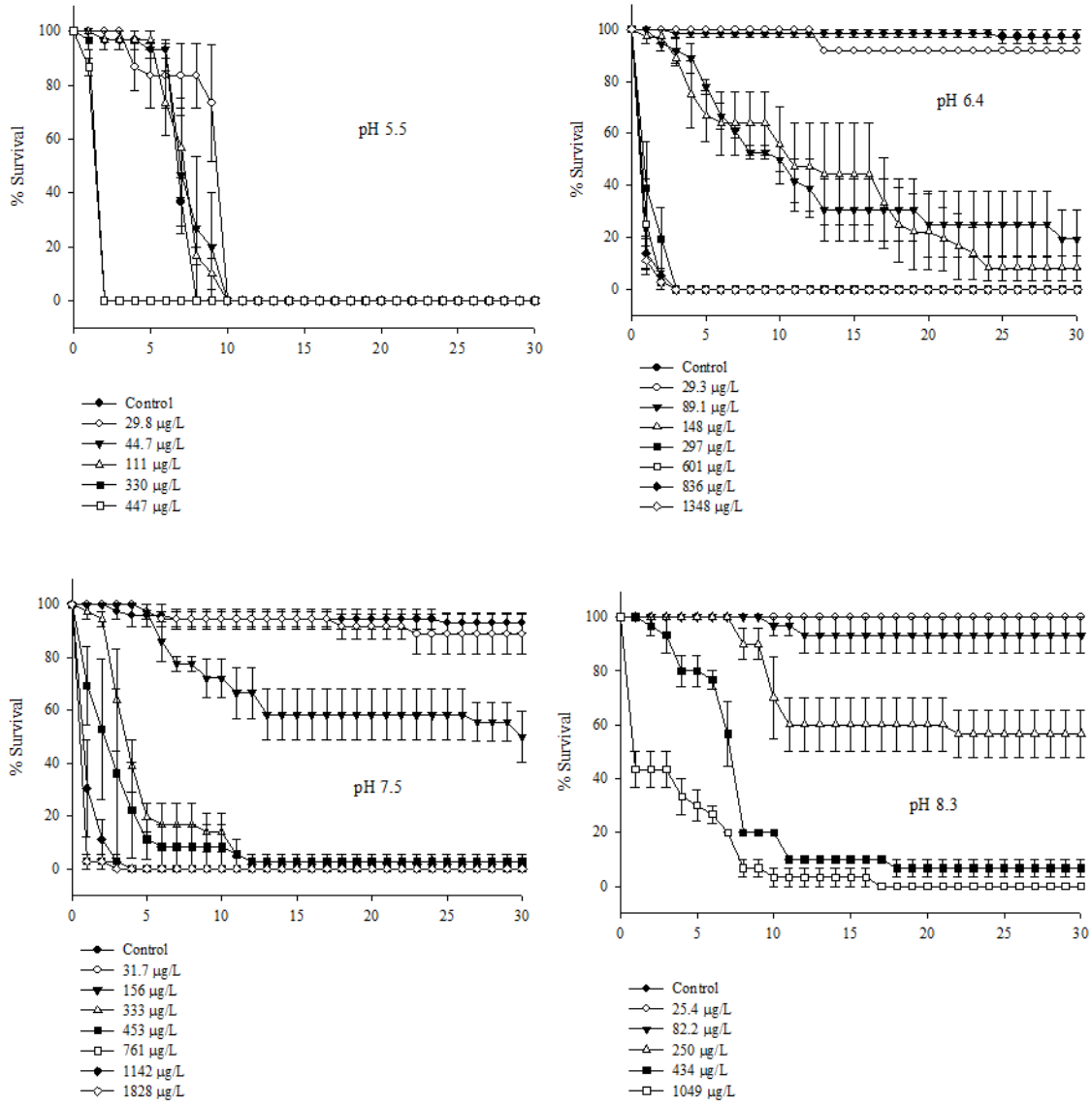


Figure 2.4. Cumulative fathead minnow mortality during 30-d Pb exposures in waters of varying pH expressed as mean percent survival ± SEM.



CHAPTER 3

TOXICOGENOMICS OF WATER CHEMISTRY INFLUENCE ON CHRONIC LEAD EXPOSURE TO THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

3.1. Summary.

Establishment of water quality criteria (WQC), intended to protect aquatic life, continues to rely principally on water hardness (i.e. Ca^{2+}) for lead (Pb) despite growing evidence that other chemical parameters also strongly influence toxicity. To more clearly define the water chemistry parameters mediating Pb toxicity we evaluated the effects of hardness as $CaSO_4$ and dissolved organic carbon (DOC) as humic acid during chronic (150 d) exposures to the fathead minnow. Measured Pb concentrations ranged from 157±5 nM (33±1 µg/L) Pb in base water to 177±7 (37±1 µg/L) and 187±7 nM (39±1 µg/L) Pb in $CaSO_4$- or HA-supplemented water, respectively. Fish were collected at 2, 4, 10, 30, 63, 90 and 150 d of exposure. Traditional toxicological endpoints were examined alongside gene expression analyses to help clarify the underlying mechanisms of Pb toxicity and to identify candidate molecular markers that might ultimately serve as robust indicators of exposure and possibly effect. Addition of $CaSO_4$ did not prevent whole body Pb accumulation whereas DOC afforded strong protection (about half the amount accumulated by fish in base water) suggesting that current, hardness-based WQC are likely inaccurate for predicting chronic Pb effects in aquatic systems. Custom-made microarrays were co-hybridized with base water samples ±Pb up to the 30 d time point. Quantitative PCR was employed to verify gene expression responses and to extend analysis to the $CaSO_4$ and HA treatments and the 150 d time point. Identification of four

genes by microarray analysis revealed clear Pb-induced responses over time: glucose-6-phosphate dehydrogenase, glutathione *S*-transferase, ferritin and β-globin. Results obtained by qPCR were in strong agreement with microarray data by regression analysis (r^2=0.82, slope=1.28). The associated pathways implicated herein for these genes provide further evidence supporting roles for anemia and neurological disorders in chronic Pb toxicity. Effects of water chemistry on Pb accumulation and gene expression responses were in close parallel, though alterations in ionoregulatory and morphological endpoints were not observed. Whereas DOC was protective against Pb accumulation and gene expression changes, Ca^{2+} was not. Additionally, several hypothesis-driven genes (ECaC, DMT-1, and ALA-D) were examined by qPCR but revealed no or small Pb-induced responses that did not suggest an influence due to water chemistry. These findings should help pave the way toward development of a new chronic Pb BLM and a Pb-responsive gene expression profile for fathead minnows, both of which would greatly aid future environmental monitoring and regulatory strategies for Pb.

3.2. Background.

Despite significant reductions in use, most notably in paint production and as a fuel additive, lead (Pb) continues to enter the environment primarily by anthropogenic means, retaining its status as a priority pollutant (USEPA, 2006). While the focus has turned towards remediation with regards to preventing human exposure, much is still needed in the way of determining appropriate measures to monitor and protect the aquatic environment, particularly with regards to point source pollution. In many cases, water quality criteria (WQC) continue to rely principally on water hardness (i.e. Ca^{2+}) despite

growing evidence that other chemical parameters (e.g. pH, salinity and dissolved organic carbon (DOC)), which may vary greatly on a local basis, also strongly influence Pb toxicity (Macdonald et al., 2002, Grosell et al., 2006a). Efforts to improve WQC for metals have given rise to several toxicity models designed to encompass the influences of all major water chemistry parameters. The most widely accepted model, the Biotic Ligand Model (BLM), is currently used by the USEPA to set WQC for copper. In essence, the BLM accounts for site-specific water conditions by considering the competitive effects from other cations and complexation with organic/inorganic agents that prevent the metal from interacting with the site of toxic action (Paquin et al., 2002). The benefits of a site-specific approach to setting regulatory standards would seem agreeable to industry and regulators alike and ideally encourage greater policy compliance.

There is no demonstrated biological need for Pb, thus uptake and toxicity is likely mediated through the mimicry of other cations (Ballatori, 2002), the most probable candidate being Ca^{2+} given the strong evidence that Pb acts as a Ca^{2+} antagonist (Busselberg et al., 1991; Rogers and Wood, 2004). However, the identification of a specific ligand for Pb remains elusive. As in mammals, the principal effects of chronic Pb exposure to fish are presumably hematological (Hodson et al., 1978a), neurological (Davies et al., 1976) and renal (Patel et al., 2006) impairment. Studies have also examined reproduction and behavior (Holcombe et al., 1976; Weber, 1993), though the nature of the observed effects are unclear with respect to the influence of water chemistry.

Another factor lending uncertainty to the regulatory decision-making process is that metals and other toxicants are commonly present as mixtures in the environment. Genomic approaches are well suited to address such problems, filling in where more conventional methods prove insufficient to pinpoint key environmental stressors or elucidate the contributions and additive effects from multiple toxicants. Furthermore, microarrays provide opportunities not only for establishing the molecular basis of toxicity, but potential for gaining insights into modes of action and higher order effects. Thus, defining toxicant-specific mechanisms that link signature gene expression profiles to chronic effects would greatly aid in monitoring and diagnosing water quality and also prioritizing higher-tier tests in ecological risk assessment. The significance of genomics in this regard was recently addressed by the USEPA as outlined in the Interim Genomics Policy (Dix et al., 2006).

We undertook this study to garner a more comprehensive understanding of chronic Pb toxicity by integrating traditional toxicological endpoints with gene expression analyses using one of the long-standing USEPA vertebrate test organisms, the fathead minnow. Specifically, we sought to achieve 3 goals: (1) further examine the influence of Ca^{2+} and DOC on the chronic toxicity of waterborne Pb, (2) identify Pb-responsive genes and determine whether expression changes reflect the influence of ambient water chemistry in modulating toxicity and (3) gain additional insights into the specific mechanisms underlying chronic Pb toxicity. A time course of multiple endpoints including molecular, ionoregulatory, and morphological effects were analyzed. Microarray analysis revealed four genes in particular that may provide additional clues to the molecular mechanisms involved in response to sublethal Pb exposures. Although no

ionoregulatory or morphological effects were observed at the Pb concentrations employed, whole body burdens differed in a manner that was closely paralleled by gene expression responses with respect to the influence of the different water chemistries. Whereas DOC was protective against Pb accumulation and gene expression changes, Ca^{2+} was not. The observed lack of protection by increased Ca^{2+} is an important finding given that Pb WQC are hardness-based, suggesting the current approach is likely inaccurate for predicting chronic effects of Pb exposure. These findings should help pave the way toward development of a new chronic Pb BLM and a Pb-responsive gene expression profile for fathead minnows, both of which would greatly aid future environmental monitoring and regulatory strategies for Pb.

3.3. Materials and methods.

3.3.1. Experimental animals.

Fathead minnows (*Pimephales promelas*) were obtained from Aquatic BioSystems, Inc. (Fort Collins, CO) at <24h post-hatch on arrival, distributed evenly among 18 1 L plastic beakers (~70 fish per beaker, 3 replicates per treatment) and gradually acclimated to test media without Pb for one week. Fish were reared in these chambers under flow-through conditions for the first 30 d of exposure, beyond which the remaining were pooled from each replicate and maintained in single 6 L plastic containers. Fish were fed freshly-hatched *Artemia* nauplii once daily for 1 month followed by 1 week of an *Artemia* and Tetramin flake food mixture and then flake food only thereafter. Prior to feeding, any fish that had perished were removed, the mortality recorded and leftover food and feces siphoned out.

3.3.2. Chronic lead exposures.

To obtain the 3 test media (Table 3.1), gravity flow-through conditions were employed as previously described (Grosell et al., 2006a). Briefly, a base water of 2:1 deionized water:dechlorinated Virginia Key tap water was adjusted for hardness or DOC by the addition of $CaSO_4$ or Aldrich humic acid (HA) stock solutions, respectively. Following one week of acclimation, Pb exposures were carried out for 150 d by dispensing concentrated $PbNO_3$ solutions via Mariotte bottles to test media targeting final concentrations of approximately 170 nM (~35 μg Pb/L). All chemicals used for modifying our target parameters were obtained from Sigma-Aldrich (St. Louis, MO). Average water temperature throughout the exposures was 22°C±1.

3.3.3. Water chemistry and whole animal lead and ion accumulation.

Fish were collected and euthanized at 2, 4, 10, 30, 63, 90 and 150 d of exposure for analysis of whole body ion and Pb composition as previously described (Grosell et al., 2006a). Sample sizes were 18, 18, 12, 12, 10, 7 and 10, respectively (except for 150 d $CaSO_4$±Pb n=6). To ensure sufficient material for analytical purposes, 6 larval fish from each beaker were pooled into a single tube at 2 and 4 d due to the small sizes at these time points. For the remaining time points, sizes were large enough to allow for collection of each fish into a single tube. Significant mortality, likely due to a pathogen infection, was observed in the $CaSO_4$ (no Pb) group around 45 d of exposure that was abated by malachite green treatment. To ensure adequate numbers for the full term exposure we elected not to collect fish from this group at 90 d.

Water chemistry was also analyzed once or twice per week. Samples were measured for Pb concentration after filtering through a 0.45 μm cellulose syringe filter (Acrodisc, Pall Life Sciences, MI) and acidification to 1% HNO_3 (Fisher Scientific, trace metal grade). All Pb measurements were performed by graphite furnace atomic absorption spectroscopy (Varian 200Z, Varian, Australia). Concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+} were determined by flame atomic absorption spectroscopy (Varian, 220FS, Varian, Australia), Cl^- and SO_4^{2-} by anion chromatography (DIONEX DX120, CA), and total CO_2 using a Corning 962 carbon dioxide analyzer (UK). To measure DOC, high temperature catalytic oxidations were performed using a Shimadzu total organic carbon-VCSH analyzer (Kyoto, Japan) (Hansell and Carlson, 2001).

3.3.4. RNA extractions.

Except for 150 d, from which six fish were collected for both $CaSO_4$ treatments and 9 for all others, pooling and sample numbers were the same as above. Fish were euthanized, placed into cryotubes and immediately snap-frozen in liquid nitrogen. Total RNA was isolated from whole fish using RNA STAT-60 solution (Tel-Test, Friendsworth, TX) and a Polytron homogenizer. From each isolate 10 μg was treated with DNase I to remove any traces of genomic DNA (Turbo DNA-free kit; Ambion, Austin, TX). Integrity of RNA was confirmed by gel electrophoresis prior to cDNA synthesis.

3.3.5. Microarray hybridization and analysis.

Arrays were constructed using 5000 randomly picked clones from a cDNA library representing the full fathead minnow life history (Wintz et al., 2006). For each time point equal amounts of RNA from all no Pb controls were pooled and hybridized with each of 3 biological replicates corresponding to the respective Pb-exposures described above. Synthesis of Cy5/Cy3-labeled cDNA was accomplished using the 3DNA Array 900 kit (Genisphere, Hatfield, PA). All hybridizations were performed per the manufacturer's instructions and repeated using dye-swapped cDNAs. An ArrayWoRx Biochip Reader (Applied Precision, Issaquah, WA) and GenePix software version 3.01 (Molecular Devices, Sunnyvale, CA) were used to scan and quantify hybridization signals, respectively.

3.3.6. 5' RACE.

To obtain poly(A) RNA as template for RACE reactions, aliquots from the 30 d total RNA samples were pooled and processed using the MicroPoly(A) Purist kit (Ambion, Austin, TX). Poly(A) RNA (1 μg) was reverse transcribed and amplified using the BD SMART RACE cDNA Amplification Kit (BD Biosciences, Franklin Lakes, NJ; Table 3.2.). Touchdown PCR cycling conditions were as follows: 5 cycles of 94°C for 30 sec, 69°C for 30 sec and 72°C for 3.5 min followed by 5 and 20-25 cycles as previously except at 67°C and 65°C annealing temperatures, respectively. Products were gel-purified, cloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced.

3.3.7. cDNA synthesis and primer design.

For qPCR, cDNA was synthesized from 1 μg DNase I-treated total RNA using the SuperScript II First-Strand System (Invitrogen, Carlsbad, CA). Following RNase H treatment, all reactions were diluted tenfold in TE buffer. To obtain fathead minnow-specific sequences ~1.5 kb of the epithelial calcium channel (ECaC) and 326 bp of δ-aminolevulinic acid dehydratase (ALA-D) were cloned by degenerate PCR; all qPCR primers were designed from the coding regions of each target gene (Table 3.2.).

3.3.8. Quantitative PCR.

Experiments were carried out in the MX4000 instrument (Stratagene, La Jolla, CA) with AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) and SYBR Green I dye (Sigma, St. Louis, MO) for detection. A preliminary experiment was performed using the geNorm approach (Vandesompele et al., 2002) to examine the suitability of several "housekeeping" genes for normalization purposes, namely 18S ribosomal RNA, elongation factor 1α (EF1α) and ribosomal protein L8. EF1α proved the strongest candidate, exhibiting stable expression across randomly selected sub-samples from all treatments and developmental time points (data not shown). Because this gene also adhered best to the criteria of amplifying at a similar cycle (i.e. <10) to those of the genes of interest we chose to use EF1α as our target for normalization. All reactions were optimized to establish PCR efficiencies of >95%. Amplicon identities were confirmed by sequencing and a single, corresponding melting peak was verified following all amplifications. Cycling was as follows: 95°C for 5 min followed by 40 cycles at 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. Six reactions per

treatment representing duplicate runs of 3 biological replicates were performed. Because fish were no longer segregated into replicate beakers beyond 30 d, biological replicates for 150 d consisted of cDNA pools from randomly selected fish. Fold changes were calculated from the log-transformed C_T values and expressed relative to the treatment-matched no Pb controls using a modification of the delta-delta-C_T method (Livak and Schmittgen, 2001) to account for amplification efficiencies as previously described (Vandesompele et al., 2002).

3.3.9. Statistical analysis.

Except for microarray analysis, differences were deemed statistically significant at $P<0.05$ by two way analysis of variance (ANOVA) using pairwise multi-sample comparison corrections (Bonferroni *t*-test) as appropriate. Data are presented as means ± 1 standard error of the mean (SEM). An α-outlier-generating model approach was employed for determination of statistically-significant changes in gene expression by microarray analysis as previously described (Loguinov et al., 2004).

3.4. Results.

3.4.1. Influence of water chemistry on lead toxicity.

Measured water chemistries were in accordance with our target nominal values and were consistently maintained across treatments (Table 3.1.). Lead concentrations were stable within treatments, but differed slightly between with mean values ranging from 157±5 nM (33±1 μg/L) Pb in base water to 177±7 (37±1 μg/L) and 187±7 nM (39±1 μg/L) Pb in $CaSO_4$- or HA-supplemented water, respectively. Lead concentrations

in control treatments remained below the detection limit (0.5 µg/L). Strong protection against Pb accumulation was clearly afforded by the increase in DOC concentration with fish typically exhibiting about half the amount accumulated by fish from base water (Figure 3.1A.), whereas elevation in $CaSO_4$ failed to prevent Pb accumulation. These results generally held true with respect to the influence of water chemistry when the same data were plotted as a function of Pb content per fish to account for an apparent growth dilution effect initiated around 10 d (Figure 3.1B.). Lead continued to accumulate as the fish grew, though again to a lesser degree in the presence of HA.

Whole body ion concentrations remained relatively stable across test media irrespective of Pb addition (Table 3.3.). Ontogenic changes in whole body ion concentrations independent of Pb were examined by comparing overall means for each time point. There were 2 immediately apparent observations: a spike in 10 d concentrations (129.4±3.8 µmol/g Ca^{2+}, 105.7±2.5 µmol/g K^+, 62.3±1.4 µmol/g Na^+) and opposite trends displayed by Ca^{2+} and K^+ concentrations over time. Whereas Ca^{2+} increased from 90.4±3.5 µmol/g at 2 d to 125.6±2.8 µmol/g at 150 d (mean 112.0±1.4 µmol/g), K^+ decreased from 110.4±3.7 µmol/g to 85.5±1.4 µmol/g (mean 89.1±1.0 µmol/g) over the same period. Conversely, Na^+ remained stable with a mean concentration of 49.4±0.8 µmol/g (excluding 10 d data). Taken together, however, the results indicate a lack of substantial ionoregulatory disturbance in response to either Pb or water chemistry.

3.4.2. Morphological and growth effects.

We regularly monitored fish for development of spinal curvature and/or black discoloration and peripheral erosion of the caudal fin as these endpoints were previously reported in rainbow trout chronically exposed to Pb (Davies et al., 1976, Holcombe et al., 1976). Arbitrary scoring of spinal curvature by eye could not be correlated to Pb exposure or test media (data not shown). Furthermore, at no time was convincing evidence of caudal fin abnormalities apparent. There was no indication of Pb-induced mortality; a few minor, transitory growth effects due to Pb or water chemistry were observed (632±41 mg overall mean weight at 150 d, Table 3.4.).

3.4.3. Identification of lead-responsive genes using microarrays.

Analysis of expression profiles revealed four candidate genes exhibiting significant responses to Pb (GEO accession no. GSE8404). The cDNAs were identified either directly or after cloning the corresponding full length transcripts by 5' RACE as ferritin heavy chain, glutathione *S*-transferase alpha (GST), glucose-6-phosphate dehydrogenase (G6PD), and β-globin (Table 3.2.). Except for β-globin all exhibited increased expression in response to Pb that remained fairly consistent over time (Figure 3.2A.). The greatest magnitude of change was displayed by GST at all time points which exhibited a pattern that was closely mirrored by G6PD expression. Ferritin was the most stably expressed for the entire duration but was induced to a lesser extent than GST and, except at 30 d, G6PD as well. The only gene that showed decreased levels due to Pb was β-globin, evident most profoundly at 30 d with minor reductions or no change occurring at earlier time points.

