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CHRONIC EFFECTS OF LEAD EXPOSURE ON *ATHERINOPS AFFINIS*

(TOPSMELT): INFLUENCE OF SALINITY AND ORGANISM AGE

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

PROGRAM IN BIOLOGY

BY

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Abstract

Pb (lead) appears in the environment as a consequence of both natural and anthropogenic processes. Mining, smelting, coal burning, lead acid batteries, and cement manufacturing substantially release Pb into aquatic environments. The goal of this study was to determine the influence of salinity and organism age on chronic toxicity of Pb to *Atherinops affinis* (topsmelt) in support of development of a species sensitivity distribution (SSD). Species sensitivity distributions assist in ecological risk assessments and establishing quality criteria for contaminants. Three chronic exposure studies were conducted for 28 days in a water flow-through testing system. Survival, standard length, dry weight, and tissue Pb concentration were measured and lethal concentrations (LC), effective concentrations (EC), and bioconcentration factor (BCF) were calculated. In general, increasing salinity and organism age decreased Pb toxicity. The 28-day LC50 values for larval fish at 14 and 28 parts per thousand (ppt) salinity were 15.1 and 79.8 µg/L dissolved Pb, respectively, whereas the 28-d LC50 for juvenile fish was 167.6 µg/L dissolved Pb at 28 ppt salinity. Using standard length, the EC10 values for larval fish were 16.6 and 82.3 µg/L dissolved Pb at low and high salinity, respectively. The dry weight EC25 for low and high salinity were 15.6 and 46.9 µg/L dissolved Pb, respectively. The BCF was higher with the lower salinity study (2.00) in comparison to the higher salinity study (0.73). This is likely due to competition between salt ions and Pb at biotic ligand binding sites as well as lowered Pb speciation rates.

CHAPTER ONE

INTRODUCTION

Metal pollution has been an environmental issue for many decades. The introduction of heavy metals to marine ecosystems is concentrated near densely populated coastal areas and industrialized regions. Lead (Pb) has become a metal of concern due to its popular application in a variety of industries. Some metals, such as Na, Ca, and K, are essential to biological function; whereas, Pb is considered a nonessential metal and therefore not required for any known physiologic process.

Pb occurs in the environment as a consequence of both naturogenic and anthropogenic processes. Activities such as mining, smelting, coal burning, manufacturing continue to contribute substantial amounts of Pb to aquatic environments [1]. According to the International Lead Association, the world production of lead has increased from 4.602 million tons in 1970 to 11.134 million tons in 2016 [2]. The top three countries for Pb production in 2012 were China, Australia, and the United States, which contribute 52, 11.5, and 6.9 percent of total lead production, respectively [3]. The principal use of lead worldwide, accounting for 85.1% of Pb use, is the production of Pb acid batteries. Pb acid batteries are a critical component of storage technologies for renewable energy sources such as solar cell and wind turbines as well as a portion of automotive vehicles [3]. Pb production is increasing and with it risks for environmental contamination are increasing as well. Naturogenic aquatic Pb exposure occurs

when the metal is leached from sediment and soil into aquatic environments; whereas, anthropogenic Pb enters surface waters principally through atmospheric deposition and industrial effluent discharge [4]. Pb and other heavy metals are amongst the most persistent contaminants because they cannot be broken down into smaller particulates, therefore, Pb contaminants are useful markers of environmental change.

According to the United States Environmental Protection Agency, the national recommended water quality criteria for marine environments is 210 µg/L for acute Pb exposure and 8.1 µg/L for chronic Pb exposure [6]. Background concentrations of marine Pb in northeastern Pacific surface waters range between 17 and 49 picomolar (pM) or 0.0035 and 0.01 µg/L [5]. The amount of Pb dissolved within a water column is directly related to the likelihood of a toxic event.

The approximate order of increasing toxicity of heavy metals to fish is Co, Al, Cr, Pb, Ni, Zn, Cu, Cd, and Hg [7]. Marine Pb research is limited partly due to the toxicant's relatively low toxicity and low solubility in marine waters [14] [15]. The abundance of dissolved ionic salts in marine environments causes Pb to precipitate out of solution and greatly reduces the bioavailability of Pb. However, the toxicity of any given metal varies for many biotic and abiotic reasons.

Metals exist in marine environments as a variety of species and may be present in different oxidation states. Speciation determines free ion activity, but interactions at the biotic ligand must also be evaluated when determining bioavailability [8]. Although Pb has been isolated in different oxidation states (II, III, IV); oxidation state II is the most prevalent in marine environments [9]. The free metal Pb (Pb^{2+}) ion is thought to be the primary metal species that

causes toxicity in aquatic organism's due to its ability to substitute for bivalent essential metals like Ca^{2+} [10]. Lead availability and water chemistry are paramount when understanding toxicity.

When Pb is introduced into an aquatic environment it takes on many different chemical species. The total analytical concentration of a given metal in water is the sum concentration of free Pb ions, Pb complexes, and Pb-suspended solid species. For example, the total molar concentration of lead, ΣPb, in water may equal:

$\Sigma Pb = mPb^{2+} + mPbOH+ + mPbCO3 + mPbHCO_3^+ + mPbNO_2 + mPb$ (suspended solids)
[10]

The concentration of Pb^{2+} in most marine waters is less than the sum of the concentrations of other Pb-complexes. [11]. Water quality parameters such as pH, alkalinity, dissolved organic carbon (DOC), and salinity affect Pb speciation and therefore bioavailability. Specifically, an increase in value in any of the aforementioned parameters may decrease the bioavailability of ionic metals and decrease toxicity.

Increased pH decreases ionic Pb, making Pb less bioavailable and therefore less toxic. Between pH 7 and 9, lead in seawater is mainly speciated with CO_3^{2-} and to a lesser extent with Cl^-. At values near 7, $PbCO_3$ and $PbCl^+$ are present in nearly equal amounts and there is an increased amount of $PbCl_2$. However, as pH increases, $PbCO_3^+$ becomes the predominant species [12]. Increased alkalinity, as well as an increase in pH, will result in the formation of Pb carbonate species (e.g., $PbCO_3$, $PbCO_3$, $PbHCO_3^+$). These compounds are known to be less bioavailable, and therefore less toxic than the free metal Pb (Pb^{2+}) ion. Pb will also complex with

DOC and form non-bioavailable forms (e.g., Pb-DOC) [13]. Therefore, elevated concentrations of DOC will also decrease Pb bioavailability and toxicity.

Lead salts are poorly soluble in marine environments [14] [15]. The abundance of dissolved ionic salts can cause Pb to precipitate out of solution and reduces the bioavailability of Pb. Anions, such as chloride and sulfate, are in greater abundance in high saline environments and thus more likely to bond with cationic Pb. The presence of marine salts also reduces the bioavailability of Pb due to the increased cationic competition for binding sites at biotic ligands.

A biotic ligand is the site of toxic action during metal exposure. The biotic ligand in fish is typically the gill, where metals induce ion regulatory impairments [8]. Increased salinity within an environment will result in fewer interactions between Pb and biotic ligands at the gills due to a greater cation presence. This competition makes Pb less bioavailable to aquatic organisms and, therefore, decreases toxicity.

Pb toxicity is believed to incorporate both osmoregulatory and ionoregulatory stress as mechanisms of toxicity to *A. affinis* [14]. Ionic mechanism of action for Pb mainly arises due to its ability to substitute other bivalent and monovalent cations, such as Ca^{2+}, Mg^{2+}, Fe^{2+}, and Na^+.[16]. Once substituted, Pb stresses fundamental biological processes of the body. Lead may bind to and interact with many of the same enzymes as these metals but, due to its differing chemistry, does not properly function as a cofactor, thus interfering with the enzyme's ability to catalyze its normal reaction [17]. Lead inhibits two major enzymes of the heme biosynthetic pathway: delta-aminolevulinic acid dehydratase and ferrochelatase [17]. These interactions occur at molecular levels and are a result of the ability of Pb to mimic and displace cations during physiologic processes such as Ca^{2+} adenosine triphosphate (ATP) pumps [17].

It is likely that Pb blocks calcium discharge from cells by substituting for calcium in Ca^{2+} ATPase [17]. Ca^{2+} ATPase is a form of p-type ATPase that transfers calcium after a muscle has contracted. The result is a decreased calcium plasma concentration and eventual hypocalcemia. The ionic mechanism contributes principally to neurological deficits as Pb encounters the blood-brain barrier (BBB) [16]. The increased Pb within the BBB causes observable manifestations of lead toxicity, which in fish includes lordoscoliosis, a combined backward and lateral curvature of the spine, and muscle spasms [18]. Pb may inhibit the release of neurotransmitters by blocking the entry of calcium into nerve terminals and by competing with calcium for uptake by calcium channels [17].

Earlier life stages of fish tend to be more sensitive to Pb than fish of later developmental stages [18]. The increased sensitivity to metals for younger fish is attributed to a variety of reasons. A greater uptake of toxicant from the environment may occur in earlier life stages due to a less effective homeostatic mechanism to deal with the toxicants. Younger fish contain less developed organs, e.g., liver and kidney, each of which have an important role in detoxification and elimination of toxicants.

Studies show that larval *A. affinis* are amenable to toxicity testing at estuarine salinities from 5 ppt to 33 ppt [19]. Although *A. affinis* tolerate lower salinities they prefer marine conditions. *A. affinis* are abundant in California, Washington, and Oregon within coastlines and estuaries. *A. affinis* reproductive events usually occur in dry summer months at spawn sites such as harbors and estuaries [20]. During a spawn migration, *A. affinis* encounter a warm thermocline in the shallow estuary environment and the fish eggs respond to this warmer thermal gradient and hatch [20]. *A affinis* is currently a non-standard organism for toxicology testing; therefore,

research is still pending on its behavior and physiology. It is important to consider the effect of salinity on toxicity of species whose choice of habitat is dependent on life cycle and spawn events.

Studies on Pb toxicity in the aquatic environment have been conducted; however, most studies focused on freshwater environments using standard organisms [20]. Marine Pb exposure research limitations are a result of its relatively low toxicity and low solubility in marine waters [14]. I was unable to generate acute Pb toxicity values because of the low toxicity and solubility of Pb. Other dissolved marine metals have been found to be more soluble and therefore more toxic to *A. affinis*. Acute *A. affinis* LC50 values for Cu and Ag are reported as 212μg/L and 259μg/L, respectively [21] [22].

 Metal studies have been conducted using acute testing procedures; however, chronic testing procedures are more indicative of Pb contaminated marine environments. Pb, like other metal contaminants, cannot be broken down and remains in the environment for extended periods of time. Therefore, chronic tests are more appropriate to evaluate environmental risk of Pb in marine ecosystems. Pb chronic toxicity data for multiple species are needed for development of chronic species sensitivity distribution (SSD)-an important step for risk assessment and setting environmental quality criteria for contaminants.