3.4.4. Influence of water chemistry on lead-induced gene expression.

Quantitative PCR was employed to verify microarray results and to determine if the Pb-induced gene expression responses reflected the influence of water chemistry on whole body Pb accumulation. Results obtained by qPCR and microarray were in strong agreement as evident by regression analysis (r^2=0.82, slope=1.28; Figure 3.2B.). For the most part, qPCR gene expression responses strongly paralleled the influence of water chemistry on Pb accumulation (Figure 3.3). Ferritin, G6PD, and GST all showed decreased induction in fish from the HA treatments which displayed lower Pb accumulation. Conversely, $CaSO_4$ typically did not afford protection at the gene expression level, as also seen for Pb accumulation. The expression pattern for β-globin was not reflective of the Pb accumulation data.

In addition to the genes identified by microarray analysis, two others were selected for qPCR analysis based on their putative roles in Ca^{2+} transport (Shahsavarani et al., 2006) and route of Pb entry in mammals (Bressler et al., 2004; Gunshin et al., 1997) (ECaC and divalent metal transporter-1 (DMT1), respectively). Expression of ALA-D was also examined because Pb is known to inhibit activity of this enzyme (Hodson, 1976; Warren et al., 1998). An increase in ECaC expression was evident in response to Pb only within the $CaSO_4$ treatments and only at 10 d and beyond (Figure 3). Expression of DMT1 and ALA-D revealed no statistically significant changes irrespective of Pb exposure, and no gene examined in this study revealed a change due to water chemistry alone (data not shown).

3.5. Discussion.

3.5.1. Chronic lead toxicity and influence of Ca^{2+} and DOC.

While Ca^{2+} did not protect against Pb accumulation when compared to moderately soft base water, HA clearly did. These results are in agreement with previous Pb gill binding experiments using rainbow trout and may be explained by the ≥2 orders of magnitude differences in calculated Pb binding affinities for organic matter (log *K* 8.4) vs. gill (log *K* 6.0) and vs. Ca^{2+} gill binding (log *K* 4.0) (Macdonald et al., 2002). These are important findings given that current WQC are hardness-based (USEPA, 2006) and provide further support that a reevaluation of the means by which chronic Pb criteria are established is warranted.

3.5.2. Lead-responsive genes identified by microarray.

The results from our custom fathead minnow arrays elicited four Pb-responsive genes that displayed clear patterns of expression that could be verified by qPCR. Several factors may have contributed to the small number of pronounced Pb-induced responses. First, we specifically chose to expose fish to low Pb concentrations to target the most sensitive responses. Thus, the concentrations used may have approached levels that induce gene expression changes reflecting lower threshold responses. Secondly, the cDNA library was of limited complexity (some redundancy) leading to an array composition of much less than 5,000 unique cDNAs (Wintz et al., 2006). Thirdly, RNA samples were pooled from multiple fish leading to a possible masking of subtle responses to Pb by native inter-individual variability. Additionally, whole fish were used out of practicality and to examine global expression responses. However, this may have

imposed a greater challenge to detecting relatively small toxic responses occurring in specific organs. Admittedly, these are potential drawbacks, but the genes garnered by such an approach of presumably reduced sensitivity are more likely to serve as robust indicators of low-level chronic exposure and effects.

What is most striking about the present findings is that the expression responses strongly reflected the influence of water chemistry on toxicity and that the genes fit together well in a biochemical scheme (see below). The changes in ferritin, G6PD, and GST expression all mirrored the Pb accumulation data indicating these genes may hold potential as sensitive indicators of Pb accumulation, though lack of a clear toxicological link points to the need for further study. Nevertheless, given that the genes can all be linked within specific pathways suggests that when used together they may offer indication of a Pb-specific response.

3.5.3. Toxic mechanisms: biochemical pathways implicated in lead toxicity.

The four genes revealed by microarray analysis link pathways previously implicated in anemia (Figure 3.4.). Induction of G6PD and GST indicate recruitment of the pentose phosphate shunt commonly employed by erythrocytes to combat oxidative stress (Lachant et al., 1984). Deficiency in G6PD, a common genetic disorder in humans, results in hemolytic anemia due to an inability to defend against oxidative stress (Beutler, 1994). The condition arises because NADPH, as required for GST-mediated glutathione conjugation (and which would otherwise be available from normal levels of G6PD production), is not sufficiently maintained to detoxify harmful free radicals. Our microarray experiments also revealed decreased β-globin expression in response to Pb in

base water at 30 d, perhaps further supporting a reactive oxygen species (ROS)-mediated hemolytic loss of erythrocytes. A parallel to these findings may be found with a previous observation of hematocrit reduction followed by recovery occurring near the same times of exposure as in our experiments (Hodson et al., 1978) (Figure 3.3.).

The inhibitory effect of Pb on ALA-D, a key enzyme in the heme synthesis pathway, has been well documented (Hodson, 1976; Schmitt et al., 2002; Warren et al., 1998) and studies have linked a role for this inhibition to ferritin Fe^{2+} release (Oteiza et al., 1995; Rocha et al., 2003). However, species-specific inconsistencies have been reported and clear links to higher order effects have proved elusive (Hodson et al., 1977; Schmitt et al., 2002). The absence of an ALA-D response by qPCR in the present study is in agreement with a 29 d time course analysis using juvenile rainbow trout exposed to Pb concentrations bracketing those employed herein which revealed no change in whole body enzymatic activity (Burden et al., 1998). This, in addition to increased ferritin expression, is perhaps expected as a feedback response to free Fe^{2+} displaced by accumulating ALA substrate and/or released during hemolysis (Figure 3.4.). Lead is also known to bind ferritin directly displacing reactive iron in the process (Kelada et al., 2001). Therefore, it is likely that at least 2 major pathways may lead to anemia as a result of Pb exposure in the fathead minnow: (1) ROS-induced hemolysis and (2) reduced heme synthesis due to ALA-D inhibition.

Ferritin Fe^{2+} displacement and increased ROS production may play a role in neurological impairment as well. Evidence suggests a role for ferritin Fe^{2+} dysregulation in human neurological disorders (Ke and Ming Qian, 2003; Berg and Hochstrasser, 2006) and that ferritin may represent a dominant means of Fe^{2+} delivery to the brains of

mammals (Fisher et al., 2007). Thus, it may be interesting to further investigate the significance of Fe^{2+} dysregulation in the neurological effects commonly observed in Pb-exposed fish.

3.6. Conclusions.

Traditional toxic biomarkers (e.g. metallothionein) often lack specificity and thus may provide little in the way of relevance, mechanistic insight, or ability to predict long-term outcomes of ecological significance. As Pb appears to be rather unique among metals with respect to its role in anemia, it would seem the genes identified by our microarrays may narrow the focus for targeting additional genes in associated pathways. Although the above results more clearly defined the influences of Ca^{2+} and HA on chronic Pb accumulation, the lack of an observed physiological or morphological effect points to the need for additional study if relevant diagnostic capabilities for ecological assessment are to be achieved. Additionally, it will be of interest to further evaluate the influence of other water chemistry parameters such as pH and alkalinity. In any event, the role of DOC in mediating toxicity should garner greater consideration in the development of a chronic BLM for Pb. The results reported herein point to the power of microarray technologies in elucidating underlying toxic mechanisms and for further revealing the means by which Pb may elicit detrimental effects to fish and potentially higher organisms as well.

Table 3.1. Chemistry of test media (in µM except for hardness which is expressed as mg/L as calculated by APHA Standard Methods; *n*=6 except for pH *n*=22 and for DOC as noted in parentheses).

	$[Na^+]$	$[K^+]$	$[Ca^{2+}]$	$[Mg^{2+}]$	$[Cl^-]$	$[SO_4^{2}-]$	$[CO_2]$	[DOC]	Hardness	pH
Base water	235±8	25±1	241±12	53±1	355±27	20±6	332±11	82±1(3)	30±1	7.5±0.1
500 µM Ca^{2+}	251±12	23±1	618±41	45±2	284±12	414±41	305±7	74±2(3)	68±4	7.4±0.1
4 mg/L humic	242±7	26±1	247±21	54±2	341±13	16±7	345±25	149±6(14)	31±2	7.6±0.1

Table 3.2. Primers used for qPCR and cloning. Accession numbers for fathead minnow sequences from which primers were designed are provided.

Primer	Accession No.	Sequence (5'→3')	Product Size (bp)
EF1α-F	AY643400	GACCACTGAGGTTAAATCTGT	142
EF1α-R		GTCGTTCTTGCTGTCTCCAG	
Ferritin-F	EF628371[a]	GAACGTCAATCAGGCTCTGC	163
Ferritin-R		GTTGCCAGCATCCATCTTGG	
G6PD-F	EF628372[a]	CAATGCATGAGCACCAAAGG	124
G6PD-R		GGTAAATCTGTTCCTCGGTG	
GST-F	EF628373[a]	GACGTTCATCTTCTGGAAGC	152
GST-R		GAGGCTTTCTCGCACTGC	
β-globin-F	EF628375[a]	CTATGCTGAACTGAGTGTGC	129
β-globin-R		TGGACTGCAGGTGTGAATG	
ALA-D-F	EF628376[a]	GCACCCTACTCATCCACTG	114
ALA-D-R		GTCATAGCCTCCATAACTGC	
ECaC-F	EF628374[a]	GTAATACCATCCTGCATCTGC	125
ECaC-R		GTTGGGAATCATGTCTAGTGG	
DMT1-F	AF190773	CCATCGCTTTCAATCTGCTG	113
DMT1-R		CTTCAGACCGTACTTGTCCAG	
dALA-D-F[b]	N/A	TTCMGAGAYGCTGCHCAGTC	326
dALA-D-R[b]	N/A	ATGATGATRTCAGCWCCTGC	
dECaC-F[b]	N/A	CTCATCAAYGAGCCCATGAC	1480
dECaC-R[b]	N/A	GYGTCCTCCAGAGCTCGTC	
7B20-GSP[c]	N/A	AACAGAGGGATGTGGCATGTCATACC	2526
8N17-GSP[c]	N/A	CTAAGGGTGTCAATCATTCTGACCATGAC	1245
8J9-GSP[c]	N/A	AACATCAGATGATCCTCTGTGCATAAGCAG	593

Abbreviations: forward primer (F); reverse primer (R); gene specific primer (GSP).
[a]Sequences obtained during the course of this study.
[b]Degenerate primers designed from conserved nucleotide regions across multiple fish species were used to obtain fathead minnow ALA-D (*Danio rerio* (BC092804), *Coryphaenoides armatus* (AJ609236), *Tetraodon nigroviridis* (CR668921)) and ECaC (*Oncorhynchus mykiss* (AY256348) and *Danio rerio* (AY325807)) sequences.
[c]Primers used for 5' RACE designed from sequences identified by microarray analysis. Product sizes include 45 bp corresponding to Clontech Universal Primer A sequence at 5' end.

Table 3.3A. Fathead minnow whole body Ca^{2+} concentrations (µmol g^{-1}).

	Exposure time (days)[a]						
	2(18)	4(18)	10(12)	30(12)	63(10)	90(7)	150(10)
Base water -Pb	87±3	108±4	134±5	97±4	101±10	108±9	126±5
Base water +Pb	110±5	105±4	126±4	99±3	104±4	118±7	124±4
$CaSO_4$ –Pb	96±8	122±4	125±6	104±4	103±8	N/A	137±8
$CaSO_4$ +Pb	95±4	104±2	130±7	108±3	88±5	118±2	145±11
Humic –Pb	77±4	93±6	121±17	106±3	96±3	125±6	122±6
Humic +Pb	78±10	94±4	140±11	94±4	96±5	110±7	113±7

Table 3.3B. Fathead minnow whole body K^+ concentrations (μmol g^{-1}).

	Exposure time (days)[a]						
	2(18)	4(18)	10(12)	30(12)	63(10)	90(7)	150(10)
Base water -Pb	112±8	108±5	114±3	83±2	75±1	69±4	85±2
Base water +Pb	127±6[b]	96±3	101±5[b]	80±1	75±1	76±2	83±1
$CaSO_4$ –Pb	114±9	111±4	104±3[c]	86±1	74±1	N/A	83±3
$CaSO_4$ +Pb	117±6	108±3	101±5	88±1	73±1	76±2	73±8
Humic –Pb	98±5	96±7	105±5[c]	84±1	77±2	83±2	88±2
Humic +Pb	94±9	96±2	109±11	87±1	74±1	78±2	95±3

Table 3.3C. Fathead minnow whole body Na^+ concentrations (μmol g^{-1}).

	Exposure time (days)[a]						
	2(18)	4(18)	10(12)	30(12)	63(10)	90(7)	150(10)
Base water -Pb	50±1	49±1	66±2	48±2	50±1	39±3	45±1
Base water +Pb	52±3	47±2	62±2	50±1	53±2	54±4[b]	55±1[b]
$CaSO_4$ –Pb	51±2	53±1	61±2	52±1	47±1	N/A	50±3
$CaSO_4$ +Pb	49±1	48±2	60±3	50±1	49±2	50±2	52±6
Humic –Pb	47±3	46±1	62±4	57±5[c]	49±1	46±1	54±4
Humic +Pb	50±1	47±1	62±6	50±1	46±1	48±3	50±3

[a]Numbers in parenthesis denote *n*-numbers except for 150 d $CaSO_4$ ± Pb (*n*=6). [b]Denotes $P<0.05$ vs. treatment-matched -Pb control at same time point. [C]Denotes $P<0.05$ vs. base water -Pb at same time point.

Table 3.4. Summary of mean ± SEM waterborne Pb concentrations (nM) and body mass (mg) during 150 day exposures ±Pb in different test media.

		Exposure time (days) [a]						
Medium	[Pb]	2(18)	4(18)	10(12)	30(12)	63(10)	90(7)	150(10)
Base water -Pb	-1.2±1.6[b]	3.1±0.1	2.9±0.2	7.2±0.7	41±2	151±24	243±27	735±114
Base water +Pb	157±5	2.8±0.1	3.7±0.4	7.2±0.8	50±1	116±22	178±29	605±95
$CaSO_4$ –Pb	-1.0±1.7[b]	2.5±0.4	4.0±0.4	6.6±0.8	52±1	144±19	N/A	783±75
$CaSO_4$ +Pb	177±7	3.6±0.2	3.2±0.5	7.7±0.9	50±1	155±19	227±34	606±172
Humic –Pb	-0.4±1.7[b]	2.2±0.3	2.8±0.3	6.9±0.9	57±5	87±12	234±44	588±82
Humic +Pb	186±7	3.2±0.5	3.5±0.1	9.8±1.0[c]	50±1	149±22[c]	256±39	526±75

[a]Numbers in parenthesis denote *n*-numbers except for 150 d $CaSO_4$ ± Pb (n=6). [b]Measured values are below detection limit. [c]Denotes $P<0.05$ vs. corresponding control at same time point.

Figure 3.1. Influence of water chemistry on whole body Pb accumulation by fathead minnows during 150 d exposures. Lead accumulation is expressed per wet weight (A) and per fish (B). All treatments +Pb exhibited statistically significant differences from corresponding control except for 2 d and 4 d time points in (B). Statistically significant difference for [a]HA +Pb or [b]$CaSO_4$ +Pb compared to base water +Pb.

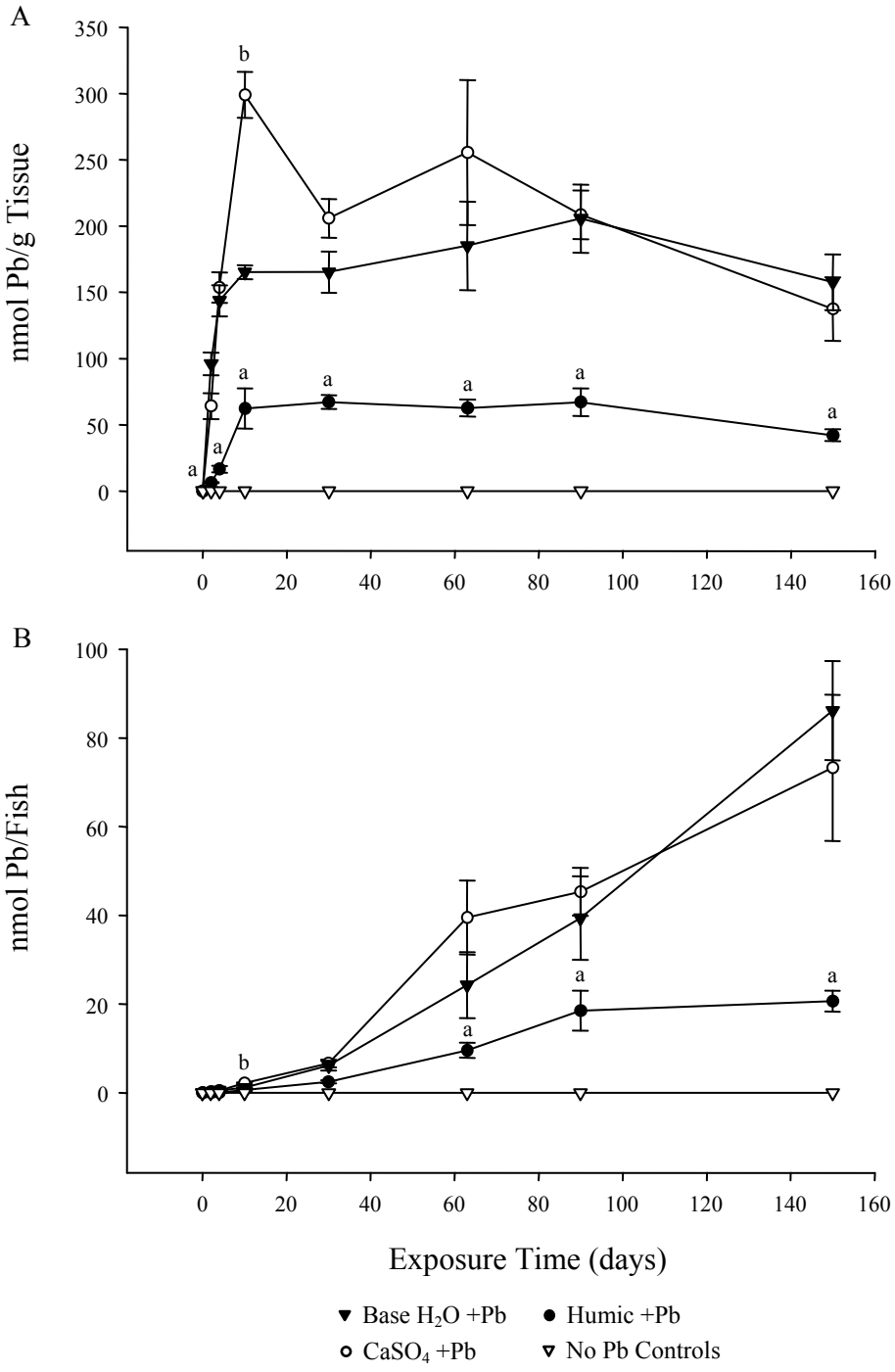


Figure 3.2. Time course of fold changes in mRNA expression ± SEM by microarray using fathead minnows exposed to 157±5 nM (33±1 µg/L) Pb versus no Pb controls in base water (A). Correlation of microarray and qPCR data comparing fold changes in gene expression from base water ±Pb samples for all time points out to 30 d (r^2=0.82; slope=1.28) (B).

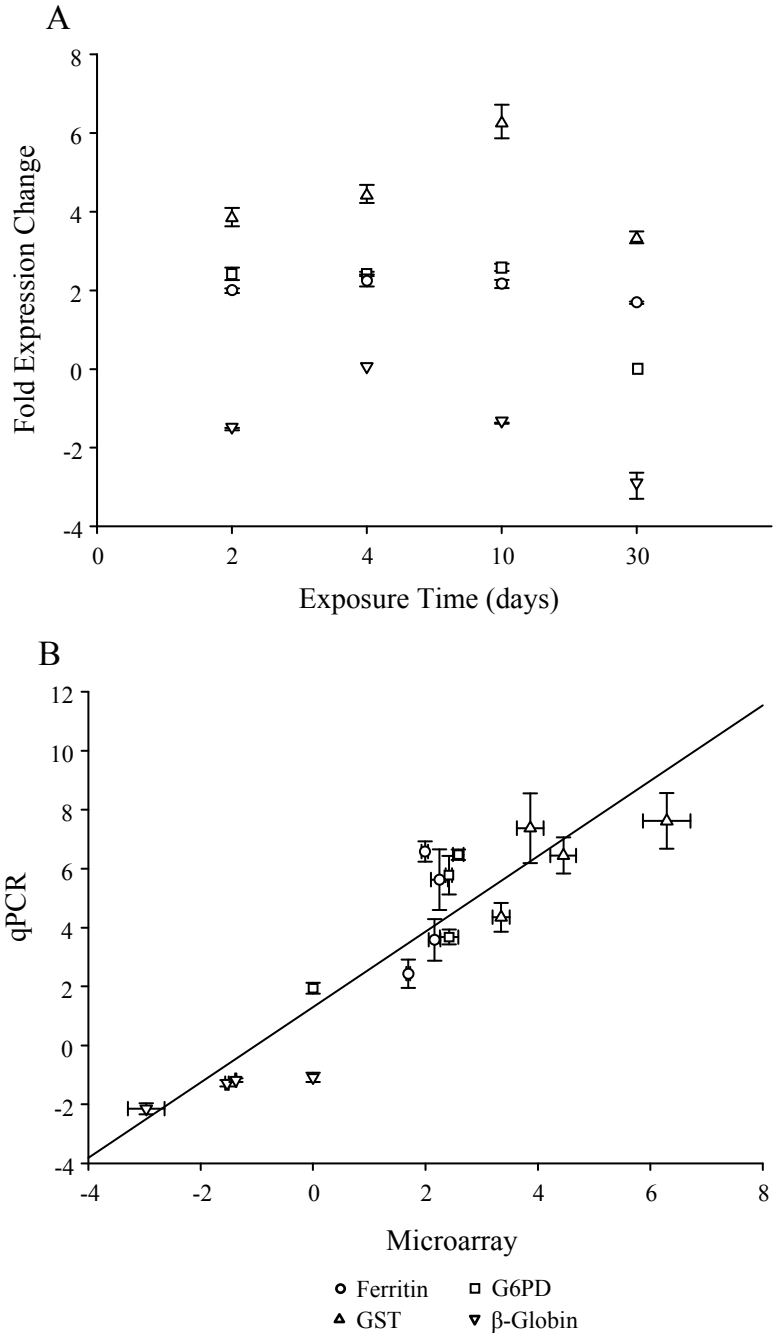


Figure 3.3. qPCR analysis of chronic Pb-induced gene expression changes in different water chemistries. Data normalized to EF1α is expressed relative to treatment-matched controls. Ferritin (A), β-globin (B), G6PD (C), GST (D), ECaC (E). [a]Statistically significant difference from corresponding control. [b]Statistically significant difference from base water +Pb.

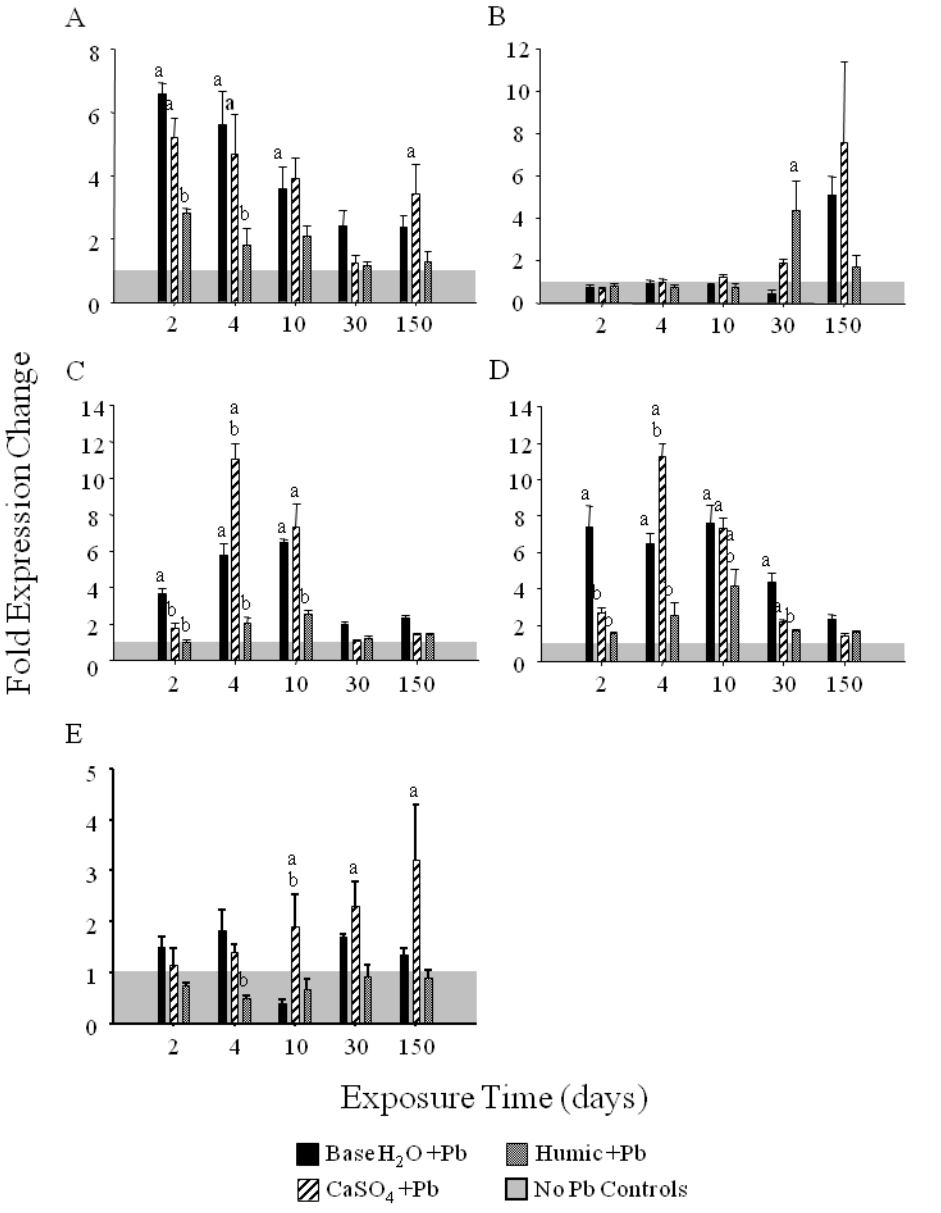
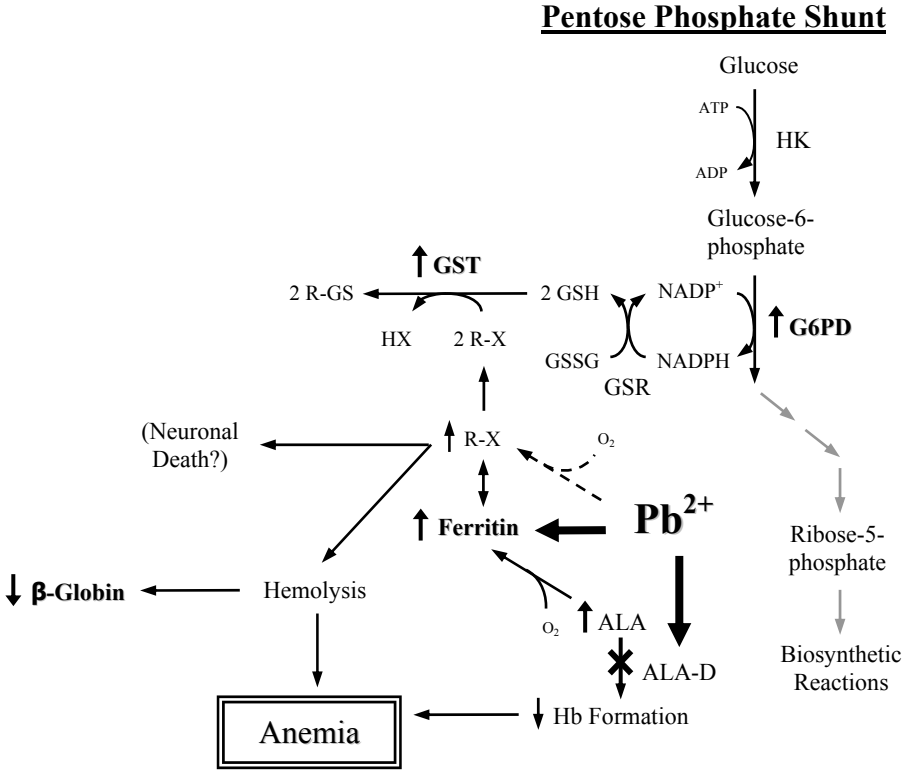


Figure 3.4. Pathways likely affected by Pb illustrating potential roles in hematological and neurological dysfunction. Genes identified by microarrays are shown in bold. Inhibition of ALA-D incurs detrimental effects by impaired heme synthesis and increased ALA levels. Binding of Pb and/or ALA to ferritin promotes release of reactive oxygen species (R-X) leading to oxidative stress, hemolysis (and an apparent decrease in β-globin expression due to the loss of erythrocytes) and potentially neuronal death. Ferritin and the pentose phosphate shunt enzymes GST and G6PD increase as compensatory and detoxification responses, respectively. Abbreviations: hexokinase (HK), hemoglobin (Hb).



CHAPTER 4.

INFLUENCE OF BICARBONATE AND HUMIC ACID ON EFFECTS OF CHRONIC WATERBORNE LEAD EXPOSURE TO THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

4.1. Summary.

Historically, the USEPA has only considered water hardness when establishing acute and chronic water quality criteria (WQC) for lead (Pb) in freshwater. Yet, recent evidence suggests that hardness may not be protective during chronic Pb exposure and that other factors (e.g., dissolved organic carbon (DOC) and alkalinity) influence toxicity. In fact, we have recently shown that Ca^{2+} (as $CaSO_4$) does not protect against Pb accumulation in fathead minnows (*Pimephales promelas*) during chronic exposures whereas DOC as humic acid (HA) clearly does. To more clearly define the water chemistry parameters mediating chronic Pb toxicity we carried out 300 d exposures to study the influence of DOC and alkalinity on Pb accumulation and toxicity to fathead minnows at 2 different Pb concentrations (170 and 580 nM (35 and 120 µg/L)). Alkalinity was adjusted by addition of 500 µM $NaHCO_3$ and DOC by addition of 4 mg/L HA. Fish were collected at 4, 30, 150 and 300 d of exposure to measure growth and Pb accumulation. Breeding assays (21 d) were performed at the end of these exposures to assess reproductive and larval behavioral endpoints. To determine whether effects were acute or chronic, switched breeding exposures were performed in which control breeders were transferred to either high or low Pb conditions and Pb-exposed breeders transferred to tap water without Pb. Mortality and growth effects were observed primarily in the

high Pb treatments and within the first 10 d of exposure. Strong protection against Pb accumulation was afforded by increased DOC at both Pb concentrations. Increased alkalinity also appeared to moderately reduce Pb accumulation although not to the level of statistical significance. Tissue distribution of Pb was analyzed at 300 d and was found to accumulate mostly in bone, gill, intestine and kidney. Unexpectedly, high Pb reduced total reproductive output and increased average egg mass in the HCO_3^- and DOC treatments but not in the control water (+Pb) treatments. No statistically significant differences in egg hatchability or egg Pb accumulation were observed. Results from switched exposures suggest that embryo Pb accumulation arose from acute exposure to embryos rather than parental transfer. Finally, prey capture assays revealed potential Pb-induced motor/behavioral impairment in 10 d old F1 larvae exposed to high Pb in all water treatments.

4.2. Background.

Lead (Pb), a non-essential metal, is of primary interest to the USEPA (Fairbrother et al., 2007) ranking behind only copper (Cu) as one of the most highly reported causes of metal impairment to water quality (Reiley, 2007). As with other metals, the toxicity of Pb can vary greatly depending on effects that differences in local water quality may have on its speciation. Previous studies have shown that acute toxicity of Pb decreases with increasing hardness or alkalinity/pH (Davies et al., 1976; Schubauer-Berigan et al., 1993; Stouthart et al., 1994). For hardness, the protective effect is most likely due to antagonistic binding of Ca^{2+} to a shared channel for Pb at the gill (Busselberg et al., 1991; Rogers and Wood, 2004). Alkalinity, on the other hand, affords protection by the

formation of Pb carbonate complexes that sequester free ionic Pb, presumably rendering it unavailable for uptake:

(Eq. 1) $Pb^{2+} + CO_3^{2-} \rightarrow PbCO_3$;

(Eq. 2) $Pb^{2+} + 2CO_3^{2-} \rightarrow Pb(CO_3)_2^{2-}$

More recently, it has become clear that other influences, such as complexation by dissolved organic carbon (DOC) or other organic/inorganic species, may also be important in determining Pb toxicity (Macdonald et al., 2002). In the case of DOC, protection is attributed to the high number of binding sites (carboxyl, phenolic, amino and sulfhydryl groups) that chelate metals and other cations from the water (Filella and Town, 2000).

The influence of water chemistry on chronic Pb toxicity is less clear than for acute toxicity due to the relative paucity of chronic studies. Consequently, acute toxicity data is heavily relied upon for establishing chronic water quality criteria (WQC) leading to potentially uncertain and/or inappropriate levels of environmental protection. From the limited studies available it would seem that hardness and increased pH/alkalinity are protective against chronic Pb toxicity in fish (Davies et al., 1976; Hodson et al., 1978b). However, since $CaCO_3$ contributes significantly to both hardness and alkalinity, and changes in these parameters commonly co-vary in natural waters and laboratory experiments, there remains uncertainty as to the protective contribution of each.

One of the main reasons that chronic studies evaluating reproductive toxicity in fish are lacking is the time and effort required to perform such experiments. Hence, we previously conducted short-term exposures to identify the likely key water chemistry parameters influencing chronic Pb toxicity prior to undertaking exposures through

reproductive maturity. These efforts demonstrated protective effects by Ca^{2+} (as $CaSO_4$) and DOC (as Aldrich humic acid (HA)) against acute Pb toxicity (Grosell et al., 2006a), as well as against chronic Pb accumulation by HA but not Ca^{2+} in fathead minnows (Mager et al., 2008). Reproduction was not evaluated in the latter study and, aside from Pb-induced transcriptional responses, no other toxic effects were observed under the conditions examined. Still, these findings shed some doubt as to the protective influence of Ca^{2+} on chronic Pb toxicity while further supporting DOC as an important protective component that warrants greater consideration. However, because $CaSO_4$ was used to explicitly study the effects of increased hardness without increasing alkalinity in these experiments, the influence of alkalinity alone on chronic Pb accumulation and toxicity remains unclear.

Having narrowed the field for potential key water chemistry parameters, we proceeded with the present study aimed primarily at investigating water chemistry influences on Pb-induced reproductive effects. Specifically, we again evaluated the influence of HA to determine whether the protection against whole body Pb accumulation observed previously translated into protection against full-term reproductive effects. We also investigated the effect of increased alkalinity (as $NaHCO_3$) in lieu of Ca^{2+} given its previous failure to protect against chronic Pb accumulation. The reproductive endpoints of fecundity, hatchability, egg mass, egg Pb accumulation and attachment of eggs to breeding substrate were monitored. Additionally, growth, Pb accumulation and potential Pb-induced neurological impairment in larval offspring were assessed.

4.3. Materials and methods.

4.3.1. Experimental design.

The main goal of this study was to examine the influence of DOC (as HA) and alkalinity (as $NaHCO_3$) on the reproductive toxicity of chronic Pb exposure to fathead minnows (Figure 4.1.). To this end, Pb exposures were administered in 3 different laboratory waters (described below) to 8 d old fathead minnow larvae for 230-300 d and subsequently through 3 sequential rounds of 21 d breeding assays. During these breeding assays eggs were counted daily to assess fecundity and collected for hatchability or determination of egg mass and Pb accumulation. As Pb is a known neurotoxin (White et al., 2007), we also sought to evaluate potential Pb-induced behavioral/motor impairment in F1 offspring hatched from eggs produced during the breeding assays. Therefore, eggs collected for hatchability were exposed to Pb for 10 d post-hatch after which larvae were analyzed using a prey capture assay. Whole body and tissue Pb burdens were also measured to determine whether the influences of water chemistry on Pb accumulation were predictive and consistent with observed chronic toxicity. For completion, standard endpoints of mortality and growth were also monitored.

4.3.2. Experimental animals.

Fathead minnow (*Pimephales promelas*) embryos were obtained from Aquatic BioSystems, Inc. (Fort Collins, CO; <24 h post-hatch on arrival), distributed evenly among 1 L plastic beakers and placed under a 5 mL/min flow-through water supply (~75-85 fish per beaker, 3 replicates per treatment). After the first 20 d of exposure 1 L beakers were replaced with 21 L aquaria and flows increased to 70 mL/min for the

remainder of the experiment. Fish were fed once daily an *ad libitum* diet of freshly-hatched *Artemia* nauplii for the first month followed by *Artemia* and Tetramin flake food for 1 week and then flake food only thereafter. Leftover food, feces and dead fish (if any) were removed daily prior to feeding. Fish were maintained on a 16 h:8 h light:dark photoperiod with an average water temperature of 22±1°C.

4.3.3. Chronic lead exposures.

Dechlorinated Virginia Key tap water was modified by nominal addition of 500 μM $NaHCO_3$ or 4 mg/L HA using a gravity flow-through approach as previously described (Grosell et al., 2006a; Table 4.1.). Larvae were gradually acclimated to different water chemistries for one week prior to initiation of Pb exposures at 8 d of age. Concentrated $PbNO_3$ solutions were dispensed at 1 mL/min via Mariotte bottles into 2 separate mixing chambers for each water chemistry targeting final "low" Pb concentrations of ~170 nM (35 μg /L) and "high" Pb concentrations of ~580 nM (120 μg/L). Using a hardness representative of the present study (92 mg/L) both Pb concentrations exceeded current hardness-based calculations for chronic Pb WQC (11 nM (2.3 μg/L)) and our high Pb exposure also exceeded that calculated for acute Pb WQC (285 nM (59 μg/L)) (USEPA, 2006). In sum, 9 different treatments were tested including controls (Figure 4.1.). High Pb exposures were initiated 100 d following that of low Pb exposures for reasons discussed in Results and Discussion (see *4.4.1 Water chemistry*). All chemicals used for maintaining test conditions were obtained from Sigma-Aldrich (St. Louis, MO).

Whole body lead accumulation was determined from acid-digested fish collected at 4, 30, 150 and 300 d of exposure as previously described (Grosell et al., 2006a); *n*=18 (3 pools of 6 fish/tube), 12, 12, and 6, respectively. For the 300 d time point, whole body Pb burdens were estimated by summing the Pb accumulated in individual tissues harvested for determination of internal Pb distribution and dividing by the total body mass of origin. Tissues were collected from 3 males and 3 females and included gills, brain, testes, ovaries, anterior intestine, liver, kidneys and the remaining carcass.

4.3.4. 21 day breeding assays.

Breeding pairs were selected randomly and evenly from replicate tanks toward the end of the exposures and maintained in flow-through conditions matching water chemistries and Pb concentrations to those leading up to reproductive assays. Aquaria (21 L) were partitioned in half with a screen divider to accommodate two separate breeding pairs per tank. Due to space limitations these assays were conducted in 3 sequential rounds beginning at approximately 210, 250 and 280 d of exposure. Two replicate tanks (4 breeding pairs) per treatment were used in each round for a total of 12 breeding assays per treatment. Each pair was provided with a breeding substrate modeled after previously described methods (Thorpe et al., 2007).

Breeding substrates were removed daily for egg counts/collection and replaced with clean substrates. Eggs were counted as either "attached" (adhered to PVC substrate) or "detached" (found in lower collection tray) and summed to obtain total daily output (designated as a single clutch). Eggs were collected for either mass/Pb accumulation or hatchability/prey capture (Figure 4.1.). Those collected for mass/Pb accumulation were

briefly rinsed in deionized water, dried by decanting and siphoning off surrounding water, counted and gently transferred to pre-weighed eppendorf tubes. Acid digests and Pb measurements were performed as above. Eggs collected for hatchability and prey capture were transferred to hatching chambers containing 1.9 L of treatment water and vigorously aerated.

Following the initial 3 rounds of reproductive assays additional experiments were arranged to determine whether any observations were due to chronic Pb exposure to adults or acute Pb exposure to embryos. To this end, breeding assays were conducted as previously (tap water only) with the exception that breeding pairs were switched to opposing exposure conditions (i.e. breeding fish from control conditions transferred to low or high Pb tap water and low or high Pb-exposed breeders transferred to control tap water).

4.3.5. Prey capture assays.

To examine potential neurological effects on offspring, eggs from the breeding assays were allowed to hatch. Following hatch, larvae were maintained for 10 d in ~1 L treatment-matched water (daily static-renewal) and fed freshly hatched *Artemia* nauplii daily until 48 h prior to the prey capture assays. The assay was initiated by placing a single fathead minnow larva into a small weight boat (4 cm L x 4 cm W x 0.8 cm H) containing 5 mL of treatment water. Ten *Artemia* nauplii were then introduced and the number ingested within a 5 min period was scored. Times were recorded at the 1st, 5th and 10th ingestions if completed. For those ingesting less than 5 of the *Artemia*, the

number remaining at the end of 5 min was noted. A total of 8-14 larvae were tested from each of 2-3 replicate beakers.

4.3.6. Water chemistry.

Dissolved Pb concentrations were measured from water samples passed through a 0.45 μm cellulose syringe filter (Acrodisc, Pall Life Sciences, MI) and acidified to 1% HNO_3 (Fisher Scientific, trace metal grade) via graphite furnace atomic absorption spectroscopy (Varian 200Z, Varian, Australia). Concentrations of major cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) and anions (Cl^- and SO_4^{2-}) were determined by flame atomic absorption spectroscopy (Varian 220FS, Varian, Australia) and anion chromatography (DIONEX DX120, CA), respectively, total CO_2 by a Corning 962 carbon dioxide analyzer (UK) and DOC by high temperature catalytic oxidations using a Shimadzu total organic carbon-VCSH analyzer (Kyoto, Japan) (Hansell and Carlson, 2001). Water pH was measured using a PHM201 meter (Radiometer, Copenhagen, Denmark) fitted with a combination glass electrode. Water chemistry and temperature was typically analyzed on a bi-weekly schedule (Table 4.1.) and dissolved Pb was measured once or twice a week (Table 4.2.).

4.3.7. Calculations and statistical analysis.

Data are presented as means ± 1 standard error of the mean (SEM). Analysis of variance (ANOVA) followed by Student's *t*-test of individual means with multi-sample comparison corrections (Bonferonni) as appropriate was used for statistical analysis as indicated in tables and legends. In all cases, differences were deemed statistically

significant at $P \leq 0.05$. Lead speciation calculations were performed using the biotic ligand model (HydroQual, Inc., Version 2.3.3).

4.4. Results and discussion.

4.4.1. Water chemistry.

In a previous 150 d study (Mager et al., 2008) we used a 2:1 deionized water:dechlorinated tap water mixture to achieve a moderately soft base water for investigating hardness and DOC effects on Pb toxicity. Due to the much larger scale and duration of this study, we chose instead to use full strength tap water to eliminate the difficulties associated with higher flow demands of deionized water. Accordingly, this led to a base water with approximately 3 fold higher concentrations of all water constituents and higher pH. Overall, water chemistry measurements were consistent with our target nominal values and were stable across treatments except for the modified parameters (Table 4.1.).