This study's objective is to quantify the chronic effects of Pb exposure on *A. affinis;* specifically, the influence of salinity and organism age on Pb toxicity on *A. affinis*. *A. affinis* is not a standard species for toxicology testing; however, using the organism for toxicity testing is becoming more common especially in the Pacific coastal environment of California [21] [20]. *A. affinis* is particularly appropriate for use in toxicity testing because of its relative sensitivity to a

variety of toxicants and amenability to laboratory culture [19]. The fish is also of high ecological importance as a primary and secondary consumer within the coastal food web [20]. *A. affinis* embryonic development, rearing, and physiology are similar to that other atherinids used widely in toxicity testing [21]. The similarities among these attributes make protocol adaptation from one species to another feasible.

CHAPTER TWO

MATERIALS AND METHODS

Experimental design

Multiple 28-day flow through system toxicity tests were conducted in synthetic salt water to evaluate the chronic toxicity of Pb to *A. affinis*. The first objective was to determine the influence of salinity on Pb toxicity to less than 3-d old *A. affinis* fry. Two 28-d chronic exposure experiments were conducted at two salinities- 14 ppt, representative of an estuarine environment, and 28 ppt, representative of a coastal marine environment. The purpose of this objective was to establish both sublethal and lethal toxicity values for *A. affinis* at two salinity levels to explore the influence of salinity on Pb toxicity.

The second objective of the study was to determine the influence of *A. affinis* age on Pb toxicity. Two 28-d chronic exposure experiments were conducted using two life stages: less than 3-d old and 2.5-month old. The 3-d old *A. affinis* fish are within the fry stage of their life cycle and the 2.5-month old *A. affinis* are within the juvenile stage. The 3-d old fish are classified as fry because they are no longer attached to a larval yolk sac and must procure their own food, whereas the 2.5-month fish are classified as juvenile because they haven't developed sexual characteristics. The purpose of this objective is to establish sublethal and lethal toxicity values for two life stages of *A. affinis*.

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Chronic exposure concentrations (Table 1) were chosen based on a preliminary acute study with *A. affinis*. Experiments for the first objective with low and high salinity and fish fry had five treatments and a control. The experiment with high salinity and juvenile fish was limited to two treatments and a control.

Table 1. Nominal Control (CTL) and Treatment (T1-T5) Pb concentrations in µg/L for *A. affinis* chronic exposure tests.

Treatment	14 ppt ≤ 3-days old	28 ppt ≤ 3-days old	28 ppt- 2.5-month old
CTL	0	0	0
T1	25	50	160
T2	50	100	320
T3	100	200	N/A
T4	150	400	N/A
T5	200	600	N/A

Toxicity testing

All experiments were performed using 2004 ASTM (American Society for Testing and Materials) Standard Guide for Conducting Early Life-Stage Toxicity Tests [23]. Test water was prepared using 16-18 MΩ MilliQ water (Barnstead E-pure) and an addition of Crystal Sea Laboratory Bioassay Formula (Marine Enterprises, Baltimore, Maryland, USA) to achieve a test dependent salinity. All test chambers were washed with nitric acid, and then rinsed with 16 – 18 MΩ MilliQ water to avoid any interference from contamination. The test with 2.5-month old juvenile fish had 2 metal concentrations; all other tests had five metal concentrations. Four replicates per concentration and control with 10 fish per replicate were used.

Fish were purchased from Aquatic Biosystems (Fort Collins, Colorado, USA) and priority shipped to the Loyola Ecotoxicology Laboratory. Upon arrival, the shipment's water quality parameters were recorded and the fish were fed live brine shrimp and inspected for health. Fish that exhibited abnormal behavior or anatomy were euthanized while the remaining observationally healthy fish were acclimated to laboratory conditions over a period of 24h. All tests were conducted at $18 \pm 1^{\circ}C$ with a photoperiod of 16h light and 8h dark to replicate common environmental conditions for *A. affinis*. Once acclimated, chronic Pb toxicity experiments were conducted in a flow-through, self-renewing, exposure system [24]. Consistent treatment water renewal was achieved with a flow-through system set at pre-determined time intervals.

Test solutions were prepared at least 24 hours before organism exposure to allow ample time for solution to equilibrate. Test solution treatments were prepared by aliquoting a test dependent quantity of Pb stock solution to Crystal Sea Bioassay formula synthetic salt water. Synthetic salt water was prepared by dissolving an equivalent amount of Crystal Sea salt in 16-

18 MΩ MilliQ water and continuously aerated for at least 24 h before use. Pb stock solution

(10,000 µg/L) was prepared by dissolving a desired amount of $Pb(NO_3)_2$ in 18MΩ water and

acidified with HNO_3 to a pH approximately of 4 to maximize Pb desorption. Prior to use,

concentration of Pb stock solution was verified by a NeXion 300S Inductively Coupled Plasma

Mass Spectrometer (IC-PMS) (Perkin Elmer, Oak Brook, IL, USA).

Water quality parameters such as dissolved oxygen (DO), pH, temperature, and

conductivity were measured one hour after preparing test solutions and once a day during all

subsequent test days. DO and temperature were measured using a YSI 550A dissolved oxygen

meter (YSI Inc., Yellow Springs, Ohio, USA). An Accumet AP 110 pH meter (YSI Inc., Yellow

Springs, Ohio, USA) was used to measure pH. Salinity was measured using a YSI 30

conductivity meter (YSI Inc., Yellow Springs, Ohio, USA). Alkalinity was calculated via sample

titration with 0.02N H_2SO_4 and measured on Day 0 (D0), D7, D14, D21, and D28. Average DO,

pH, and temperature were 7.60 ± 0.76 mg/L, 7.96 ± 0.05, and $18.2 \pm 0.13^{\circ}$ C, respectively.