We chose to study the effects of Pb at 2 different concentrations, one "low" concentration similar to that used in our previous study (~170 nM, 35µg/L Pb) and a "high" concentration to maximize the likelihood of observing toxicity. For the latter, we aimed for the highest Pb concentration that could be achieved without inducing mortality to such an extent that the number of fish remaining would be insufficient for sample collection throughout the course of the experiment. The first 2 attempts using measured dissolved Pb concentrations of 1690 nM (350 µg/L) and 1110 nM (230 µg/L) both resulted in excessive mortality within the first 10 d of exposure. Due to the time invested for these initial attempts our final high Pb exposure of 530 nM (110 µg/L) trailed behind

our low and control exposures by 100 d. However, no effect of exposure starting time was discernable on growth or reproduction by two-way ANOVA. Final mean Pb concentrations for both low and high exposures were moderately lower than targeted, but comparable across treatments (Table 4.2.).

4.4.2. Mortality and growth.

Most of the mortality (≥95%) occurred during the first 10 d of exposure, primarily within the high Pb treatments. Humic acid and HCO_3^- protected against 10 d Pb-induced mortality to nearly the same extent where cumulative percent mortalities were 28.9% (tap water), 20.0% (HCO_3^-) and 19.3% (HA), respectively. Additionally, some adult mortality of note occurred during the breeding assays from which 14 of 108 fish died prior to completion of the 21 d. Female breeders accounted for 11 of these deaths due most likely to male aggression (attacks against females were commonly observed) whereas males likely succumbed to natural mortality after spawning (Andrews and Flickinger, 1973).

Protection was less evident for growth which was inhibited to a similar extent at 4 d by high Pb regardless of water treatment (Table 4.3A.). Growth had recovered in all cases by 30 d and no further differences were observed at any time point beyond. Average fish masses at 30 and 150 d of exposure were 41±2 mg and 1311±45 mg, respectively (n=108). Masses were also recorded by sex after each round of reproduction at 230 d, 270 d and 300 d of exposure (Table 4.3B.). Mean values were 3766±123, 4489±137 and 4623±167 mg for males, respectively (n=32-35); 1547±53,

1889±105 and 1700±59 mg for females (*n*=28-35), respectively. No statistically significant growth differences were observed at any age due to water chemistry alone.

Sex ratios were determined at the end of the study from all fish remaining after the 150 d time point (Table 4.3B.). In all of the tap water exposures as well as the HCO_3^- controls ratios were close to 1. Sex ratios trended towards higher numbers of males in HCO_3^- and HA treatments with Pb, though the differences were not statistically significant. Also, the apparent shift in the HA treatments with Pb were in reference to a lower baseline of males:females in the HA control water when compared to tap water controls.

4.4.3. Whole body lead accumulation.

As in our 150 d study, we investigated the potential mitigating effects of DOC on Pb accumulation by the nominal addition of 4 mg/L HA. This resulted in a similar base water increase of 60 µM DOC as previously (Table 4.1.). Assuming a background DOC comprised of 10% HA (Santore et al., 2001), a 60 µM measured increase (assumed to be 100% HA) would translate into a 3.4 fold increase in the humic acid component of the DOC. However, it should be noted that addition of 60 µM DOC in the present study is a smaller proportional increase than that in our previous 150 d study due to the higher starting background levels in undiluted tap water vs. 33% tap water (257±8 µM DOC vs. 82±1 µM DOC, respectively). Nevertheless, this increase was sufficient to provide strong protection against Pb accumulation for both low and high Pb concentrations when compared to tap water controls (Figure 4.2A.). Thus, these findings further illustrate the substantial protective effect of relatively small increases in DOC against whole body Pb

accumulation by fathead minnows. This is perhaps best explained by the much higher calculated affinity (~250 fold) of Pb for organic carbon over the gill (Macdonald et al., 2002).

In the alkalinity treatment we increased the HCO_3^- concentration by 500 μM using $NaHCO_3$ which raised the total CO_2 concentration from 701±11 μM to 1191±23 μM and pH from 8.1±0.1 to 8.3±0.1 relative to the tap water control (Table 4.1.). At many of the time points examined alkalinity appeared to reduce whole body Pb burdens at both Pb concentrations, though only at 300 d in the high concentration was this difference statistically significant (Figure 4.2A.). Furthermore, these reductions were typically not to the extent of that observed with HA. Differences between the protective effects of HA and alkalinity may be resolved by the greater complexing capacity of HA due to the high number of strong Pb binding sites available per mg of carbon (Macdonald et al., 2002). Conversely, Pb is removed in a nearly linear fashion by increased carbonate complexation at higher alkalinities (Davies et al., 1976; Macdonald et al., 2002). Thus, one might expect that a nearly 1.7-fold increase in total CO_2 at a pH close to 8 as reported herein would lead to a proportional decrease in Pb accumulation. However, this was not observed, suggesting that some inorganic forms of Pb, which increase with increasing alkalinity/pH, might be available for uptake (see *4.4.5 Role of Pb speciation* below). Whatever the mechanism, clearly an increase in HCO_3^- concentration does not necessarily lead to a proportional protective effect against Pb accumulation in fathead minnows.

The apparent decrease in Pb accumulated on a per mass basis with age beyond the 30 d time point, as evident in Figure 4.2A., can likely be attributed to a growth dilution

effect when the same data are plotted per number of fish as shown in Figure 4.2B. These results suggest that the mass of tissues not accumulating Pb likely grow at a rate greater than those tissues that do accumulate Pb. This is not surprising given that Pb primarily targets bone (see below) which will account for less proportional mass as the fish grows.

4.4.4. Internal distribution of lead.

The internal distribution of Pb at 300 d was consistent with previous findings by our lab in fathead minnows (Grosell et al., 2006a) and by others in salmonids (Hodson et al., 1978b; Holcombe et al., 1976; Varanasi and Gmur, 1978). On a Pb concentration basis, Pb accumulated mostly in the gill, kidney, anterior intestine and carcass with far less found in the brain and liver (Figure 4.3A-B.). In contrast, the relative contribution of total Pb accumulated was accounted for mostly by the carcass, representing 95% (Figure 4.3C-D.), which previous work by our lab has shown is predominantly a reflection of skeletal accumulation (Grosell et al., 2006a). While whole body Pb burdens did not differ across water treatments at 300 d in low Pb, the gill and kidney individually accumulated less Pb in the HA treatment when compared to the tap water control (Figure 4.3A.). In the high Pb treatments, Pb accumulation in the carcass mirrored that observed at the whole body level with significantly less found in HCO_3^- and HA treatments (Figure 4.3B.).

High accumulation by the intestine is somewhat surprising and suggests that some dietary Pb exposure likely occurred. It is conceivable that Pb was ingested following rapid adsorption to food. Mount et al. (1994) found increased Pb accumulation in rainbow trout fed a diet of *Artemia* previously exposed to waterborne Pb for 24 h and

others have clearly demonstrated that dietary Pb can be taken up and accumulated by the intestine (Alves and Wood, 2006; Ojo and Wood, 2007). Alternatively, secretions into the intestine from other organs (e.g., bile from the liver) may have resulted in apparent intestinal Pb accumulation.

Because sex was easily discernable at 300 d, equal numbers of each (n=3; 6 total) were collected to examine differential Pb accumulation between males and females. No statistically significant differences with respect to sex were found at the whole body level, although females did accumulate more Pb in intestine, kidney and brain in the high Pb treatments, but not low Pb treatments, when tested by one-way ANOVA (data not shown).

4.4.5. Role of lead speciation.

Since metal bioavailability in general is considered to be strongly tied to speciation, calculations were performed to determine concentrations and relative abundances of the major Pb species within each of the different water treatments using the biotic ligand model (HydroQual, Inc.; Paquin et al., 2002) and are summarized in Table 4.4. Clearly, the vast majority of Pb (> 90%) was organically bound regardless of water treatment, although as Pb concentrations increased the proportions of the major inorganic and free ion species also increased. Not surprisingly, the treatments exhibiting the largest percentages of free Pb^{2+} ion were the tap water treatments while the HCO_3^- treatments had the highest percentages of Pb-carbonate complexes. The latter would also account for the highest overall percentages of inorganic Pb species observed in the HCO_3^- treatments.

Interestingly, HA reduced all inorganic forms of Pb to the greatest extent except for the free Pb^{2+} ion which was reduced to a similar extent as that achieved with HCO_3^-. Given that fish appeared to accumulate more Pb from the HCO_3^- than from the HA treatments suggests that some of the inorganic Pb other than the free Pb^{2+} ion was bioavailable. For example, Pb could be similar to Cu, for which the monohydroxide form is believed to be bioavailable (USEPA, 2007). Thus, $PbOH^+$ which increases with increasing alkalinity/pH above ~pH 6.5 in fresh water (Stumm and Morgan, 1996) might similarly contribute to Pb accumulation. Finally, in light of our reproductive and behavioral results, our speciation data suggests that inorganically and organically complexed Pb exerts chronic toxicity in a manner not reflected by whole body Pb accumulation, though it is unclear at this time how this might occur.

4.4.6. Fecundity.

Unexpectedly, Pb effects on fecundity were observed in the HCO_3^- and HA treatments but not in the tap water controls (Table 4.5.). For the HCO_3^- treatments, low and high Pb concentrations reduced 21 d total reproductive output. This effect appeared related to both reduced clutch size and reduced number of clutches produced when compared to treatment-matched controls, although only the former difference was statistically significant (high Pb only). It should be noted, however, that addition of HCO_3^- alone actually increased reproductive output, suggesting that increased alkalinity may promote greater fecundity but also greater sensitivity to Pb. Surprisingly, HA reduced total reproductive output at the high Pb concentration. This effect was apparently due more to a reduced number of clutches laid than to clutch size. Because

one member of the breeding pair occasionally perished before 21 d, total reproductive output was also analyzed as eggs per female per day to eliminate any potential artifact or bias in the data due to mortality. This correction had little influence, however, as differences were similar to those previously described (Figure 4.4.).

To determine whether effects were acute or chronic, switched breeding exposures were carried out in which control breeders were transferred to either high or low Pb conditions and Pb-exposed breeders transferred to tap water without Pb. These experiments were not performed in the high alkalinity and DOC waters, as we anticipated Pb effects far more likely to occur in tap water. Since this was not the case it is difficult to conclude whether the effects observed in the HCO_3^- and HA treatments were due to acute or chronic Pb exposure. However, the findings with switched exposures in tap water were intriguing in that fecundity was often reduced acutely regardless of direction of transfer (Table 4.5.; Figure 4.4B.). These data suggest that fathead minnows may experience reduced reproductive output, at least temporarily, with abrupt fluctuations in Pb exposure. From the present study it cannot be concluded whether any of the observations on fecundity are the result of physiology or behavior; nevertheless, results from the switching experiments may hold particular relevance since in natural environments pollutant loads are often pulsatile.

Overall, fecundity in our control treatments (Figure 4.4.) was low compared to that of Tyler and colleagues (Harries et al., 2000; Thorpe et al., 2007) who reported mean values ranging from 43 to 112 eggs/female/d, but comparable to more commonly reported mean values approximating 20 eggs/female/d by others (for examples see Jensen et al., 2001; Sellin and Kolok, 2006; Watanabe et al., 2007). Thorpe et al. (2007)

suggested that higher fecundity observations might be accounted for by the use of screened trays placed beneath the breeding substrates to collect detached eggs. Although our counts were certainly higher than would have been without a collection tray, our daily reproductive output per female was still less than in the studies with higher fecundity. Water chemistry or other environmental factors and inter-facility variation may represent important factors in explaining these discrepancies.

Indeed, as we have shown in the present study, simple differences in water chemistry can influence reproductive output. This was evident in our HCO_3^- treatment which led to significantly higher fecundity when compared to the control tap water treatment (Table 4.5.). It may be that the higher buffering capacity of the HCO_3^- treatment confers greater overall reproductive fitness, or possibly that higher alkalinity leads to greater sperm motility and therefore greater fertilization and water hardening of eggs. Of course the latter would be reflected in the number of eggs counted only if a loss of unfertilized eggs (by disintegration or paternal ingestion, for example) occurred prior to counting. However, the higher base-line fecundity in the HCO_3^- treatment was decreased by addition of Pb perhaps by countering in some manner any such potential beneficial effects of HCO_3^- to egg fertilization and water hardening.

4.4.7. Egg mass.

Perhaps most surprising from this study was the effect of high Pb on fecundity in the presence of HA and increased alkalinity while no Pb effects were detectable in tap water + Pb only exposures. Consistent with the reduced reproductive output for the HCO_3^- and HA treatments with high Pb was the increase in average egg mass from the

same treatments (Table 4.6.). It is tempting to speculate that, when exposed to Pb, more energy may be directed toward producing fewer, higher quality eggs than in similar water conditions without Pb. Given that no effects on hatchability were observed in the HCO_3^- and HA treatments with high Pb (see below), the greater egg masses in these same groups may support this possibility. No differences were observed for any of the tap water experiments including switched exposures aimed at revealing potential acute effects (Table 4.6.).

4.4.8. Egg lead accumulation.

When exposed to Pb, eggs accumulated similar amounts irrespective of Pb concentration or water chemistry (Table 4.6.). It is possible that Pb accumulated directly from acute waterborne exposure to sperm and/or eggs or, alternatively, was transferred from chronically exposed parents. To evaluate the contributions of each, eggs were analyzed for Pb accumulation from the switched breeding experiments carried out as previously described. Lead accumulated to a great extent (nearly 40 nmol Pb/g) in eggs from control fish transferred to Pb exposure for breeding, indicating that direct acute exposure to the eggs accounts for most of the accumulation rather than parental transfer (Table 4.6.). Nevertheless, a discrepancy seems to exist as egg Pb concentrations were not as high as in the non-switched Pb exposures and the difference seems unlikely accounted for by parental transfer given the low Pb accumulated in eggs from Pb-exposed parents bred in control water. However, the results of Weber (1993) support direct environmental exposure to eggs as the source of Pb accumulation. Furthermore, previous work has shown that waterborne Pb can enter through the zona radiata

(Rombough, 1985) though superficial adsorption to the egg surface could also account for the observed accumulation.

4.4.9. Egg attachment to breeding substrate.

It might reasonably be expected that failure of eggs to attach, or remain attached, to a breeding substrate might lead to drifting or other transport away from a defended nest. Such detached eggs may be subjected to greater predation and infection and thus reduced viability in the wild. Therefore, we recorded separately the number of eggs attached to the PVC breeding substrate and those detached to see if Pb and/or water chemistry had any influence. For the most part, egg attachment was low with nearly 70-80% detached in both tap water and HCO_3^- treatments (Table 4.6.). In comparison, HA promoted attachment (to ~50%) and this result was not influenced by addition of Pb. Humic acid is known to bind biological surfaces, thereby potentially facilitating egg attachment to surfaces with HA staining/deposition. At low levels, Pb negatively impacted attachment slightly in tap water control and HCO_3^- treatments, but this effect was not consistent at the high Pb concentrations. Interestingly, switching either Pb-exposed fish to control water or vice versa seemed to improve egg attachment (Table 4.6.).

4.4.10. Hatchability.

No statistically significant differences in egg hatchability as a function of water chemistry or Pb treatment were observed (data not shown). In fact, percent hatchability

was high overall with a mean±SEM of 89.8±1.4%, *n*=32 (93.5±1.6%, *n*=11 for HA; 87.6±3.2%, *n*=10 for HCO_3^-; 88.1±2.1%, *n*=11 for tap water controls).

4.4.11. Behavior/motor function of offspring.

The impact of Pb as a neurotoxin in mammals is well recognized and has been the focus of much research. Far less effort has centered on Pb neurotoxicty in fish. Weber examined juvenile fathead minnows feeding on *Daphnia magna* (1991) and reproductive behavior of adult fathead minnows (1993) during shorter exposures (4 week) to higher Pb concentrations (2415-4831 nM (500-1000 µg/L)) than that of the present study and found various Pb-induced alterations on feeding and behavior. Other neurological effects include lordoscoliosis and black discoloration of the caudal peduncle as observed in species of trout (Davies et al., 1976; Holcombe et al., 1976). Because fish are more sensitive to toxicants and starvation at early life stages, we decided to investigate Pb effects on prey capture ability of 10 d old larvae. Furthermore, by using F1 offspring we hoped to distinguish effects due to chronic parental Pb exposure from those arising from direct acute waterborne Pb exposure.

Hatched larvae from 21 d breeding experiments were cultured for 10 d by static-renewal in treatments matching those of their respective parents. Initially, the test design was simply to record the time to completion for 5 and 10 ingestions out of 10 possible *Artemia* nauplii in 5 mL of test water within a time limit of 5 min. However, introduction of the *Artemia* typically induced a stress response of frantic swimming followed by a period of rest (typically 1-2 min) during which the larva would remain stationary and unresponsive to *Artemia* that appeared clearly within range of easy and rapid detection.

Usually, a presumed recovery to "normal" swimming (i.e. slower, more controlled) would then commence followed shortly thereafter by active foraging behavior, seemingly upon first notice of the *Artemia* after rest. Often, a larva would not ingest all of the 10 *Artemia*, or even half in some cases, within the given 5 min time period. Considering the variability in startle swimming duration and recovery period, and failures of some larvae to complete all 10 ingestions, we decided to use the interval between the 1^{st} and 5^{th} ingestion as likely representing the most reproducible endpoint to assess prey capture ability.

The percentages of larvae ingesting half (5) of the possible *Artemia* are shown in Table 4.7. Fractions were also included to indicate the number of tests performed per treatment and the number of larvae completing 5 ingestions for the analysis depicted in Figure 4.5. High Pb concentrations significantly increased the duration between the 1^{st} and 5^{th} ingestions in both HA and HCO_3^- treatments, but had no effect on this time in the tap water controls (Figure 4.5.). However, far fewer larvae completed the 5^{th} ingestion in high Pb tap water (44%) compared to the other high Pb treatments indicating protective effects from DOC and HCO_3^- for this endpoint (Table 4.7.). Yet, larvae from control breeders hatched in high Pb tap water were more similar to controls (78% completed), suggesting that the reduced ingestion in high Pb tap water may be due to chronic parental Pb exposure rather than acute larval exposure. Finally, for reasons unknown, larvae seemed to perform faster and ingest more prey in water of higher DOC content (without Pb).

To summarize, high Pb concentrations altered feeding performance in all water treatments, although the influence of water chemistry differed depending on the endpoint

examined. Larvae from DOC and HCO_3^- treatments with high Pb required more time to ingest 5 *Artemia* than those from treatment-matched controls. However, larvae from these same high Pb treatments more frequently completed 5 ingestions than those from tap water with high Pb. Thus, regardless of the exact nature of the effect, these findings indicate that fathead minnows chronically exposed to Pb may produce offspring with ecologically-relevant behavioral impairment. However, clarifying these effects and understanding the role of water chemistry will require more research.

4.5. Conclusions.

We have demonstrated that increased HCO_3^- and DOC (as HA) protect against chronic Pb accumulation by fathead minnows. Yet paradoxically these same parameters appear to augment reproductive toxicity at high Pb concentrations. Indeed, the influences of HCO_3^- and HA on the effects of Pb exposure throughout this study were unexpected and somewhat puzzling. However, these influences were consistent across several of the endpoints examined lending credence to the connectivity of the combined impacts of Pb and water chemistry. Since Pb in either its carbonate- or DOC-bound form is believed to be, for the most part, biologically unavailable the chronic toxicity observed in the HCO_3^- and HA treatments is difficult to explain. Furthermore, in the absence of Pb, addition of HCO_3^- or HA may actually improve various aspects of fathead minnow reproduction. Specifically, HCO_3^- may increase basal fecundity and HA may improve egg attachment to a breeding substrate as well as the ability of hatched larvae to capture prey. In the end, the unexpected nature of the observed Pb effects on reproduction, and their apparent interactions with water chemistry which cannot be explained at present, suggest that more

research is needed if we are to understand the true nature of chronic waterborne Pb toxicity to fathead minnows.

Table 4.1. Chemistry of test media (mean±SEM in µM except for hardness (Hard.) which is expressed as mg/L as calculated by APHA Standard Methods; *n*=22 except for pH *n*=29, CO_2 *n*=34, and DOC *n*=25). Water temperature mean ± SEM throughout the exposures was 22±1°C.

	$[Na^+]$	$[K^+]$	$[Ca^{2+}]$	$[Mg^{2+}]$	$[Cl^-]$	$[SO_4^{2}$-]	$[CO_2]$	[DOC]	Hard.	pH
Control tap H_2O	2528 ±180	100 ±5	619 ±28	274 ±16	2280 ±133	180 ±18	701 ±11	257 ±8	91 ±4	8.1 ±0.1
+500 µM $NaHCO_3$	3066 ±184	102 ±4	639 ±23	275 ±13	2206 ±153	169 ±16	1191 ±23	257 ±12	93 ±3	8.3 ±0.1
+4 mg/L humic	2573 ±176	104 ±4	636 ±22	278 ±13	2249 ±145	167 ±18	690 ±23	318 ±8	93 ±3	8.0 ±0.1

Table 4.2. Summary of nominal and mean±SEM measured waterborne Pb concentrations over full duration of study (in nM and (µg/L), *n*=71 for controls and 75-78 for +Pb).

	Nominal	Tap	HCO_3^-	Humic
Control		0.8±0.3 (0.2±0.1)	1.3±0.3 (0.3±0.1)	1.4±0.4 (0.3±0.1)
Low Pb	170 (35)	137±5 (28±1.1)	148±6 (31±1.2)	144±7 (30±1.4)
High Pb	580 (120)	506±23 (105±4.8)	547±22 (113±4.6)	542±22 (112±4.5)

Table 4.3A. Summary of mean±SEM body masses (mg; *n* are denoted in parentheses) during first 150 days of exposure ±low and high concentrations of Pb in different test treatments.

	Exposure time in days		
Treatment	4 (18)	30 (12)	150 (12)
Tap control	4.2±0.5	46±3	1334±146
Tap + low Pb	3.5±0.2	33±3	1426±99
Tap + high Pb	2.5±0.3[a]	43±7	1344±133
HCO_3^- control	3.8±0.1	37±4	1082±156
HCO_3^- + low Pb	3.9±0.3	34±4	1276±126
HCO_3^- + high Pb	2.8±0.1[a]	56±7[a]	1274±148
Humic control	4.1±0.3	34±5	1341±164
Humic + low Pb	3.9±0.1	37±3	1399±100
Humic + high Pb	2.8±0.3[a]	50±7	1317±161

[a]Denotes statistically significant difference vs. corresponding control and low Pb at same time point as determined by two-way ANOVA.

Table 4.3B. Summary of mean±SEM body masses (g) following each of 3 rounds of 21 d reproduction assays in different test media (*n*=4 unless noted otherwise in parentheses) and final sex ratios (male:female, *n*=3) at conclusion of study (includes pairs used for reproduction).

		Round (approximate age)			
Treatment	Sex	1 (230 d)	2 (270 d)	3 (300 d)	Ratio
Tap control	♂	4.23±0.47	4.60±0.47	4.78±0.41	0.99±0.12
	♀	1.40±0.21 (3)	1.77±0.07 (3)	1.40±0.06 (3)	
Tap + low Pb	♂	3.13±0.42	4.95±0.15	5.43±0.50	0.95±0.08
	♀	1.53±0.08	1.55±0.15	1.87±0.03 (3)	
Tap + high Pb	♂	3.70±0.25 (3)	2.83±0.47 (3)	4.40±0.46	1.00±0.04
	♀	1.48±0.19	2.15±0.57	1.45±0.14	
HCO_3^- control	♂	4.00±0.48	4.23±0.34	4.03±0.09 (3)	0.96±0.20
	♀	1.55±0.16	2.80 (1)	1.78±0.21	
HCO_3^- + low Pb	♂	4.28±0.30	5.05±0.31	5.73±0.68	1.41±0.19
	♀	1.67±0.03 (3)	1.80±0.50	1.75±0.23	
HCO_3^- + high Pb	♂	3.55±0.25	3.53±0.49	4.33±0.55	1.37±0.19
	♀	1.40±0.11	1.78±0.33	1.65±0.10	
Humic control	♂	3.38±0.25	4.95±0.42	4.88±0.43	0.76±0.05
	♀	1.63±0.19	2.00±0.21	2.03±0.14	
Humic + low Pb	♂	4.08±0.35	4.35±0.21	4.10±0.07	0.90±0.11
	♀	1.80±0.06	1.88±0.12	1.77±0.03 (3)	
Humic + high Pb	♂	3.55±0.29	4.75±0.36	3.80±0.27	1.10±0.10
	♀	1.53±0.34 (3)	1.73±0.13	1.57±0.32 (3)	

No statistically significant differences in mass within sex were determined by two-way ANOVA.

Table 4.4. Concentrations (in nM) for major Pb species within each test media as predicted by the biotic ligand model. Values for $Pb(OH)_2$, $Pb(OH)_3^-$, $PbSO_4$, $PbCl^+$ and $PbCl_2$ were < 0.03 nM (< 0.01%) in all cases. Relative abundances expressed as the percentage of total dissolved Pb are provided in parentheses.

	Pb^{2+}	$PbOH^+$	$PbCO_3$	$Pb(CO_3)_2^{2-}$	Total Organic Pb
Tap Control	4.9×10^{-4} (0.061)	7.6×10^{-4} (0.095)	0.020 (2.53)	1.9×10^{-4} (0.023)	0.78 (97.3)
Tap Low Pb	0.091 (0.066)	0.14 (0.103)	3.8 (2.74)	0.035 (0.025)	133 (97.1)
Tap High Pb	0.42 (0. 084)	0.66 (0. 131)	18 (3.46)	0.16 (0. 032)	487 (96.3)
HCO_3^- Control	5.3×10^{-4} (0.040)	1.3×10^{-3} (0.099)	0.058 (4.43)	1.4×10^{-3} (0.111)	1.2 (95.3)
HCO_3^- Low Pb	0.065 (0.044)	0.16 (0.108)	7.1 (4.81)	0.18 (0.120)	140 (94.9)
HCO_3^- High Pb	0.30 (0.056)	0.75 (0. 136)	33 (6.09)	0.83 (0. 152)	512 (93.6)
Humic Control	6.4×10^{-4} (0.046)	7.9×10^{-4} (0.057)	0.021 (1.47)	1.5×10^{-4} (0.011)	1.4 (98.4)
Humic Low Pb	0.072 (0.048)	0.089 (0.060)	2.3 (1.56)	0.017 (0.011)	146 (98.3)
Humic High Pb	0.31 (0.058)	0.39 (0. 071)	10 (1.86)	0.072 (0.013)	531 (98.0)

Table 4.5. Fathead minnow 21 d reproductive output per breeding pair (mean±SEM). Parentheses indicate total values for 3 rounds combined except for switched exposures (2 rounds combined).

	Avg. Tot. # Eggs Laid	Avg. # Clutches	Avg. Clutch Size
Tap Control	334±68 (4006)	3.5±0.5 (42)	95.4 ±9.6
Tap + low Pb	416±62 (4991)	4.5±0.4 (54)	90.8 ±8.3
Tap + high Pb	320±44 (3834)	3.0±0.4 (36)	109.3 ±11.9
Tap (low Pb breeders)[c]	349±44 (2789)	4.3±0.5 (34)	82.03 ±10.34
Tap (high Pb breeders)[c]	134±47 (1073)[b]	1.9±0.4 (15)[b]	71.5 ±11.1
Tap + low Pb (cont. breeders)[c]	149±38 (1195)	2.8±0.4 (22)	52.9 ±9.9[b]
Tap + high Pb (cont. breeders)[c]	97±41 (774)[b]	1.6±0.7 (13)[b]	59.5 ±13.9
HCO_3^- Control	546±68 (6557)[b]	4.1±0.3 (49)	134.0 ±10.4[b]
HCO_3^- + low Pb	345±69 (4140)[a]	3.2±0.5 (38)	112.2 ±11.6
HCO_3^- + high Pb	256±42 (3067)[a]	3.0± 0.3 (36)	85.2 ±8.8[a]
Humic Control	467±77 (5600)	4.1± 0.5 (49)	114.3 ±9.7
Humic + low Pb	413±67 (4950)	3.0± 0.5 (36)	137.7 ±9.6
Humic + high Pb	195± 49 (2336)[a]	2.1± 0.4 (25)[a]	92.6 ±12.7

[a-b]Denotes statistically significant differences as determined by Student's *t*-test as follows: vs. [a]treatment-matched control; [b]vs. tap control.
[c]Denotes switched breeding exposures in which control breeders were transferred to either high or low Pb conditions and Pb-exposed breeders transferred to tap without Pb.

Table 4.6. Effect of Pb and water chemistry on fathead minnow egg mass (*n*=2-6 clutches) and egg Pb accumulation (*n*=4-12 clutches) and attachment to PVC breeding substrate[f] (*n*=13-54) (mean±SEM).

	Egg Mass (mg)	Pb Accum. (nmol/g)	% Detached
Tap Control	1.52±0.09	0.26±0.15	80.8±3.3
Tap + low Pb	1.55±0.07	75.9±28.1[a]	87.0±3.0[b]
Tap + high Pb	1.59±0.10	92.6±17.8[a]	72.7±4.3
Tap (low Pb breeders)[e]	1.41±0.10	0.58±0.41	41.3±4.5[b]
Tap (high Pb breeders)[e]	1.64±0.10	1.21±1.54	66.2±5.8[b]
Tap + low Pb (cont. breeders)[e]	1.46±0.12	35.9±8.9[b]	73.3±5.7[d]
Tap + high Pb (cont. breeders)[e]	1.53±0.02	39.2±10.9[b, c]	61.6±10.0
HCO_3^- Control	1.61±0.08	0.99±1.16	74.4±3.3
HCO_3^- + low Pb	1.50±0.07	59.5±22.2[a]	83.4±2.8
HCO_3^- + high Pb	1.81±0.12[a]	93.0±33.1[a]	63.5±4.7
Humic Control	1.52±0.07	0.36±0.14	51.4±4.6[b]
Humic + low Pb	1.47±0.08	72.7±21.9[a]	50.7±4.4
Humic + high Pb	1.99±0.10[a]	85.9±12.2[a]	44.7±5.3

[a]Denotes statistically significant differences from treatment-matched control as determined by two-way ANOVA with Pb concentration and water chemistry as variables.
[b-c]Denotes statistically significant differences as determined by Student's *t*-test as follows: [b]vs. control tap water; [c]vs. tap + high Pb; [d]vs. tap + low Pb.
[e]Denotes switched breeding exposures in which control breeders were transferred to either high or low Pb conditions and Pb-exposed breeders transferred to tap without Pb.
[f]Proportions of detached eggs per clutch were arcsine transformed to obtain normal distribution of data.

Table 4.7. Effect of Pb and water chemistry on ability of 10 d old fathead minnow larvae to ingest 5 of 10 *Artemia* nauplii in 5 mL of treatment water within 5 minutes. Number of clutch replicates shown in parentheses.

	% Ingesting Half	Fraction
Tap Control	87.5	21/24 (2)
Tap + low Pb	84.4	27/32 (3)
Tap + high Pb	43.5	17/33 (3)
Tap + high Pb (switched)[a]	77.8	14/18 (2)
HCO_3^- Control	61.5	16/26 (2)
HCO_3^- + low Pb	79.2	19/24 (2)
HCO_3^- + high Pb	70.0	14/20 (2)
Humic Control	92.3	24/26 (2)
Humic + low Pb	95.7	22/23 (2)
Humic + high Pb	80.0	26/30 (3)

[a]Denotes switched breeding exposures in which control breeders were transferred to high Pb conditions.

Figure 4.1. Flow chart of overall experimental design highlighting the major endpoints of the study. Fathead minnows were exposed to 0, 145 nM Pb (30 µg Pb/L) or 530 nM Pb (110 µg Pb/L) in different water chemistries to investigate the influence of alkalinity and DOC on chronic Pb toxicity. Lead exposures were administered to 8 d old fathead minnow larvae for 230-300 d and through 3 subsequent rounds of 21 d breeding assays. Eggs were counted daily (fecundity) and collected for hatchability or determination of mass and Pb accumulation. Eggs collected for hatchability were also exposed to Pb through 10 d post-hatch after which larvae were used in prey capture assays to evaluate behavior/motor impairment.

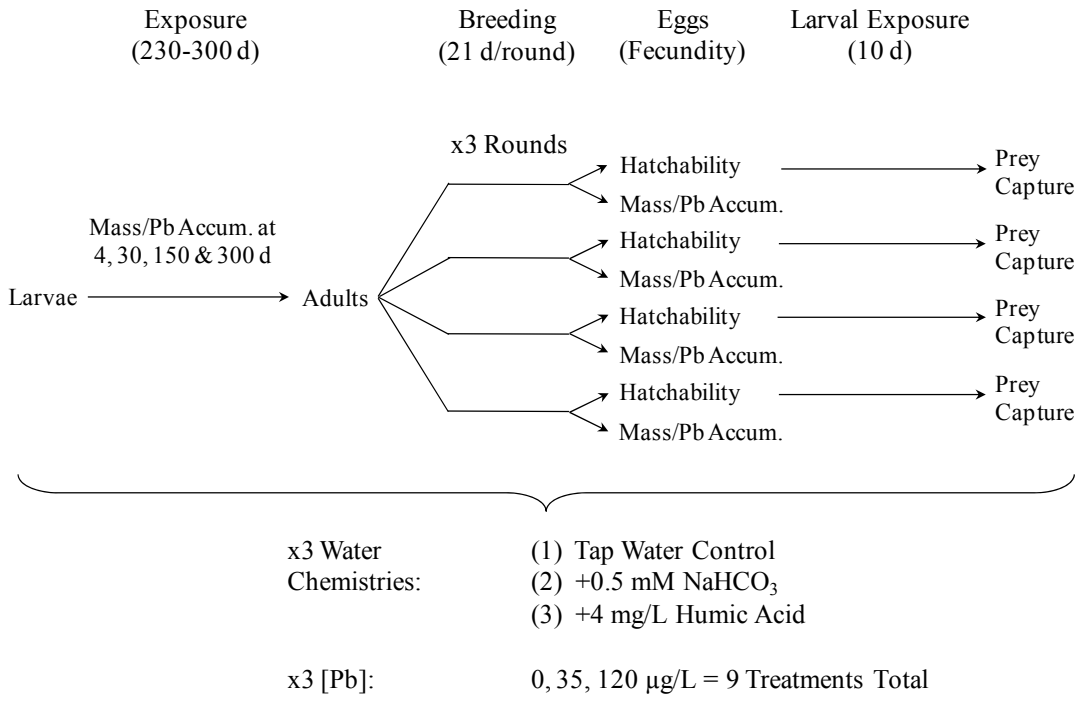
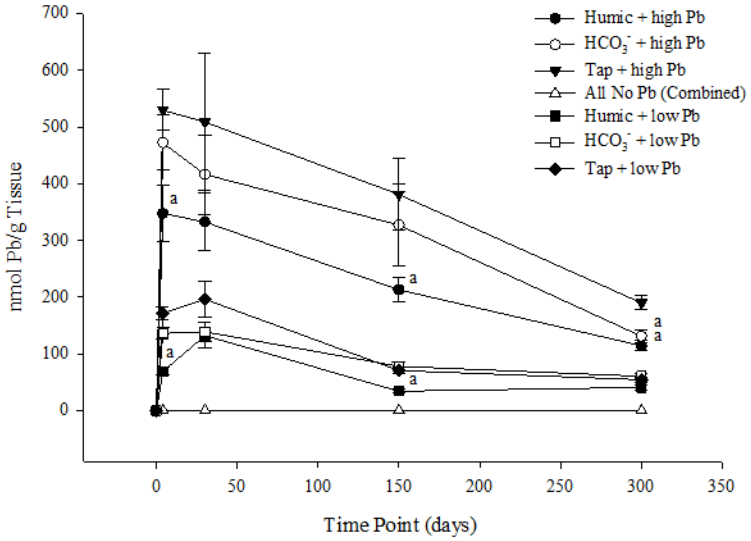


Figure 4.2. Influence of HCO_3^- and HA on whole body Pb accumulation in fathead minnows exposed to either low (145 nmol/L) or high (530 nmol/L) Pb concentrations. Lead burdens are reported per wet weight of tissue (A) and per fish to illustrate an apparent growth dilution that effect persists to 300 d in all cases except for HCO_3^- and HA high Pb treatments (B). Mean±SEM, n=3 for 4 d, n=12 for 30 and 150 d, and n=6 for 300 d. Statistically significant difference from [a]tap water + Pb at same time point and Pb concentration determined by a two-way ANOVA with time and water chemistry as variables followed by Bonferroni-corrected Student's t-test. All Pb-exposed fish at all time points accumulated significantly more Pb than unexposed controls (not indicated by symbols).

(A)



(B)

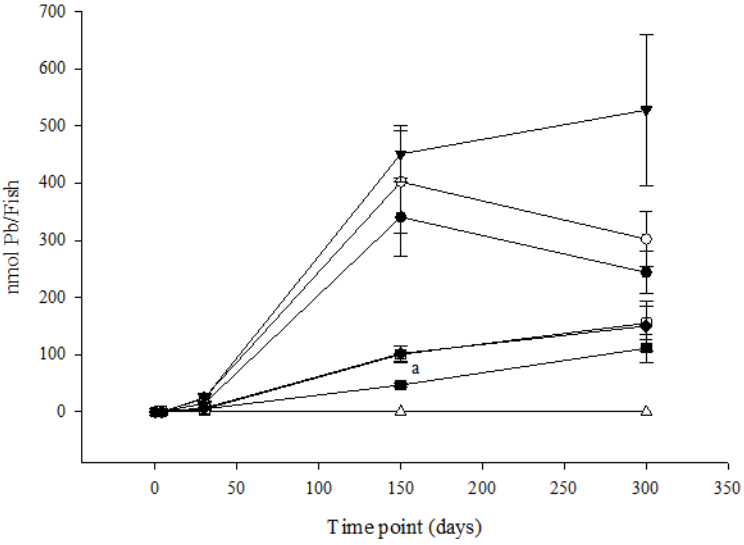


Figure 4.3. Influence of HCO_3^- and HA on Pb accumulation (A-B) and internal distribution (C-D) of selected fathead minnow tissues following 300 d exposures to either low (145 nmol Pb/L; left side) or high (530 nmol Pb/L; right side) Pb concentrations. Mean±SEM, *n*=6, except for testes and ovaries in lower panels *n*=3. Statistically significant difference by Student's *t*-test from [a]tap water + Pb at same Pb concentration.

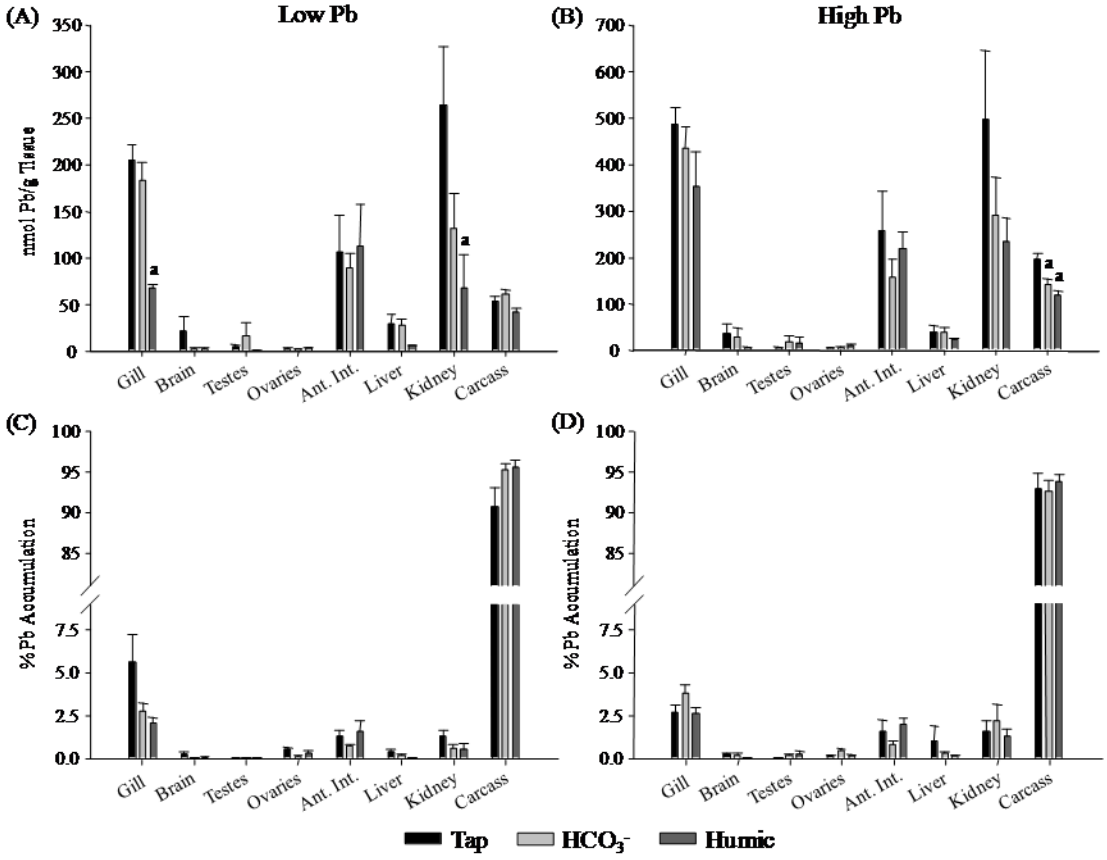
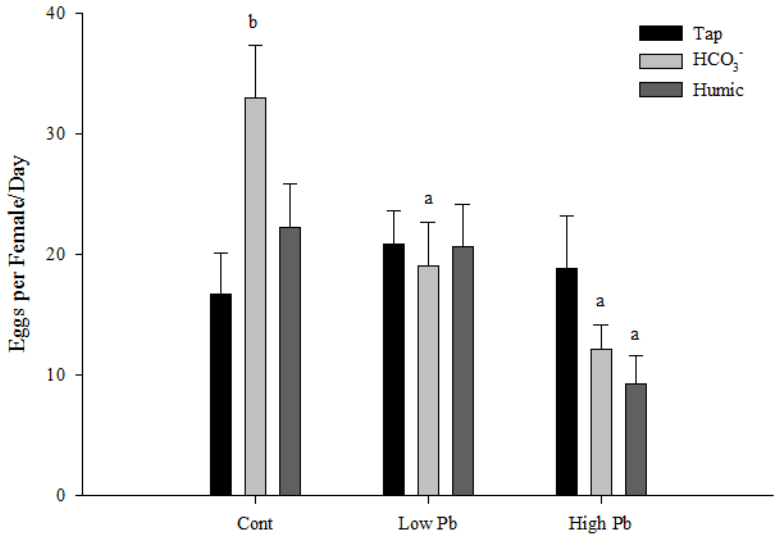


Figure 4.4. Influence of HCO_3^- and HA on Pb-induced effects on fathead minnow fecundity. Fish were exposed to either low (145 nmol Pb/L) or high (530 nmol Pb/L) Pb concentrations. Data are presented as total number of eggs per breeding pair normalized to the number of days each breeding pair was together (i.e. days lost to mortality not included). Mean±SEM, n=12 in all cases. Statistically significant difference from treatment-matched control (a) or from tap control (b) as determined by Student's t-test.