Average salinity was 14.09 ± 0.141, 27.98 ± 0.576, and 27.96 ± 0.303 ppt for the low salinity

fry, high salinity fry, and high salinity juvenile studies, respectively. Average alkalinity was

58.18 ± 5.02, 105.04 ± 8.08, and 114.47 ± 3.98 mg $CaCo_3$ for the low salinity fry, high salinity

fry, and high salinity juvenile studies, respectively.

Two ages of test organisms, less than 3-d old fry and 2.5-month old juvenile, were used.

Fry *A. affinis* were younger than 1-d old by the time they arrived. The fish were then acclimated

to laboratory conditions for at least 24-h but no longer than 2 days prior to tests. Juvenile *A.*

affinis were 1-month-old upon arrival and subsequently acclimated to laboratory conditions for

1.5 months. During acclimation, fish were fed daily with freshly hatched brine shrimp (Brine

Shrimp Direct, Ogden, UT, USA). Test organisms were fed at least two hours prior to test initiation and every day of testing (D1-D28) with freshly hatched brine shrimp. Fish were then randomly distributed into test chambers one or two at a time. Fish were only used for testing if they appeared healthy.

All tests were conducted using a flow through testing system (Image 1). Large aerated treatment water basins were placed above the test chamber and a series of peristaltic pumps (Cole Palmer, Vernon Hills, IL, USA) aliquoted 83.4 mL to each discrete replicate every 30 minutes (Image 2). A hole was bored into each replicate to fit a modified silicone stopper. Once the water level within a replicate exceeded 2 L, treatment water would flow through the modified silicone stopper and out of the test chamber. This ensured fresh treatment water remained within replicates. The flow- through system replaced the total volume of each discrete replicate, 2 L, once every 12-h. The peristaltic pumps (Aqua Logic Inc., San Diego, CA, USA) were scheduled to turn on every 30 minutes. The water bed was kept at 18ºC using a water chiller (Pentair Aquatic Eco-Systems, Inc., Apopka, FL, USA).
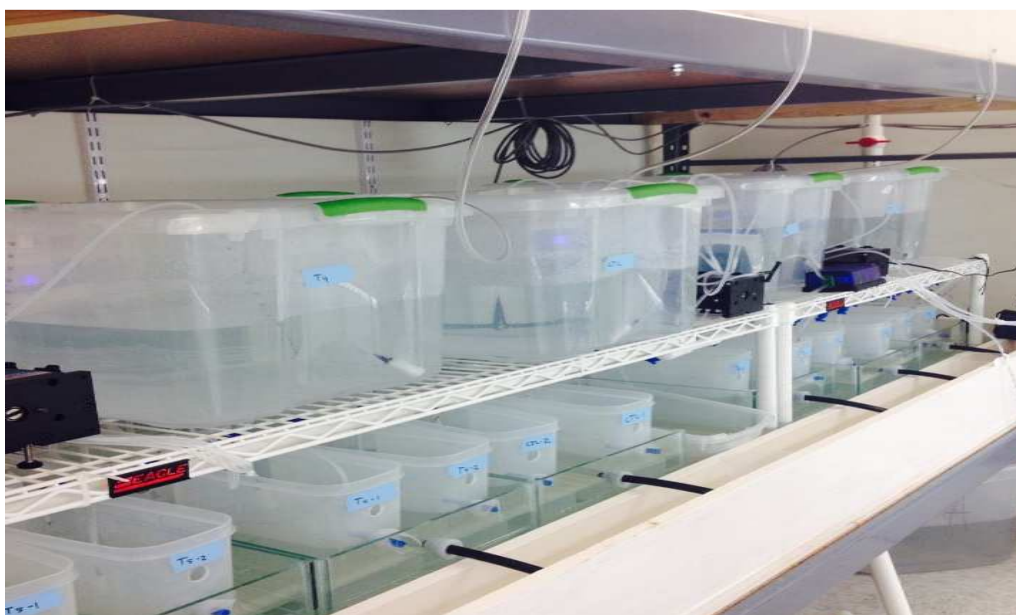
Image 1. Chronic toxicity flow-through testing system.
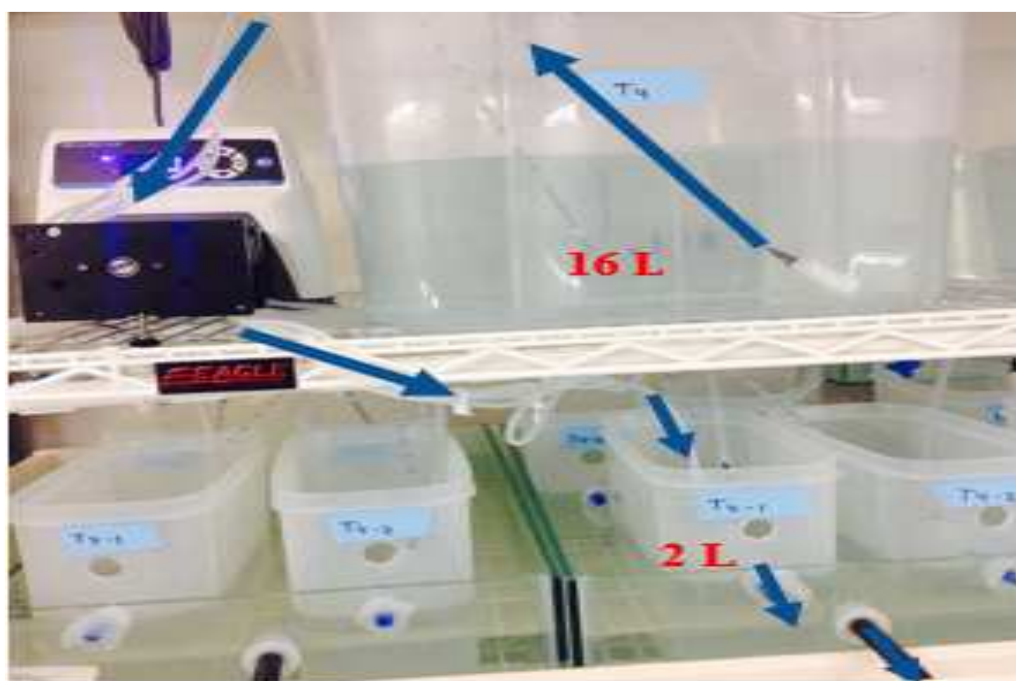
Image 2. Experimental flow-through system depicting the flow of treatment water (arrows) from basins to respective replicates. Peristaltic pumps aliquot 83.4 mL to each discrete replicate every 30 minutes which equates to 2 L every 12 hours.