(A)



(B)

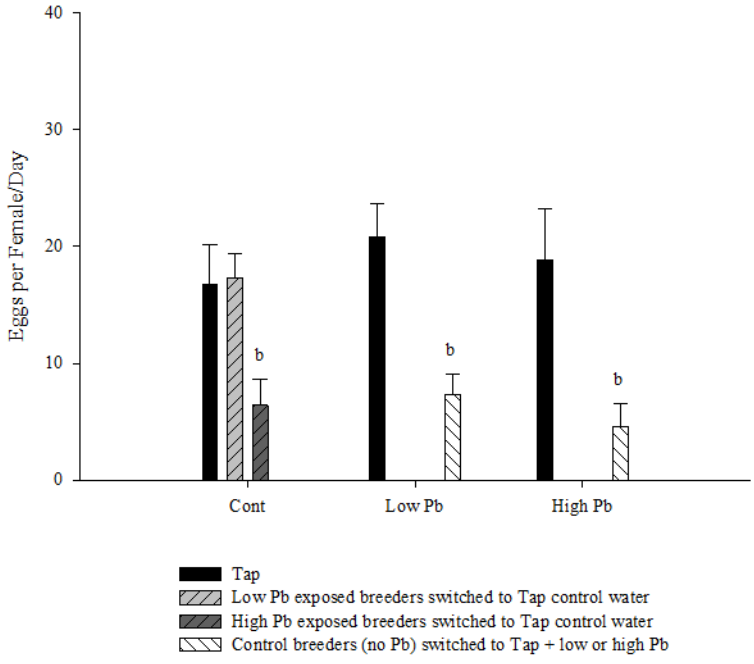
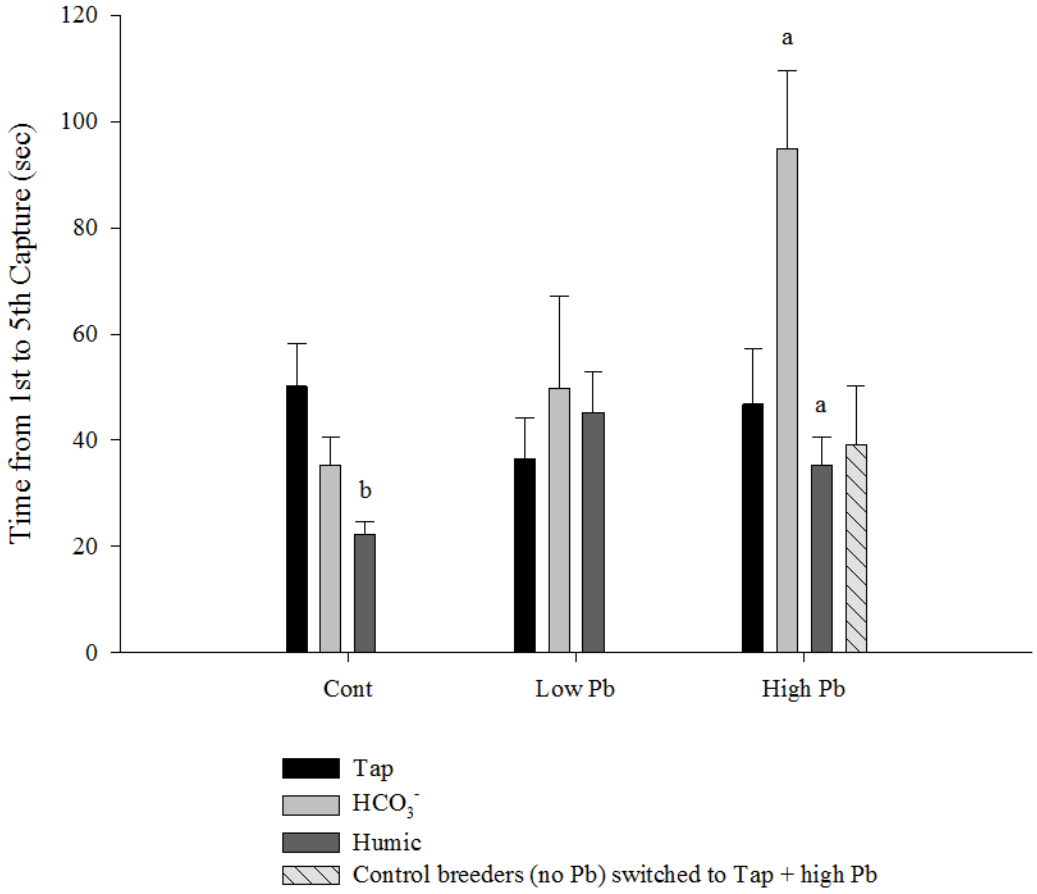


Figure 4.5. Lead and water chemistry influence on 10 d larval fathead minnow prey capture ability. Values represent mean±SEM durations between 1st and 5th ingestions; *n*=13-26. Eight-14 larvae were tested from each of 2-3 replicate beakers. Those not completing 5 ingestions were analyzed separately (see Table 4.7.). Statistically significant difference from [a]treatment-matched control or from [b]tap water control as determined by Student's *t*-test.



CHAPTER 5.

THE EFFECTS OF ACUTE AND CHRONIC WATERBORNE LEAD EXPOSURE ON THE SWIMMING PERFORMANCE AND AEROBIC SCOPE OF FATHEAD MINNOWS (*PIMEPHALES PROMELAS*)

5.1. Summary.

While there is considerable evidence indicating the effects of sublethal Pb exposure are consistent between mammals and fish, involving primarily hematological and neurological dysfunction, links to higher order effects of ecological relevance (e.g. swimming performance) remain poorly defined. In the present study, fathead minnows (*Pimephales promelas*) were subjected to an incremental velocity test using swim tunnel respirometery for analysis of aerobic scope and swimming performance, as critical aerobic swim speed (U_{crit}), following chronic exposures (33-57 d) to Pb concentrations of 0.9±0.4, 157±18 or 689±66 nmol L^{-1} Pb and an acute exposure (24 h) to 672±35 nmol L^{-1} Pb (means ± SEM). Blood samples were subsequently collected to measure hemoglobin (Hb) concentrations for an assessment of Pb-induced anemia. Neurological impairment was evaluated indirectly using a cost of transport (COT) analysis derived from data collected during swimming respirometery. Fish from the acute 672±35 nmol L^{-1} Pb treatment (24.4±1.2 BL sec^{-1}) and chronic 689±66 nmol L^{-1} Pb treatment (24.6±0.9 BL sec^{-1}) exhibited reduced U_{crits} compared to control fish (27.6±0.8 BL sec^{-1}). Oxygen consumption results revealed a reduced aerobic scope in the acute Pb treatment (8.6±2.6 μmol O_2 g^{-1} h^{-1} vs. 22.6±3.8 μmol O_2 g^{-1} h^{-1} from controls) that was attributed to a decrease in maximum oxygen consumption rates (38.8±0.8 μmol O_2 g^{-1} h^{-1} vs. 54.0±4.2

μmol O_2 g^{-1} h^{-1} from controls). While no statistically significant differences were observed for hemoglobin concentrations or COT, several trends are discussed that may support a subtle chronic neurological impairment to U_{crit}. These findings suggest that the mechanisms of impaired swimming performance arising from acute and chronic Pb exposure are different and may involve a transition from an acute effect of reduced oxygen uptake at the gill, potentially owing to morphological alterations that cause an increase in diffusion distance, to chronic effects on motor function.

5.2. Background.

Lead (Pb) is a non-essential heavy metal that has been used for a variety of applications throughout human history owing to its unique chemical and physical properties (e.g. low melting temperature, high malleability and resistance to corrosion). Today, most concern for Pb entering aquatic environments is from point-source discharges related to mining and smelting of Pb ores, largely for use in the production of storage batteries; however, natural sources such as erosion and atmospheric fallout from volcanoes and forest fires also contribute (Nriagu and Pacyna, 1988; World Health Organization, 1995). Lead has been shown to have toxic effects on a variety of freshwater organisms with sensitivity as low as 19 nmol L^{-1} (4 μg L^{-1}) (Grosell et al., 2006b). With respect to fish, the acute toxicity of Pb is putatively due to a mucus-induced respiratory asphyxiation under extreme conditions (Carpenter, 1927; Westfall, 1945) and the disruption of Ca and Na homeostasis in more environmentally relevant Pb concentrations (Birceanu et al., 2008; Patel et al., 2006; Rogers et al., 2003). The chronic toxicity of Pb, on the other hand, is generally consistent between fish and

mammals, involving primarily neurological (Davies et al., 1976; Holcombe et al., 1976) and hematological (Hodson et al., 1978a) dysfunction.

Such sublethal effects of Pb could lead to higher order effects, such as reduced swimming performance, with important ecological ramifications. For example, the neurological effects of Pb potentially involve disruption of the coordinated sensory-motor responses required for capturing prey and eluding predation. Indeed, there are several lines of evidence to this effect. Lead has been shown to increase feeding duration (Mager et al., 2010b; Weber et al., 1991; Weis and Weis, 1998), number of feeding miscues (Weber et al., 1991; Weis and Weis, 1998) as well as a reduced ability to avoid predation (Weis and Weis, 1998). Additionally, the Pb-induced developmental abnormality of lordoscoliosis commonly observed in salmonids is likely a direct result of neurological damage (Davies et al., 1976). The effect has significant implications for reproductive success as spawning mobility becomes severely limited as a result of the spinal curvature (Holcombe et al., 1976).

Impaired swimming performance could also arise from Pb-induced hematological effects that cause a reduction in oxygen carrying capacity (anemia). Lead-induced anemia is presumably the result of two separate, but related, effects, namely reduced heme synthesis and the destruction of mature erythrocytes (Goyer and Clarkson, 2001). The former is largely due to inhibition of the enzyme, delta-aminolevulinic acid dehydratase (ALAD, also known as porphobilinogen synthase), while the latter appears to occur as a result of reactive oxygen species- (ROS-) mediated hemolysis. Inhibition of ALAD not only impairs heme synthesis but also contributes to oxidative stress due to the accumulation of ALA which has been linked to the production of ROS via oxidative

interactions with oxyHb (Monteiro et al., 1989) and ferritin (Oteiza et al., 1995; Rocha et al., 2003). Lead can also form ROS such as hydrogen peroxide and singlet oxygen via lipid peroxidation events (Sanders et al., 2009) thereby adding to the oxidative stress involved in hemolysis. Such ROS may also contribute to neuronal death, thus providing another possible mechanism for Pb-induced sensory-motor and behavioral impairment (Sanders et al., 2009).

From our recent studies investigating the toxicity of Pb to the fathead minnow (*Pimephales promelas*), a number of effects were observed supporting hematological and neurological dysfunction as potential toxic mechanisms of Pb. A time course toxicogenomic analysis of fathead minnows exposed to 170 nmol L^{-1} Pb (35 μg L^{-1} Pb) for 150 d revealed several genes indicating responses likely involved in ROS detoxification and anemia (Mager et al., 2008). In support of a neurological effect of Pb, a subsequent full life cycle (>300 d) investigation revealed an impaired prey capture ability in F1 larval fathead minnows exposed to 580 nmol L^{-1} Pb (120 μg L^{-1} Pb) (Mager et al., 2010b). We therefore undertook the present study to investigate whether the potential hematological and neurological effects of chronic Pb exposure suggested by our previous studies translated into higher order effects on swimming performance (as critical aerobic swim speed) and aerobic scope as assessed using a swim tunnel respirometer and the incremental velocity test (Blazka et al., 1960). Hemoglobin (Hb) concentrations were measured to evaluate potential hematological responses to Pb, whereas oxygen consumption data were used to generate cost of transport (COT) values as an indirect assessment of motor function impairment by Pb. That is, we assumed that a Pb effect on motor function would lead to less efficient swimming and therefore greater COT. To

facilitate a direct comparison with our previous studies (Mager et al., 2010b; Mager et al., 2008), Pb exposures in the present study targeted the 170 and 580 nmol L^{-1} Pb (35 and 120 μg L^{-1} Pb) concentrations used previously.

5.3. Materials and methods.

5.3.1. Experimental animals.

Fathead minnows (*Pimaphales promelas*) were obtained from Aquatic Biosystems, Inc. (Fort Collins, CO). Upon arrival fish were gradually acclimated to a low-ionic strength tap water by receiving a constant flow of 2:1 dechlorinated Virginia Key tap water:deionized water for 48 h and then the reverse ratio for the remainder of the experiment using a gravity flow-through approach as previously described (Grosell et al., 2006a). For the chronic Pb exposures, fish were obtained at <24 h post-hatch and fed *ad libitum* a daily diet of *Artemia* nauplii for the first 20 d, a mixture of *Artemia* plus Tetramin flake food for the next 10 d and then flake food only thereafter. For the acute Pb exposures, fish were obtained at approximately 30 d of age and were fed flake food daily *ad libitum*. Prior to feeding, the bottoms of all exposure chambers were siphoned to remove feces and any leftover food from the previous day. Fish used for swim respirometery were starved for at least 24 h prior to initiation of the experiment.

5.3.2. Lead exposures and sampling protocol.

Lead concentrations were maintained for all tests by addition of concentrated $PbNO_3$ (Sigma-Aldrich, St. Louis, MO) delivered at a constant rate of 1 ml min^{-1} to vigorously aerated mixing chambers using Mariotte bottles. Water was drained from the

mixing chambers into 2 L exposure chambers at a rate of 15 ml min^{-1}. Addition of Pb was started 2-3 d prior to introducing fish to the exposure chambers to allow time for equilibration. Chronic Pb exposures targeting nominal Pb concentrations of either 0, 170 or 580 nmol L^{-1} Pb were initiated with 8 d old larvae and continued until time of collection for swim respirometery. Beginning at approximately 41 d of age (33 d of chronic Pb exposure), 2-3 fish were swum per day (1 at a time), alternating treatments with each day to avoid a significant time lag between treatments. To obtain a minimum *n* of 8 for each Pb treatment sampling was performed over a period of several weeks for the swimming experiments and therefore the durations of chronic Pb exposures ranged from 33-57 d with a mean of 43 d (mean 51 d of age). Following completion of the chronic Pb exposures, acute Pb exposures were performed with the fish obtained at 30 d of age using 2-3 fish per day (mean 46 d of age) by transferring from control water to 580 nmol L^{-1} Pb 24 h prior to swim respirometery. Control fish were swum for both acute and chronic exposures (n = 7 from each). As no significant differences were observed between the acute and chronic control groups, data were pooled (n = 14) for all comparisons with Pb treatments.

5.3.3. Swim tunnel respirometery.

Automated intermittent flow respirometery was performed using a miniature Blazka-type variable speed respirometer with a DAQ-1 control device and the AutoResp1 version 1.7 software (Loligo Systems, Denmark) (Blazka et al., 1960; Steffenson, 1989). Briefly, the system consists of a small swim tunnel (0.17 L) submerged inside an ~8 L reservoir of well-aerated water used for flushing the tunnel after each closed cycle.

Water velocity was initially calibrated using stop-motion video and a ruler fastened above the swim tunnel to measure the velocity of a dye injected into the submerged swim tunnel at various speeds. Once placed in the swim tunnel, monitoring of the fish using a small video camera connected to a computer, as well as manual adjustments to swim speed, were performed remotely from an area partitioned off from the respirometer to avoid disturbing the fish. Oxygen measurements via a Pt100 fiber optic probe and temperature were collected using the Fibox 3 Minisensor Oxygen Meter (Loligo Systems, Denmark). Each day prior to use the system was thoroughly cleaned and the oxygen sensor was calibrated using two partial pressures of O_2. The first (maximum O_2 saturation) was established by vigorous aeration with an air stone and the second (complete absence of O_2) was achieved using a solution of 10 g L^{-1} Na_2SO_3 (Sigma-Aldrich, St. Louis, MO). Control experiments were periodically conducted using an empty swim tunnel to confirm that background microbial O_2 consumption was negligible.

Preliminary O_2 consumption tests were performed overnight to establish an acceptable duration for acclimation of the fish to the swim chamber as evaluated by the time necessary to achieve a stable routine metabolic rate. A minimal water velocity of 0.7 cm sec^{-1} was used during these tests to maintain mixing within the swim chamber without forcing the fish to exercise. From these initial experiments a stabilized routine metabolic rate was typically established by 1 – 1.5 h. Thus, prior to swimming, fish were initially acclimated at a flow rate of 0.7 cm sec^{-1} for a minimum of 1-1.5 h or longer if necessary until 2 consecutive 30 min measurements of MO_2 were approximately the same.

To measure U_{crit}, fish were exercised at 30 min intervals beginning with a flow rate of approximately 20 cm sec^{-1} with subsequent increments in flow of 10 cm sec^{-1} every interval until the fish was exhausted. Exhaustion was designated as when the fish became pinned against the back screen of the tunnel and would not regain activity after briefly decreasing flow and then returning to the last speed achieved. The duration (T, in sec) at the final swim speed (V_f, in cm sec^{-1}) was recorded and the U_{crit} (in cm sec^{-1}) calculated using the following equation originally described by Brett (1964):

(Eq. 1.) $U_{crit} = [V_f + (T/t)dV]/cm$

where t is the time interval (30 min) and dV is the increment in swim speed (10 cm sec^{-1}). Upon completion of the swimming experiment, the fish was removed and total body length measured to transform U_{crit} values to body lengths (BL) per second. As the cross-sectional areas of fish did not exceed 10% of the cross-sectional area of the swim tunnel, corrections for solid blocking effects were not made for measured swimming velocities (Smit et al., 1971; Webb, 1971). A regression equation was derived for each fish by plotting the logarithm of oxygen consumption versus swimming speed to estimate basal MO_2 (y intercept), maximum MO_2 (at U_{crit}) and aerobic scope (maximum – basal MO_2).

5.3.4. Hemoglobin concentration.

Fish used for swim respirometery were subsequently sacrificed for analysis of hemoglobin concentration. Following a blow to the head to stun the fish, the gill was lacerated with a scalpel and blood was collected using a 1-5 µL calibrated pipet (Drummond Scientific Co., Bromall, PA) pre-filled with 0.5 µL 0.5 M EDTA, pH 8 (Ambion, Austin, TX). The total volume within the pipet was then measured and the

EDTA volume subtracted to obtain the volume of blood collected. Samples were then mixed with 20 µL Drabkin's Reagent with Brij 35 (Sigma-Aldrich, St. Louis, MO), allowed to stand at room temperature for at least 15 min and then measured at 540 nM using a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE). Hemoglobin concentrations were determined from a standard curve made with human hemoglobin (Sigma-Aldrich, St. Louis, MO) spanning the range of measured experimental values.

5.3.5. Cost of transport.

To calculate COT, the oxygen consumption rate (mg O_2 kg^{-1} h^{-1}) was converted to mg O_2 kg^{-1} sec^{-1}, multiplied by the oxycaloric value of 14.1 J mg^{-1} O_2 and then divided by the corresponding swimming speed (in m sec^{-1}) to obtain the final units of J kg^{-1} m^{-1} (Videler, 1993).

5.3.6. Water chemistry.

For the chronic exposures, Pb concentrations were measured daily for the first 3 days of exposure and then once a week thereafter. All other water chemistry parameters were measured on a weekly basis. For the acute exposures, Pb concentrations were measured at the onset and completion (i.e. 24 h later) of exposure. For dissolved Pb, water samples were first passed through a 0.45 µm cellulose syringe filter (Acrodisc, Pall Life Sciences, MI), acidified to 1% HNO_3 (Fisher Scientific, trace metal grade) and concentrations measured via graphite furnace atomic absorption spectroscopy (Varian 200Z, Varian, Australia). Flame atomic absorption spectroscopy (Varian 220FS, Varian, Australia) and anion chromatography (DIONEX DX120, CA) were used to measure

concentrations of major cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) and anions (Cl^- and SO_4^{2-}), respectively. Total CO_2 (total inorganic carbon) was measured using a Corning 962 carbon dioxide analyzer (UK) and pH was measured using a PHM201 meter (Radiometer, Copenhagen, Denmark) fitted with a combination glass electrode calibrated with IUPAC standards (Radiometer, Copenhagen, Denmark) prior to each use. Concentrations of DOC were determined by high temperature catalytic oxidations using a Shimadzu total organic carbon-VCSH analyzer (Kyoto, Japan) (Hansell and Carlson, 2001).

5.3.7. Statistical analysis.

Data are presented as means ± 1 standard error of the mean (SEM). Differences were tested for statistical significance by Student's *t*-test for comparisons involving controls and acute Pb treatments, or one-way analysis of variance (ANOVA) for comparisons involving controls and chronic Pb treatments using pairwise multi-sample comparison corrections (Fisher LSD Method) as appropriate. Cost of transport comparisons were analyzed separately for acute and chronic Pb exposures using two-way ANOVA. In all cases, differences were deemed significant at $P < 0.05$.

5.4. Results.

5.4.1. Lead exposures.

Mean ± SEM values for measurements of dissolved Pb concentrations and general water chemistry parameters are provided in Table 5.1. For the high Pb treatments, measured dissolved Pb concentrations were higher than the targeted nominal value of 580

nmol L^{-1}, although mean values were similar between acute and chronic exposures. Conversely, for the chronic low Pb treatment, the measured dissolved Pb concentration was slightly lower than the targeted nominal value of 170 nmol L^{-1}.

5.4.2. Swimming performance.

Maximum aerobic swim speeds (U_{crit}) were extrapolated from data collected during swim respirometery experiments to evaluate whether acute and/or chronic Pb exposure impairs the swimming performance of fathead minnows. Fish from the acute 672±35 nmol L^{-1} Pb treatment and chronic 689±66 nmol L^{-1} Pb treatment exhibited reduced U_{crits} compared to control fish, whereas fish from the chronic 157±18 nmol L^{-1} Pb treatment were not statistically different from controls (Figure 5.1.).