Dissolved oxygen, pH, temperature, and salinity were measured every day of the test within ± 1 hour of the initial time of exposure on D0. After recording daily measurements, mortality was recorded for every replicate of each treatment. Any dead fish were collected and removed daily. After 28 days of testing, surviving individuals were euthanized with methane tricaine sulfonate (MS-222), rinsed with deionized water, and subsequently stored in 8% formalin solution for later analysis of standard length, dry weight, and Pb tissue concentrations. Standard length refers to the length of a fish measured from the tip of the snout to the last vertebrae [23].

Dry weight was calculated with desiccated fish to determine fish tissue mass while excluding fish fluid mass. Fish were washed with deionized water to remove any excess salt water or formalin. Replicate-specific aluminum pans were rinsed with deionized water and placed in a convection oven set to 60° C for 4 hours to remove any excess moisture. Fish wet weight was then recorded with in previously weighed replicate-specific pans. After wet weight was recorded, the fish were desiccated in a replicate-specific aluminum pan in a convection oven set to 60° C for 24-hours to remove any fish fluid mass. Once complete, replicate dry weights were recorded and test organisms were subsequently digested with HNO_3 based on the US EPA Method 3050B for tissue Pb analysis [10] and finally analyzed by ICP-MS.

Water samples were collected for total and dissolved metals, anions, cations, and dissolved organic carbon (DOC) analyses. Samples for DOC analysis were collected into 30 mL amber glass bottles. All other samples were collected into acid washed 15 mL polypropylene tubes. Dissolved metal, anion, cation and DOC samples were filtered using a 0.45μm Whatman™ filter (GE Healthcare Life Sciences, Piscataway, NJ, USA). Total and dissolved

metal samples were acidified with HNO₃ to about pH 2. All samples were stored at 4°C in a

refrigerator prior to analysis. Analysis of metals and cations was performed by ICP-MS.

Concentration of anions was analyzed with an Ion Chromatograph (Metro Ω, Northbrook, IL,

USA). A TOC analyzer was used to measure concentration of DOC in the test water (Shimadzu

Inc, Addison, IL, USA)

Data Analysis

After physiochemical analysis, data were used to calculate bioaccumulation factor (BCF).

Survival data were used to determine lethal concentrations (LCs) and effect concentration (ECs)

were determined with standard length and weight data. NOEC (No observed effect

concentration) and LOEC (Lowest observed effect concentration) were calculated with both

survival and growth data. All calculations were performed using measured dissolved Pb

concentrations and survival data with CETIS (Tidepool Scientific Software, McKinleyville,

California, USA), a statistical package designed for analysis of results from environmental

toxicity tests.

Pb speciation calculations were performed with all stability constants taken as

concentration constants as opposed to activity constants. This avoids the need to specify an

activity model for seawater [25]. The specific constants, logK values, for each exposure solution

were calculated and the corresponding logK values were interpolated from NIST National

Institute of Standards and Technology (NIST) tabulated stability constants over the range 0.0 to

1.0 mol/L ionic strength [26]. The ionic strength of the 28 ppt exposures were assumed to be

0.61 mol/L, and 0.31 mol/L for the 14 ppt exposures based on 32 ppt seawater having an ionic strength of 0.7 mol/L.

       With interpolated certified logK values it is also necessary to have total concentrations for each complexing agent. These anion concentrations were determined by measurement and used directly in modelling. The simultaneous equilibrium was presented in table notation and solved using a MATLAB program developed by Dr. Scott Smith at Wilfrid Laurier University. The MATLAB program was written based on the solution method of Carrayrou et al [27].

CHAPTER THREE

RESULTS

Dissolved Pb concentrations for each experiment are provided (Table 2)**.** Water quality parameters such as salinity, dissolved oxygen, temperature, pH, dissolved organic carbon, measured cations, and measured anions are shown for the low salinity fry test (Table 3), high salinity fry test (Table 4), and high salinity juvenile test (Table 5).

The average concentration ± 1 standard deviation salinity for the low salinity fry, high salinity fry, and low salinity juvenile tests were 14.1 ± 0.14 ppt, 28.0 ± 0.58 ppt, 28.0 ± 0.30 ppt, respectively. Salinity values were close to the target values of 14 ppt and 28 ppt. A higher incidence of anions and cations was found in high salinity studies due to an increased dissociation and ionization of the marine salts. The effect of marine salt abundance is also seen in alkalinity measurements of treatment waters. Average alkalinity was 58.18 ± 5.02 mg $CaCO_3$, 105.04 ± 8.08 mg $CaCO_3$, and 114.47 ± 3.98 mg $CaCO_3$ for the low salinity fry, high salinity fry, and high salinity juvenile studies, respectively. Lower saline waters are expected to have a lower alkalinity due to the lowered concentration of the key chemical species (i.e., HCO_3^-, CO_3^{2-}, and $B(OH)_4^-$) that contribute to alkalinity proportionally with increasing salinity [28].

Other variables such as dissolved oxygen, dissolved organic carbon, temperature, and pH remained relatively constant among studies. Dissolved oxygen never fell below the point at which fish may be harmed by a lack of oxygen, 5.0 mg/L [29]. Dissolved organic carbon, DOC,

remained consistent among tests. Mean temperature (± 1 standard deviation) for the low salinity

fry, high salinity fry, and low salinity juvenile tests were 18.2°C (0.29), 18.1°C (0.20), and

18.4°C (0.38), respectively; all of which were near the target temperature of 18.0°C. Mean (± 1

standard deviation) pH values were found to be nearly consistent across the low salinity fry, high

salinity fry, and low salinity juvenile tests at 7.96 (0.17), 7.92 (0.07), and 8.02 (0.09),

respectively.

Cumulative mortality graphs were generated for the high salinity fry experiment (Figure

1), low salinity fry experiment (Figure 2), and high salinity juvenile experiment (Figure 3). Per

ASTM guidelines, chronic toxicology studies must not exceed 15% mortality in control

treatments [23]. A study that exceeds this 15% threshold may have flaws in experimental design,

test conditions, or unhealthy test organisms. This threshold was not exceeded in any of the

control treatments for this study. Mortality was 100% in Treatment 4 (T4) (259 ± 23.7 µg/L Pb)

and T5 (435 ± 48.1 µg/L Pb) in the 28 ppt salinity fry study and in T3 (51 ± 3.1 µg/L Pb), T4 (80

± 7.4 µg/L Pb), and T5 (117 ± 18.7 µg/L Pb) in the 14 ppt salinity fry experiment (Table 2).

Mortality dose response curves were generated for fry in high (Figure 4) and low (Figure 5)

salinity tests.

Influence of salinity on Pb toxicity

Mortality was higher in the lower salinity *A. affinis* test than in the higher salinity test. As

a result, all LC values were higher for the high salinity test than the low salinity test (Figure 6).

The LC50 value, the lethal concentration that results in fifty percent mortality, for the 28 ppt

salinity study was 79.84 µg/L Pb; whereas, the LC50 value for the 14 ppt study was 15.14 µg/L

Pb. The mortality NOEC, the no observed effect concentration, and LOEC, the lowest observed

effect concentration, for the 28 ppt study were 46 µg/L Pb and 90 µg/L Pb, respectively.

The chosen alpha-level (α) for all significance tests was α = 0.05. A Welch's two sample

t-test confirms 46 µg/L Pb as the NOEC via comparison of control mortality to that of 46 µg/L

Pb treatment mortality (t = 0.73, df = 5.6, p = 0.5). This confirms the NOEC because mortality of

Treatment 1 (46 µg/L Pb) is not significantly different to the control mortality. A Welch's

independent sample t-test confirms 90 µg/L Pb as the LOEC via comparison of control mortality

to that of the 90 µg/L Pb mortality (t = 9.2, df = 4.8, p = <0.01). This confirms Treatment 2 (90

µg/L Pb) as the LOEC because it represents the lowest treatment which showed statistically

significant mortality in comparison to the control mortality for the 28 ppt *A. affinis* fry

experiment.

The mortality NOEC and LOEC for the 14 ppt were <14 µg/L Pb and 14 µg/L Pb,

respectively. A Welch's two sample t-test confirms the LOEC at 14 µg/L treatment (t = 4.4, df =

4.6, p = <0.01); whereas, the NOEC treatment is not applicable because the lowest treatment was

the LOEC. This result indicates an increase in Pb induced mortality to *A. affinis* when Pb

exposure occurs in a lower, 14 ppt, salinity environment in comparison to a higher, 28 ppt,

salinity environment.

A table of growth data, length and dry weight, was generated for comparative purposes

(Table 6). Results indicate a greater proportional decrease in control versus Pb treatment induced

weight loss to *A. affinis* when Pb exposure occurred in a lower, 14 ppt, salinity environment in comparison to a higher, 28 ppt, salinity environment.

The influence of salinity on sublethal effects of Pb on *A. affinis* fry length was determined (Figure 7). EC10 values, the effect concentration that results in a ten percent reduction in length compared to the control mean, for the 28 ppt salinity study were 82.30 µg/L Pb; whereas, the EC10 for the 14 ppt study was 16.36 µg/L Pb. The length NOEC and LOEC for the 28 ppt study are 46 µg/L Pb and 90 µg/L Pb, respectively. A Welch's two sample t-test confirms 46 µg/L Pb as the NOEC ($t = 0.74$, $df = 95$, $p = 0.46$) via comparison of control fry length to that of 46 µg/L Pb treatment fry length; the result is non-significant p-value >0.05. A Welch's two sample t-test confirms 90 µg/L Pb treatment is the length LOEC for the 28 ppt ($t = 3.7$, $df = 29$, $p = 0.001$) via comparison of control mortality to that of 46 µg/L treatment mortality; the result is a significant p-value <0.05.