5.4.3. Oxygen consumption.

Oxygen consumption measurements obtained during swim respirometery experiments revealed a significantly reduced aerobic scope and MO_{2max} by fish exposed acutely to 672±35 nmol L^{-1} Pb compared to controls (Figure 5.2.). While the differences were not statistically significant, there was a trend for higher basal and maximum oxygen consumption rates in fish chronically exposed to either 157±18 or 689±66 nmol L^{-1} Pb. Mean ± SEM values for masses and total body lengths of fathead minnows measured just before and after swim respirometery, respectively, are provided in Table 5.2. Fish from the chronic low Pb treatment were significantly larger than control fish both in terms of mass and body length and fish from the acute high Pb treatment were smaller in mass compared to controls. To evaluate whether differences in body mass may have

contributed to effects on oxygen consumption, one-way analyses of co-variance (ANCOVAs) were performed using the corresponding log-transformed data. A significant relationship was observed between body mass and MO_{2max} ($P = 0.0258$) and aerobic scope ($P = 0.0049$), but not between body mass and MO_{2basal} ($P = 0.9918$). However, none of the relationships were significant when the data from the acute 672±35 nmol L^{-1} Pb treatment were removed from the analyses, indicating a Pb treatment effect was confounding the initial analyses of influence of mass. Thus, although body mass was found to co-vary with MO_{2max} and aerobic scope in the acute 672±35 nmol L^{-1} Pb treatment, the relationship (decrease in MO_{2max} and aerobic scope with a decrease in body mass) was opposite to expectations for a metabolic scaling effect (i.e. increase in MO_{2max} and aerobic scope with a decrease in body mass). Hence, any potential effect due to metabolic scaling should have resulted in increased MO_{2max} in the acute Pb-exposed fish which were the smallest. Therefore, the effect of body mass is a conservative error and the observed reductions in MO_{2max} and aerobic scope can be attributed to acute Pb exposure and not the difference in body mass.

5.4.4. Hemoglobin concentrations.

Concentrations of Hb were measured from fish following swim respirometery experiments to test for an anemic response induced by acute or chronic Pb exposure. Mean ± SEM (n) concentrations (mg mL^{-1}) of fish Hb measured from the control, acute 672±35 nmol L^{-1} Pb, chronic 689±66 nmol L^{-1} Pb and chronic 157±18 nmol L^{-1} Pb treatments were 35±2 (14), 34±4 (8), 38±3 (8) and 41±4 (8), respectively. Although a trend toward an increase in Hb concentration appeared evident in fish chronically

exposed to either 689±66 or 157±18 nmol L^{-1} Pb, no statistically significant differences among the treatments were determined.

5.4.5. Cost of transport.

Metabolic rates and the corresponding swimming speeds obtained during the swim respirometery experiments were used to calculate COT as an indirect assessment of motor function impairment by Pb. Similar trends of decreasing COT with increasing swimming speed were observed regardless of treatment (Figure 5.3.). No statistically significant differences were found among any of the Pb treatments when compared to controls.

5.5. Discussion.

Results from the present study reveal a Pb-induced impairment to the swimming performance (U_{crit}) of fathead minnows following acute and chronic exposures to 672±35 and 689±66 nmol L^{-1} Pb, respectively (Figure 5.1.). However, whereas the reduced U_{crit} from the acute Pb exposure was clearly associated with a reduction in aerobic scope, the chronic effect was not (Figure 5.2.). Two types of stress have been described that could account for the reduced aerobic scope exhibited by fish from the acute Pb exposure: loading stresses that add to routine maintenance costs (MO_{2basal}) and limiting stresses that reduce MO_{2max} (Brett, 1958). Since MO_{2max} was significantly reduced, whereas MO_{2basal} was unchanged compared to controls (Figure 5.2.), the reduction in U_{crit} elicited by the acute Pb exposure appeared to reflect a limiting stress. In light of the lack of a Pb effect on Hb concentration, a potential explanation for the reduced swimming performance displayed by the acute high Pb group is a reduction in aerobic scope owing to changes at

the gill leading to increased gas diffusion distance and reduced efficiency in oxygen uptake (i.e. gill morphology, mucus secretion). Morphological alterations to the gills are common following acute metal exposures (Wood, 2001) and the limiting stress imposed on aerobic scope due to acute Pb exposure appears consistent with other metals, such as aluminum and nickel, for which effects on aerobic scope and swimming performance have been attributed to histological alterations at the gill (discussed below). Future experiments employing histological techniques should help clarify whether any potential morphological effects of Pb at the gill could account for the observed effects.

Surprisingly, the relatively large decrease in aerobic scope (62%) exhibited by fish from the acute Pb treatment translated into a rather small reduction in U_{crit} (11%). While an increase in swimming efficiency (i.e. reduced COT) could account for a partial maintenance of swimming performance in the face of a reduced oxygen consumption capacity, the results from the COT analysis did not support this possibility as statistically significant differences in COT were not observed for any of the treatments at any of the corresponding swim velocities (Figure 5.3.). In the end, the reason for the discrepancy between the magnitudes of the two effects remains unclear at this time.

In contrast to the acute impairment of Pb to swimming performance, the reduced U_{crit} exhibited by fish chronically exposed to 689±66 nmol L^{-1} Pb did not coincide with a concurrent decrease in aerobic scope. This finding may support neurological dysfunction as one possible alternative mechanism for the observed effect. Indeed, there are several lines of evidence supporting neurological dysfunction as an effect of chronic Pb exposure. A previous study revealed a likely behavioral impairment in larval offspring exposed to Pb as assessed by a prey capture assay (Mager et al., 2010b). Furthermore, it

has been suggested that the Pb-induced developmental abnormalities of lordoscoliosis and black tail commonly observed in salmonids are the result of effects to motor neurons and the sympathetic nerves controlling caudal pigment cells, respectively (Davies et al., 1976; Holcombe et al., 1976). Other effects of Pb to the nervous system of fish have been reported, including the disruption of various neurotransmitter systems (Rademacher et al., 2003; Sloman et al., 2005; Spieler et al., 1995), increased brain endocannabinoid levels (Rademacher et al., 2005) and injury to the hippocampus and optic tetum, regions of the brain controlling memory and visuomotor function (Giusi et al., 2008). Although the Pb concentration was higher than used in the present study (1450 nmol L^{-1}; 300 µg L^{-1}), Weber and Dingel (1997) found a 38% decrease in U_{crit} of rainbow trout (*Oncorhynchus mykiss*) following 1 week of Pb exposure. From separate analyses of catecholamine neurotransmitter dynamics and startle responses of Pb-exposed fathead minnows the authors concluded that the effect of Pb on the swimming performance of rainbow trout was likely due to neurobehavioral dysfunction (Weber and Dingel, 1997). In light of such neurological effects it therefore seems reasonable that neurological impairment could account for a decrease in U_{crit} by fathead minnows during chronic Pb exposures in the absence of a corresponding reduction in aerobic scope. However, other effects, such as muscle fiber atrophy, could also account for the reduced U_{crit}. Clearly, this is an area warranting further research.

Finally, it appears that fathead minnows may have an ability to recover from the acute effect of Pb on aerobic scope as fish chronically exposed to the same Pb concentration revealed an apparent restoration in aerobic scope (Figure 5.2.). It is interesting to note that the persistent decrease in β-globin mRNA expression observed for

up to 30 d of Pb exposure during our previous study was recovered from at 150 d of Pb exposure (Mager et al., 2008). Thus, as the fish from the present study were exposed for 33-57 d (mean 43 d), this period may have coincided with a transition to recovery and reversal from the effects of Pb on Hb concentration. This possibility is supported by the results of Hodson et al. (Hodson et al., 1978a) which demonstrated a recovery in hematocrits, likely owing to accelerated hemopoiesis, following an initial decline in hematocrits during the first 4 weeks of Pb exposures to rainbow trout. Hence, restoration of aerobic scope by fish chronically exposed to Pb may involve compensatory hematological responses in addition to recovery from acute histological alterations at the gill.

From the results of this study it would seem that the effects of Pb on the swimming performance of fish during chronic exposures, as well as the metabolic cost associated with acclimation to Pb, differs from that of other metals. For example, using a time course of swimming metabolism tests, Wilson et al. (1994) demonstrated that rainbow trout exposed to aluminum exhibited reductions in aerobic scope and MO_{2max} (attributed to structural alterations at the gill) as early as 1 day post-exposure that was not recovered by the end of the 36 d exposure regime, which accounted for a similar trend with respect to U_{crit}. These findings were consistent with the effects of nickel over similar time periods which were also attributed to structural changes at the gill limiting oxygen uptake (Pane et al., 2004). Copper on the other hand elicits reductions in swimming performance during sublethal exposures that are due, not to an increased diffusion distance at the gill (Taylor, 1996; Waser et al., 2009), but are rather potentially effects of hyperammonaemia and/or disruption of muscle membrane potentials due to

elevated ammonia concentrations that limit the maximum demand for oxygen (Beaumont et al., 2000). It seems unlikely, however, that a similar mechanism could account for the reduced U_{crits} in the present study in light of the apparent recovery in MO_{2max} and aerobic scope during chronic Pb exposures.

5.6. Conclusions.

In summary, we have shown that the swimming performance of fathead minnows is impaired following acute and chronic exposures to Pb concentrations approximating 680 nmol L^{-1}. Given the reduction in aerobic scope (due to a reduced MO_{2max}) in addition to the lack of evidence for hematological or neurological dysfunction, the nature of the impaired swimming performance during acute Pb exposure is potentially due to an increase in gas diffusion distance at the gill. From the chronic Pb exposures, fathead minnows appear capable of recovering from the acute effect of Pb on aerobic scope, possibly owing to acclimation and/or compensatory responses related to gill repair and/or increased hemopoiesis. These findings suggest that the mechanisms of impaired swimming performance by fathead minnows arising from acute and chronic Pb exposure are different and may involve a transition from an acute effect of reduced oxygen uptake at the gill, potentially owing to morphological alterations that cause an increase in gas diffusion distance, to chronic effects of an uncharacterized nature. Clearly, there is still much to be learned regarding the nature of the reduced aerobic scope and swimming performance in fathead minnows during acute and chronic Pb exposures. Additional experiments employing histological techniques and alternative assessments of motor

function (e.g. tail beat frequency) should help clarify the mechanisms of Pb impairment observed herein.

Table 5.1. Measured concentrations for dissolved lead (in nmol L^{-1} and µg L^{-1}) and general water chemistry parameters (in µmol L^{-1} except where indicated otherwise). Values represent mean ± SEM.

Lead Concentrations	nmol L^{-1} (µg L^{-1})
Control	0.9±0.4 (0.2±0.1)
Acute High Pb	672±35 (139±7)
Chronic High Pb	689±66 (143±14)
Chronic Low Pb	157±18 (33±4)

General Water Chemistry Parameters	
Na^+	569±16
K^+	11±0
Ca^{2+}	202±29
Mg^{2+}	54±1
Cl^-	698±92
SO_4^{2-}	47±2
Total CO_2	543±69
DOC (µmol C L^{-1})	108±4
Hardness (mg L^{-1})	26±3
pH	7.50±0.03
Temperature (°C)	21±1

Table 5.2. Mean ± SEM values for masses and total body lengths of fathead minnows used in this study.

	Mass (mg)	BL (cm)	*n*
Controls	99±7	2.2±0	14
Acute High Pb	81±8[a]	2.1±0	7
Chronic High Pb	116±4	2.3±0	8
Chronic Low Pb	134±9[a]	2.5±0.1[a]	8

[a]Significantly different from controls by one-way ANOVA.

Figure 5.1. Critical aerobic swimming speeds (U_{crit}) of juvenile fathead minnows exposed acutely to a high Pb concentration (672 nmol L^{-1}), chronically to either a high (689 nmol L^{-1}) or low (157 nmol L^{-1}) Pb concentration and control fish (means ± SEM). [a]Statistically significant from control by Student's *t*-test (acute) or one-way ANOVA (chronic).

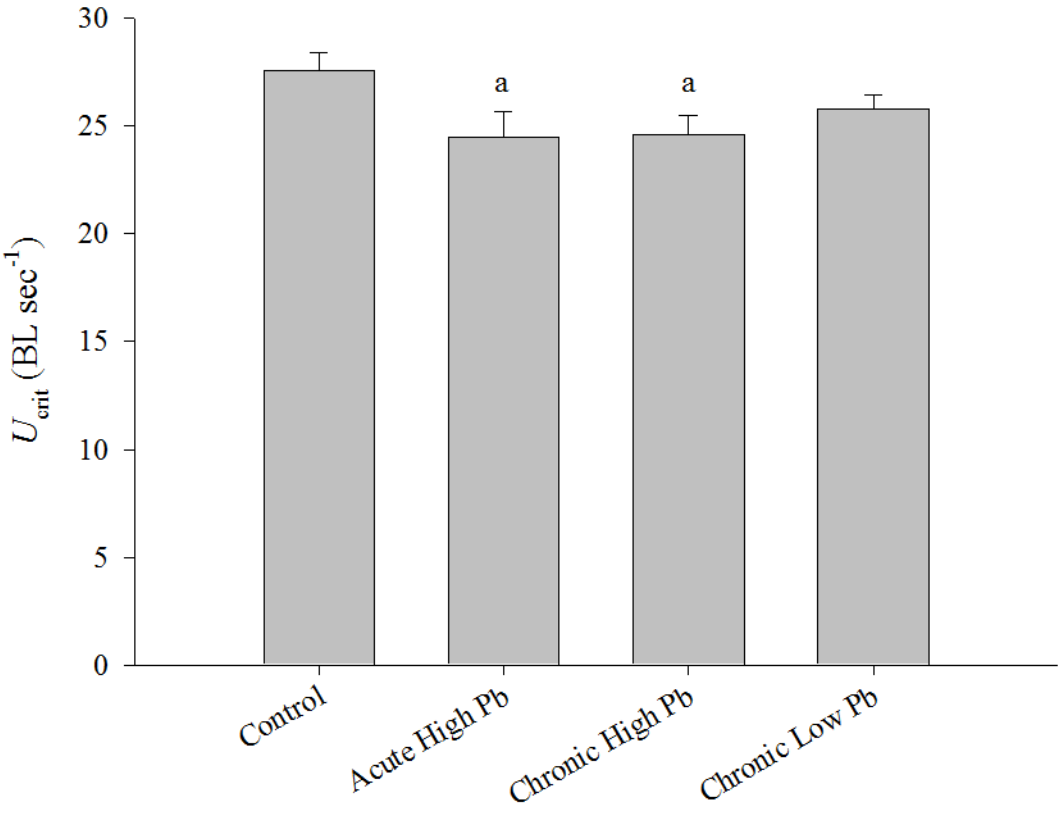


Figure 5.2. Maximum (A) and basal (B) oxygen consumption rates of juvenile fathead minnows exposed acutely to a high Pb concentration (672 nmol L^{-1}), chronically to either a high (689 nmol L^{-1}) or low (157 nmol L^{-1}) Pb concentration and control fish. Aerobic scope (C) represents the difference of $MO_{2max} - MO_{2basal}$. Data are presented as means ± SEM. [a]Statistically significant from control by Student's *t*-test.

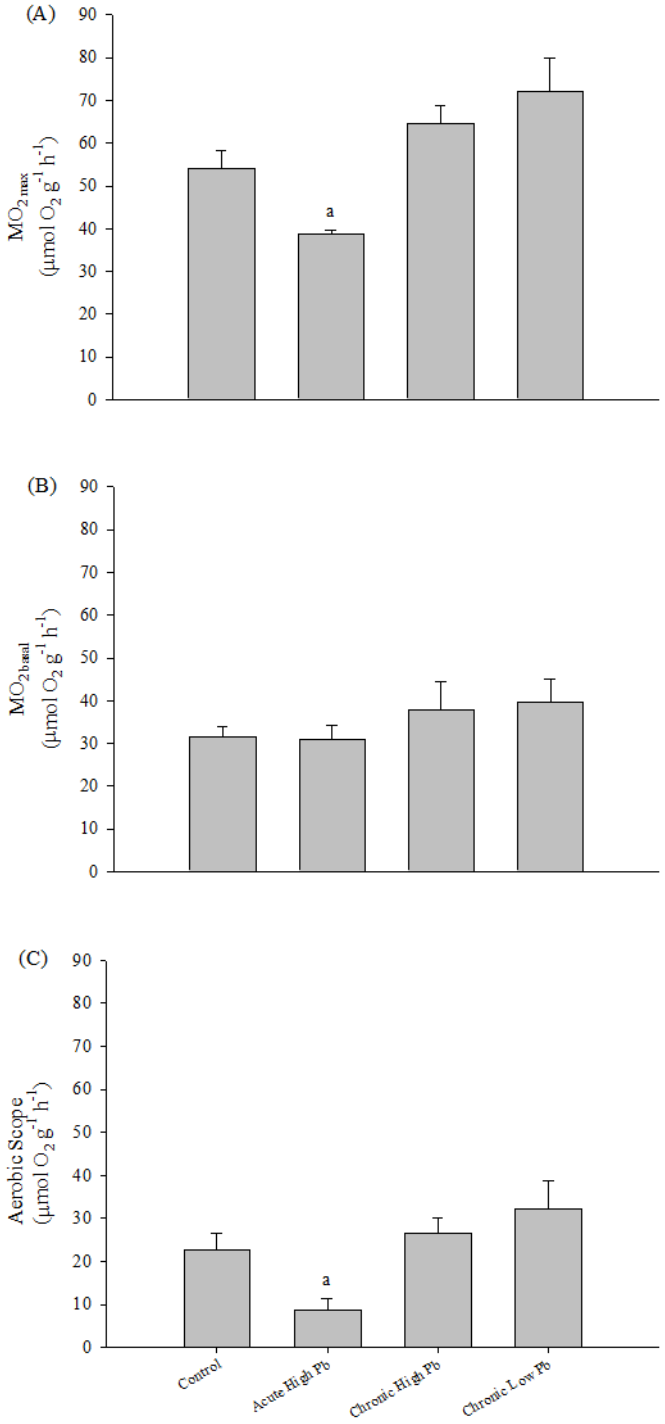
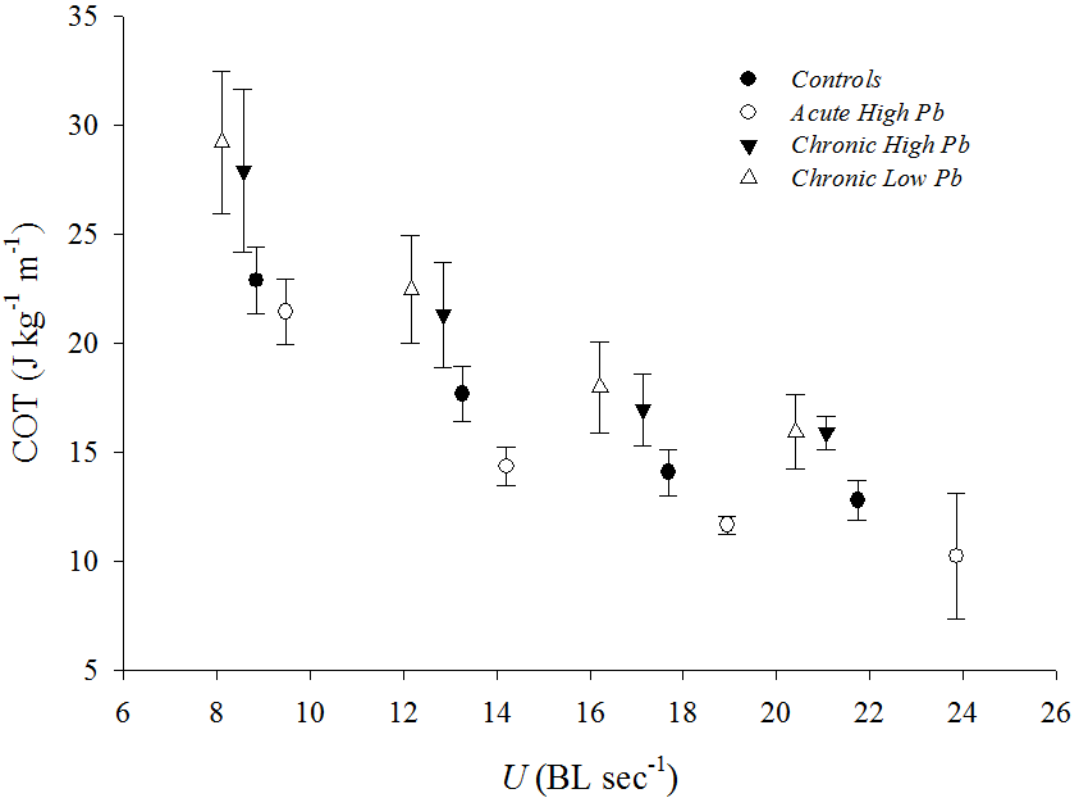


Figure 5.3. Cost of transport (COT) as a function of swimming speed (*U*) for juvenile fathead minnows exposed acutely to a high Pb concentration (672 nmol L^{-1}), chronically to either a high (689 nmol L^{-1}) or low (157 nmol L^{-1}) Pb concentration and control fish (means ± SEM).