The length NOEC and LOEC for the 14 ppt test were <14 µg/L Pb and 14 µg/L Pb, respectively. A Welch's two sample t-test confirms 14 µg/L Pb as the LOEC via comparison of control length to that of 14 µg/L Pb treatment length ($t = 2.9$, $df = 38$, and $p = 0.007$); whereas, the NOEC treatment is not applicable because the lowest treatment was the LOEC. The LOEC calculation result is significant with a p-value <0.05. This result indicated greater proportional reduction between control and treatment groups of *A. affinis* length when Pb exposure occurs in a lower, 14 ppt, salinity environment in comparison to a higher, 28 ppt, salinity environment.

The influence of salinity on sublethal effects of Pb on *A. affinis* fry dry weight were calculated (Figure 8). The EC25 dry weight value for the 28 ppt study was 6.85 µg/L Pb;

whereas, the EC25 dry weight value for the 14 ppt experiment was 15.62 µg/L Pb. The dry weight NOEC and LOEC for the 28 ppt study are <46µg/L Pb and 46 µg/L Pb, respectively. A Welch's two sample t-test confirms the LOEC at 46 µg/L Pb treatment (t = 5.0, df = 96, *p* = 0.001). The LOEC calculation result is a significant *p*-value <0.05; whereas, the NOEC treatment is not applicable because the lowest treatment was the LOEC.

The dry weight NOEC and LOEC for the 14 ppt test were <14 µg/L Pb and 14 µg/L Pb, respectively. A Welch's two sample t-test confirms the LOEC at 14 µg/L Pb treatment (t = 7.6, df = 51, *p* = 0.001). The LOEC calculation result is significant with a *p*-value <0.05; whereas, the NOEC treatment is not applicable because the lowest treatment was the LOEC. This result indicated a greater proportional reduction between control and treatment groups of *A. affinis* dry weight when Pb exposure occurs in a lower, 14 ppt, salinity environment in comparison to a higher, 28 ppt, salinity environment.

The influence of salinity on Pb tissue accumulation to *A. affinis* was determined. For the high salinity experiment, *A. affinis* tissue from Pb concentrations 46, 171, and 259 µg/L contained 34, 67, and 84 µg/L Pb, respectively. For the low salinity experiment, *A. affinis* tissue from Pb concentrations 14 µg/L and 27 µg/L contained 28 and 37 µg/L, respectively.

The effect of salinity on tissue Pb concentration was examined by means of comparison of the two experiments BCF (bioconcentration factor) values (Figure 9). Bioconcentration Factor was calculated by dividing µg Pb in tissue by the water treatment Pb concentration. The bioconcentration factor (BCF) is used to calculate the distribution of contaminants between water and biota as:

$$BCF \ = \ C\ Biota \div C\ Water$$

where *C* Biota is the total metal concentration in biota and *C* Water is the total metal concentration in water [34].

It is important to calculate the BCF because *A. affinis* play a major ecological role in aquatic food webs as a link to higher trophic levels [30]. Treatment one (14 µg/L) BCF results were higher (2.00) in the low salinity test in comparison to the high salinity test (0.73). This result indicates a greater proportional Pb uptake within the body tissue of test organisms when exposed to Pb in a lower (14 ppt) salinity environment compared to that in a higher (28 ppt) salinity environment.

Influence of organism age on Pb toxicity

A chronic, 28-d, 28 ppt salinity Pb exposure experiment was performed with juvenile *A. affinis*. The effect of organism age on toxicity was determined by comparing results from the previously conducted experiment on *A. affinis* fry to those of the *A. affinis* juvenile experiment (Figure 10). Juvenile *A. affinis* had reduced Pb mortality compared to $\leq$ 3-d-old fry when exposed to Pb. The LC25 and LC50 values for the juvenile fish were 129.5 µg/L Pb and 167.6 µg/L Pb, respectively, whereas the LC25 and LC50 values for the fry fish were 57.96 µg/L Pb and 79.84 µg/L Pb, respectively. These results indicate an increase in Pb induced mortality to *A. affinis* fry in comparison to juveniles.

Influence of water chemistry on Pb speciation

Figures 11 and 12 present measured and MATLAB modelled results of the relationship of total µg/L of Pb and dissolved µg/L Pb for 28 ppt and 14 ppt salinity exposure solutions, respectively. Figure 13 compares maximum free Pb^{2+} ion saturation points of the two salinities of interest, 14 ppt and 28 ppt, and how each affect the free Pb^{+2} ion species presence. Total Pb concentration would include Pb^{2+} as well as any other Pb species. The 14 ppt salinity becomes saturated with Pb^{2+} ions at a comparatively lower total Pb concentration value than that of the 28 ppt salinity. The result of a lower proportional Pb^{2+} to total Pb saturation point means a proportionally greater amount of toxic Pb^{2+} per total Pb µg/L within 14 ppt saline environments in comparison to that of 28 ppt saline environments.

Table 2. Pb concentration (µg/L) across Control (CTL) and Treatments (T1-T5) for 14 ppt *A. affinis* fry, 28 ppt *A. affinis* fry, 28 ppt *A. affinis* juvenile experiments.