CHAPTER 6

DISCUSSION

6.1. Influences of water chemistry on the acute toxicity of lead to *Pimephales promelas* and *Ceriodaphnia dubia.*

One of the primary goals of this dissertation was to characterize the influences of water chemistry on the acute toxicity of Pb to two of the long-standing sentinel test organisms commonly employed by the USEPA, *P. promelas* and *C. dubia*, for parameterization of an acute Pb BLM. To this end, Chapter 2 (Mager et al., 2010c) examined the acute toxicity of Pb to these species in waters modified for key parameters known to influence metal toxicity, namely hardness (as $CaSO_4$), DOM (as HA), pH (5.5-8.3) and alkalinity (as $NaHCO_3$) (Pagenkopf, 1983). Owing to various technical difficulties in performing static pH tests with *C. dubia* and funding constraints, the analysis of a pH influence on Pb toxicity was limited to *P. promelas*. The results revealed that strong protection is afforded by HA and $NaHCO_3$ against acute toxicity of Pb to *P. promelas* whereas milder protection is afforded to *C. dubia* with both parameters. Additionally, it was found that Ca is protective against the acute toxicity of Pb to *P. promelas* but not *C. dubia*. Finally, results from the pH experiments with *P. promelas* revealed an overall trend of increased acute toxicity of Pb with decreasing pH.

While these findings are consistent with expectations for the influences of water chemistry on the acute toxicity of Pb to *P. promelas*, the responses observed with *C. dubia* suggest different and potentially complex interactions between water chemistry and the organism. Although the exact mechanisms remain to be elucidated, there are several

possibilities that could help explain the different influences of water chemistry on acute Pb toxicity to these two species. For example, the direct interactions of DOM with exposed biological surfaces could lead to altered epithelial potentials with variable physiological responses affecting the uptake of Pb. Indeed, DOM has been shown to interact directly with exposed respiratory surfaces leading to alterations of epithelial potentials in fish (Galvez et al., 2008). Specific to daphnids, it has been shown that DOM as HA stimulates Na^+ uptake and that this stimulation is dependent on both ambient Ca^{2+} and pH (Glover et al., 2005; Glover and Wood, 2005). As daphnids possibly take up Na^+ via an electrogenic $2Na^+$:H^+ exchanger (Bianchini and Wood, 2008), and Ca^{2+} can substitute for Na^+ at this exchanger (Ahearn et al., 2001), the interactions among Pb^{2+}, DOM, Ca^{2+}, and Na^+ homeostasis in daphnids is likely to be quite different from that observed in fish which lack this transporter. Furthermore, as Pb is a known Ca antagonist (Audesirk, 1993; Busselberg et al., 1991), HA could promote the uptake of Pb through stimulation of the $2Na^+$:H^+ exchanger. Thus, the reduced protection of HA against acute toxicity of Pb to daphnids may reflect the counteracting effects of strong complexation and stimulated uptake across the $2Na^+$:H^+ exchanger.

The influence of Ca also revealed substantially different effects with respect to the toxicity of Pb to *P. promelas* and *C. dubia*. Indeed, perhaps most surprising, and most significant from a regulatory perspective, was the lack of protection afforded by Ca against acute Pb toxicity to *C. dubia*. It has been shown in rainbow trout that at relatively high Pb concentrations (as used in acute fathead minnow tests) Pb enters via a Ca^{2+} channel and that competition between Ca^{2+} and Pb^{2+} for uptake at this site results in reduced Pb bioavailability (Rogers and Wood, 2004). This mechanism was supported by

the apparent saturation response exhibited by *P. promelas*, but no such trend supporting a similar protective effect of Ca due to competitive inhibition of Pb was observed for *C. dubia*. Thus, it may be possible that Pb^{2+} enters *C. dubia* by a different mechanism, for example through a high affinity divalent metal transporter (DMT1) that has low affinity for Ca^{2+} (Gunshin et al., 1997; Bury et al., 2003). As a result, competitive interactions between Ca^{2+} and Pb^{2+} are possibly minimized under these conditions and Ca^{2+} does therefore not reduce Pb toxicity. Interestingly, Komjarova and Blust (2009a, b) used a stable Pb isotope to show that Pb uptake rates in *Danio rerio* and *Daphnia magna* were both inhibited in water with 2.5 mM Ca^{2+}, but not in water with 0.5 mM Ca^{2+}. These experiments were performed at a low Pb concentration (5 µg/L dissolved Pb), comparable to exposure levels in the *C. dubia* toxicity tests, and thus suggest that the lack of Ca^{2+} protection at low Pb concentrations may not apply uniformly or that Pb accumulation and acute toxicity are not strictly linked.

While the above examples may help explain the observed discrepancies with respect to the influences of water chemistry on the acute toxicity of Pb to fish versus daphnids, additional research will be needed to more fully characterize the physiological mechanisms underlying the interactions between water chemistry and acute Pb toxicity in these species. In the end, although differences in sensitivity to metals among organisms are common and have been dealt with by calibrating BLMs to toxicity data for metals such as Cu, such an approach will likely be insufficient for Pb. Rather, since it appears that water chemistry parameters which protect fathead minnows against Pb are either less effective or without effect on *C. dubia*, particularly with respect to the role of Ca^{2+}, it

seems likely that different BLMs taking these differences into account will be required to ensure adequate environmental protection of both species.

6.2. Influences of calcium and humic acid on chronic lead accumulation and transcriptional responses in *Pimephales promelas.*

The first goal of Chapter 3 (Mager et al., 2008) was to examine the influence of Ca^{2+} (as $CaSO_4$) and DOM (as HA) on the accumulation and chronic toxicity of waterborne Pb during chronic (150 d) exposures approximating a concentration of 35 μg L^{-1} Pb. Strong protection against Pb accumulation was clearly afforded by addition of HA with fish typically exhibiting about half the amount accumulated by fish from control water, whereas addition of $CaSO_4$ failed to prevent Pb accumulation. These results are in agreement with previous Pb gill binding experiments using rainbow trout and may be explained by the >2 orders of magnitude differences in calculated Pb binding affinities for organic matter (log*K* 8.4) vs. gill (log*K* 6.0) and vs. Ca^{2+} gill binding (log *K* 4.0) (Macdonald et al., 2002). However, given that current WQC are hardness-based, the observed lack of protection by Ca against Pb accumulation is an important finding and provides further support that a re-evaluation of the means by which chronic Pb criteria are established is warranted.

Previous studies have demonstrated development of spinal curvature and black discoloration and peripheral erosion of the caudal fin as chronic, sublethal effects of Pb exposure in salmonids, presumably owing to neurological dysfunction (Davies et al., 1976; Holcombe et al., 1976). Thus, to evaluate whether similar effects could be used as potential endpoints of sublethal Pb toxicity in fathead minnows, fish were periodically monitored for these effects throughout the exposures. However, convincing evidence

supporting a Pb-effect on spinal curvature or caudal fin abnormalities was not apparent at any time.

The lack of such morphological effects developing in Pb-exposed fathead minnows may reflect species-specific differences or, more likely, was related to the timing at which the Pb exposures were initiated. In experiments with both brook trout and rainbow trout, the earlier the exposure was initiated (e.g. egg or sac fry), the earlier the onset of effects was observed and the lower the concentration of Pb was needed to induce effects (Davies et al., 1976; Hodson et al., 1979; Holcombe et al., 1976). Thus, as the Pb exposures for the fish used in the experiments of Chapter 3 were initiated following 1 week of acclimation to the test waters (i.e. 8 d of age), it is possible that a critical period was missed for development of such effects to occur.

The second goal of this chapter was to identify transcriptional responses during chronic Pb exposure that might provide further insight as to the underlying toxic mechanisms of Pb. Using a toxicogenomic analysis, 4 genes in particular with pronounced Pb-induced changes in mRNA expression were identified: β-globin, glutathione *S*-transferase alpha (GST-α), glucose-6-phosphate dehydrogenase (G6PD) and ferritin heavy chain. The combined responses of these 4 genes lent further support for the Pb-induced anemia and neurological dysfunction commonly observed for both fish and mammals (Goyer and Clarkson, 2001; Sorensen, 1991) as the primary effects of chronic Pb exposure. Specifically, a decrease in β-globin mRNA was observed, suggesting an anemic response potentially arising from a ROS-mediated hemolysis induced by Pb. This notion was supported by a nearly parallel co-induction of GST-α and G6PD which likely indicated recruitment of the pentose phosphate shunt, a

biochemical pathway employed by erythrocytes to defend against oxidative stress (Lachant et al., 1984). In addition, the observed upregulation of ferritin mRNA likely reflected additional sources of oxidative stress in the form of ALA and free Fe^{2+} arising from the inhibition of ALAD by Pb (Oteiza et al., 1995; Rocha et al., 2003) and/or from free Fe^{2+} released from the breakdown of Hb. Ferritin Fe^{2+} displacement and increased ROS production may play a role in neurological impairment as well. Evidence suggests a role for ferritin Fe^{2+} dysregulation in human neurological disorders (Ke and Ming Qian, 2003; Berg and Hochstrasser, 2006) and that ferritin may represent a dominant means of Fe^{2+} delivery to the brains of mammals (Fisher et al., 2007).

The final goals of Chapter 3 were to determine whether the genes identified by microarray analysis could serve as early markers of Pb exposure and long-term effects by assessing whether the transcriptional responses reflect the influences of water chemistry on Pb accumulation and toxicity, respectively. Using a QPCR approach, it was found that the magnitude of the Pb-induced responses of these genes largely paralleled the influences of water chemistry on Pb accumulation, suggesting that these genes could serve as robust indicators of Pb exposure and accumulation in fathead minnows. However, as no detectable effects of Pb exposure on spinal curvature were observed, extending the utility of these genes to indicators of Pb effect could not be established. Nevertheless, the hematological and neurological effects suggested by the combined response of these 4 genes narrowed the focus for future endpoints of chronic effects to be studied in subsequent chapters (i.e. prey capture ability and swimming performance).

6.3. Influences of humic acid and alkalinity on the growth and reproductive effects of lead to *Pimephales promelas.*

Having narrowed the field for potential key water chemistry parameters mediating the chronic toxicity of Pb in Chapter 3, the next step was to investigate their influences on Pb-induced reproductive effects. Thus, in Chapter 4 (Mager et al., 2010b) the influence of HA was evaluated to determine whether the protection against whole body Pb accumulation observed previously translated into protection against full-term reproductive effects. The effect of increased alkalinity (as $NaHCO_3$) was also examined in lieu of Ca given its previous failure to protect against chronic Pb accumulation. Two Pb concentrations were used for the full life cycle exposures, one targeting the 35 μg L^{-1} Pb concentration used in Chapter 3 (Mager et al., 2008) and an additional higher concentration targeting 120 μg L^{-1} Pb. In addition to various reproductive endpoints (e.g. fecundity, hatchability, egg mass, egg Pb accumulation), growth, Pb accumulation and potential Pb-induced neurological impairment in larval offspring (discussed in *Section 6.4.*) were assessed.

Consistent with the findings of Chapter 3, HA afforded strong protection against Pb accumulation during exposures to both concentrations of Pb. While there was an apparent trend toward reduced Pb accumulation in fish from the $NaHCO_3$ treatments, the difference compared to controls was only significant at the 300 d time point. The different protective effects of HA and $NaHCO_3$ are likely due to the greater complexing capacity of HA due to the high number of strong Pb binding sites available per mg of carbon (Macdonald et al., 2002). Conversely, Pb is removed in a nearly linear fashion by increased carbonate complexation at higher alkalinities (Davies et al., 1976; Macdonald et al., 2002). However, a proportional decrease in Pb accumulation was not observed

with the increase in alkalinity in the $NaHCO_3$ treatment, suggesting that some inorganic forms of Pb (e.g. $PbOH^+$) might be available for uptake. Whatever the mechanism, clearly an increase in $NaHCO_3$ concentration does not necessarily lead to a proportional protective effect against Pb accumulation in fathead minnows.

Fish collected for Pb accumulation were also weighed to determine any potential growth effects. While the high Pb concentration inhibited growth early on (4 d) the effect was not influenced by water chemistry. Furthermore, the effect was transient, having recovered in all cases by 30 d and no further differences were observed at any time point beyond.

Considering the endpoint of fecundity, the influences of water chemistry appear inconsistent with respect to the effects of acute versus chronic Pb exposures to *P. promelas*. While Ca, HA and alkalinity each afforded protection against the acute toxicity of Pb to *P. promelas*, there was evidence that these parameters either do not protect against, or even enhance, the effects of chronic Pb exposure. Specifically, as revealed in Chapter 3 (Mager et al., 2008), Ca did not protect against chronic Pb accumulation or transcriptional responses during 150 d of sublethal Pb exposure, whereas HA protected against both. However, the subsequent full life cycle study described in Chapter 4 revealed that, while HA again reduced Pb accumulation, it actually enhanced reproductive toxicity. An effect of enhanced metal toxicity with DOM without a concurrent increase in Pb accumulation has been previously demonstrated with other metals on algae and cladocerans (Borgmann and Charlton, 1984; Giesy et al., 1983; Laegreid et al., 1983), but this appears to be the first report of enhanced Pb-induced reproductive toxicity with addition of DOM in fish. Furthermore, from the full life cycle

fathead minnow study it was found that alkalinity increased reproductive output in control fish, but also conferred greater sensitivity to Pb. While the mechanisms of these effects are difficult to explain at present, they clearly indicate a need for more research on the influences of water chemistry on chronic Pb toxicity and caution against inferring chronic effects of water chemistry from acute exposures.

Interestingly, there was a potential connection between egg mass and fecundity. Consistent with the reduced reproductive output for the HA and $NaHCO_3$ treatments with 120 μg L^{-1} Pb was the increase in average egg mass from the same treatments. It is tempting to speculate that, when exposed to Pb, more energy might be directed toward producing fewer, higher quality eggs than in similar water conditions without Pb. Given that no effects on hatchability were observed, the greater egg masses may support this possibility. Conversely, there was no apparent connection between egg Pb accumulation and other reproductive (or behavioral) effects, as all eggs exposed to Pb accumulated similar amounts regardless of Pb concentration or water chemistry.

Finally, the different influences of HA on mortality and fecundity during chronic Pb exposure raises the question of whether HA should be considered beneficial to fathead minnows. By multiplying the probability of survival by total reproductive output within a given water treatment, a reasonable estimate of a life history effect can be achieved. For example, the product of the survival and fecundity values of 0.807 and 2336, respectively, from the HA high Pb treatment of the present study (Table 4.5.) gives an estimated total number of individuals after one life cycle of 1885. Performing the same calculation for the tap water high Pb treatment (i.e. 0.711 survival probability multiplied by 3834 total offspring; Table 4.5.) one obtains a value of 2726 individuals following one

life cycle. The addition of HA at the high Pb concentration would therefore translate into a 31% reduction in total number of individuals following one life cycle compared to the high Pb tap water treatment. Hence, from an ecological standpoint, it must be concluded that addition of HA at the high Pb concentration is not beneficial to fathead minnows owing to a negative influence on reproductive output that outweighs the protective effect on survival.

6.4. Effects of chronic lead exposure on prey capture ability and swimming performance of *Pimephales promelas.*

To investigate whether chronic Pb exposure to fathead minnows leads to neurological and/or hematological effects of ecological significance (as suggested by the microarray-identified genes of Chapter 3) two approaches were used. The first approach was to assess for potential neurological impairment of Pb exposure by examining the feeding performance of 10 d larval F1 offspring from the reproduction studies of Chapter 4 using a prey capture assay with *Artemia* (Mager et al., 2010b). While it was found that exposure to 120 μg L^{-1} Pb altered feeding performance in all water treatments, the influence of water chemistry differed depending on the endpoint examined. Larvae from the HA and $NaHCO_3$ treatments with 120 μg L^{-1} Pb required more time to ingest all possible (i.e. 5) *Artemia* than those from treatment-matched controls. However, larvae from the same Pb treatments more frequently completed 5 ingestions than those from the tap water control treatments with the same Pb concentration (120 μg L^{-1} Pb). Thus, regardless of the exact nature of the effect, these findings indicate that fathead minnows chronically exposed to Pb may produce offspring with ecologically relevant behavioral impairment.

The second approach, and the focus of Chapter 5 (Mager et al., 2010c), was to investigate the potential neurological and hematological effects of Pb on the swimming performance and aerobic scope of *P. promelas* following chronic exposures to Pb concentrations targeting those used in previous chapters (i.e. 35 and 120 μg L^{-1} Pb) and an acute exposure of 24 h to 120 μg L^{-1} Pb. Swimming performance, as assessed by critical aerobic swim speed (U_{crit}), and aerobic scope were analyzed using the incremental velocity test and swim tunnel respirometery (Blazka et al., 1960). To assess for Pb-induced anemia, blood samples were collected to measure hemoglobin (Hb) concentrations, whereas an assessment of neurological impairment was evaluated indirectly using a cost of transport (COT) analysis derived from data collected during swimming respirometery.

The findings revealed a Pb-induced impairment to the swimming performance (U_{crit}) of *P. promelas* following acute and chronic exposures to 120 μg L^{-1} Pb. However, whereas the reduced U_{crit} from the acute Pb exposure was clearly associated with a reduction in aerobic scope, the chronic effect was not. These findings strongly suggested different mechanisms underlying the impaired swimming performances exhibited during acute and chronic Pb exposures. As an acute adverse neurological effect of Pb on swimming performance could not be discerned from the COT analysis, and Hb concentrations from fish acutely exposed to Pb were similar to controls, a possible explanation for the reduced swimming performance observed following acute Pb exposure is a reduction in aerobic scope owing to changes at the gill leading to reduced efficiency in oxygen uptake (i.e. gill morphology, mucus secretion). Future experiments employing a histological analysis of the gills should help clarify the potential effects of

Pb in this regard. While an explanation for the reduced U_{crit} following chronic exposure to the high Pb concentration remains unclear at this time, the lack of effect on oxygen consumption indicates the involvement of an alternative mechanism. In the end, these findings suggest that the impaired swimming performance arising from acute and chronic Pb exposure may reflect a transition from an acute effect of reduced oxygen uptake at the gill, potentially owing to morphological alterations that cause an increase in diffusion distance, to chronic effects of an undetermined cause.

The results of this study may call into question the ecological relevance of anemia in Pb-exposed fathead minnows. The small sizes of fish corresponding to the exposure times at which the Pb-induced molecular responses identified in Chapter 3 were most apparent (30 d or less) precluded the collection of blood for a direct assessment of Pb-induced anemia. However, if anemia were in fact occurring as a result of Pb exposure, there are several lines of evidence indicating that fathead minnows have the ability to acclimate to such effects. For example, the gene expression responses to Pb appeared to recover by 30 d in most cases, and by 150 d in all cases (Mager et al., 2008). Given the limitations on fish size imposed by the swim tunnel apparatus, Pb exposures for fish used for swim respirometery (mean 43 d) fell between the 30 d and 150 d time points used for the gene expression analyses. This period may have coincided with a transition to recovery, and potentially reversal, from the anemic effects of Pb given the recovery in β-globin mRNA expression observed at 150 d. This possibility is supported by the results of Hodson et al. (1978a) which demonstrated a similar recovery in hematocrits, likely owing to accelerated hemopoiesis, following an initial decline in hematocrits during the first 4 weeks of Pb exposures to rainbow trout. Thus at the Pb concentrations used in

these experiments, any potential hematological impairment seems to have little long-term biological relevance with respect to swimming performance.

Perhaps of greater ecological significance are the long-term neurological effects of Pb. For example, the reduced prey capture ability exhibited by larval offspring exposed to Pb described in Chapter 4 likely indicated chronic behavioral effects of Pb (Mager et al., 2010b). Furthermore, it has been suggested that the Pb-induced developmental abnormalities of lordoscoliosis and black tail commonly observed in salmonids are likely the result of effects to motor neurons and the sympathetic nerves controlling caudal pigment cells, respectively (Davies et al., 1976; Holcombe et al., 1976). Other effects of Pb to the nervous system of fish have been reported, including the disruption of various neurotransmitter systems (Rademacher et al., 2003; Sloman et al., 2005; Spieler et al., 1995; Weber and Dingel, 1997), increased brain endocannabinoid levels (Rademacher et al., 2005) and injury to the hippocampus and optic tetum, regions of the brain controlling memory and visuomotor function (Giusi et al., 2008). While the effects just described may have important ecological implications, more work will be necessary to more clearly define dose responses as well as threshold levels at which Pb impairment to various behaviors occur, particularly in the context of ambient water quality.

In the end, considering the chronic effects of Pb on the neurological and hematological parameters discussed above, it would seem that the genes identified from the microarray analysis may indeed have some utility as indicators of chronic neurological effects of ecological significance, although more research will be necessary to establish firm links to chronic outcomes of ecological relevance. Future experiments

examining the influences of water chemistry (e.g. HA and $NaHCO_3$) on behavioral and sensory-motor effects of chronic Pb exposure, in combination with concurrent gene expression analyses, should help elucidate any such potential.

6.5. Overall conclusions.

This dissertation characterized the influences of key water chemistry parameters on the acute and chronic toxicity of Pb to the freshwater organisms, *P. promelas* and *C. dubia*. Perhaps most surprising, and most significant from a regulatory perspective, was the lack of protection afforded by Ca against acute Pb toxicity to *C. dubia* and chronic Pb accumulation by *P. promelas*. These are important findings given that current WQC for Pb are hardness-based and provide further support for the need for alternative approaches to setting WQC. Indeed, the findings reported herein should facilitate the arrival of such an approach in the form of a new acute BLM for Pb. However, the different responses exhibited by these species suggest that development of separate BLMs for *P. promelas* and *C. dubia* should be considered to ensure adequate protection for both species. Importantly, the results from the experiments with *P. promelas* revealed that the influences of water chemistry are inconsistent during acute and chronic Pb exposures and caution against inferring chronic effects from acute exposures. Finally, results from the toxicogenomic analysis with *P. promelas* illustrate well the power of microarray technologies in helping elucidate underlying toxic mechanisms and for further revealing the means by which Pb may elicit detrimental effects to fish. While the microarray-identified genes exhibit a strong potential for serving as robust indicators of Pb exposure and accumulation in *P. promelas*, additional work will be necessary to firmly link these

genes to chronic outcomes of ecological relevance in the context of ambient water chemistry.

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