Treatment	14 ppt fry	28 ppt fry	28 ppt juvenile
Control	0	0	0
T1	25* (14 ± 1.2)**	50 (46 ± 10)	100 (100 ± 21.4)
T2	50 (27 ± 2.1)	100 (90 ± 20)	200 (190 ± 30.0)
T3	100 (51 ± 3.1)	200 (171 ± 22.7)	N/A
T4	150 (80 ± 7.4)	400 (259 ± 23.7)	N/A
T5	200 (117 ± 18.7)	600 (435 ± 48.1)	N/A

*Pb nominal treatment concentrations (µg/L).
**Pb measured dissolved Pb concentrations (µg/L) ± one standard deviation.

Table 3: Physicochemical water quality parameters in low salinity (14 ppt) *A. affinis* fry experiment for Control (CTL) and 5 Treatments (T1-T5).

Treatment	Nominal Con. (µg/L)	Salinity (ppt) Mean	Stdev	Temp (°C) Mean	Stdev	DO (mg/L) Mean	Stdev	pH Mean	Stdev	DOC (mg/L) Mean	Stdev	F^-	Br^-	SO_4^{2+}	Cl^- (mg/L)	Na^+	Mg^{2+}	K^+	Ca^{2+}
Control	0	14.1	0.14	18.3	0.29	7.63	0.34	7.93	0.16	2.00	0.43	NA	NA	58.7	7567	NA	541	151	130
T1	25	14.1	0.13	18.1	0.29	7.59	0.30	7.94	0.16	2.51	0.50	NA	NA	NA	7750	NA	503	138	129
T2	50	14.1	0.19	18.1	0.28	7.56	0.37	7.95	0.18	2.40	0.03	NA	NA	57.6	7557	NA	510	139	123
T3	100	14.1	0.12	18.2	0.33	7.62	0.49	7.99	0.18	2.48	0.82	NA	NA	NA	7459	NA	485	128	119
T4	150	14.1	0.14	18.2	0.30	7.52	0.42	7.97	0.17	2.00	0.62	NA	NA	NA	7489	NA	490	131	119
T5	200	14.1	0.09	18.1	0.27	7.55	0.40	7.98	0.16	2.12	0.70	NA	NA	NA	7446	NA	505	139	124
Test average	NA	14.1	0.14	18.2	0.29	7.58	0.39	7.96	0.17	2.14	0.53	NA	NA	58.18	7561	NA	507	138	125
Stdev	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	7.8	226	NA	61	24	16

N/A: Not Applicable

Table 4: Physicochemical water quality parameters in high salinity (28 ppt) *A. affinis* fry experiment for Control and Treatments as in Table 2.

eatment	Nominal Con. (µg/L)	Salinity (ppt) Mean	Stdev	Temp (°C) Mean	Stdev	DO (mg/L) Mean	Stdev	pH Mean	Stdev	DOC (mg/L) Mean	Stdev	F⁻	Br⁻	SO₄²⁺	Cl⁻	Na⁺	Mg²⁺	K⁺	Ca²⁺
															(mg/L)				
Control	0	27.9	0.60	18.1	0.14	6.86	0.59	7.89	0.06	1.79	0.26	1.20	126	6102	21766	13687	1026	359	406
T1	50	28.0	0.58	18.0	0.21	6.85	0.63	7.91	0.05	1.69	0.32	1.28	128	6499	22348	13901	1075	369	440
T2	100	28.0	0.61	18.1	0.17	6.87	0.60	7.93	0.06	NA	NA	NA	NA	NA	NA	14260	1111	380	446
T3	200	28.0	0.60	18.0	0.20	6.91	0.62	7.94	0.06	NA	NA	NA	NA	NA	NA	13945	1079	372	441
T4	400	27.9	0.56	18.0	0.26	6.92	0.57	7.93	0.06	NA	NA	NA	NA	NA	NA	14236	1093	378	438
T5	600	27.9	0.54	18.1	0.23	6.87	0.59	7.90	0.15	1.72	0.19	1.19	120	5902	20936	13231	994	349	408
Test average	NA	28.0	0.58	18.1	0.20	6.88	0.60	7.92	0.07	1.73	0.25	1.22	125	6168	21683	13877	1063	368	430
Stdev	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.05	4	304	710	383	44	12	18

N/A: Not Applicable

Table 5: Physicochemical water quality parameters in high salinity (28 ppt) *A. affinis* juvenile experiment for Control (CTL) and 2 Treatments (T1-T2).

Treatment	Nominal Con. (µg/L)	Salinity (ppt)		Temp (°C)		DO (mg/L)		pH		DOC (mg/L)		F-	Br-	SO4^2+	Cl-	Na+	Mg^2+	K+	Ca^2+
		Mean	Stdev	Mean	Stdev	Mean	Stdev	Mean	Stdev	Mean	Stdev				(mg/L)				
Control	0	27.9	0.33	18.4	0.40	8.98	0.33	8.00	0.08	2.65	0.43	0.63	NA	3088	15610	NA	1581	542	463
T1	100	27.9	0.28	18.4	0.38	8.85	0.31	8.04	0.09	2.35	0.84	NA	NA	3179	15712	NA	1739	602	512
T2	200	28.0	0.31	18.4	0.37	8.80	0.33	8.03	0.09	2.36	0.85	0.49	NA	3199	15732	NA	1801	628	530
Test average	NA	28.0	0.30	18.4	0.38	8.88	0.33	8.02	0.09	2.45	0.65	0.55	NA	3155	15685	NA	1707	591	502
Stdev	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.12	NA	90	319	NA	346	132	111

N/A: Not Applicable

Figure 1. Cumulative mortality of *A. affinis* fry exposed to Pb at 28 ppt salinity. The horizontal line at 15% indicates the maximum percent mortality allowed in control groups per ASTM guidelines.
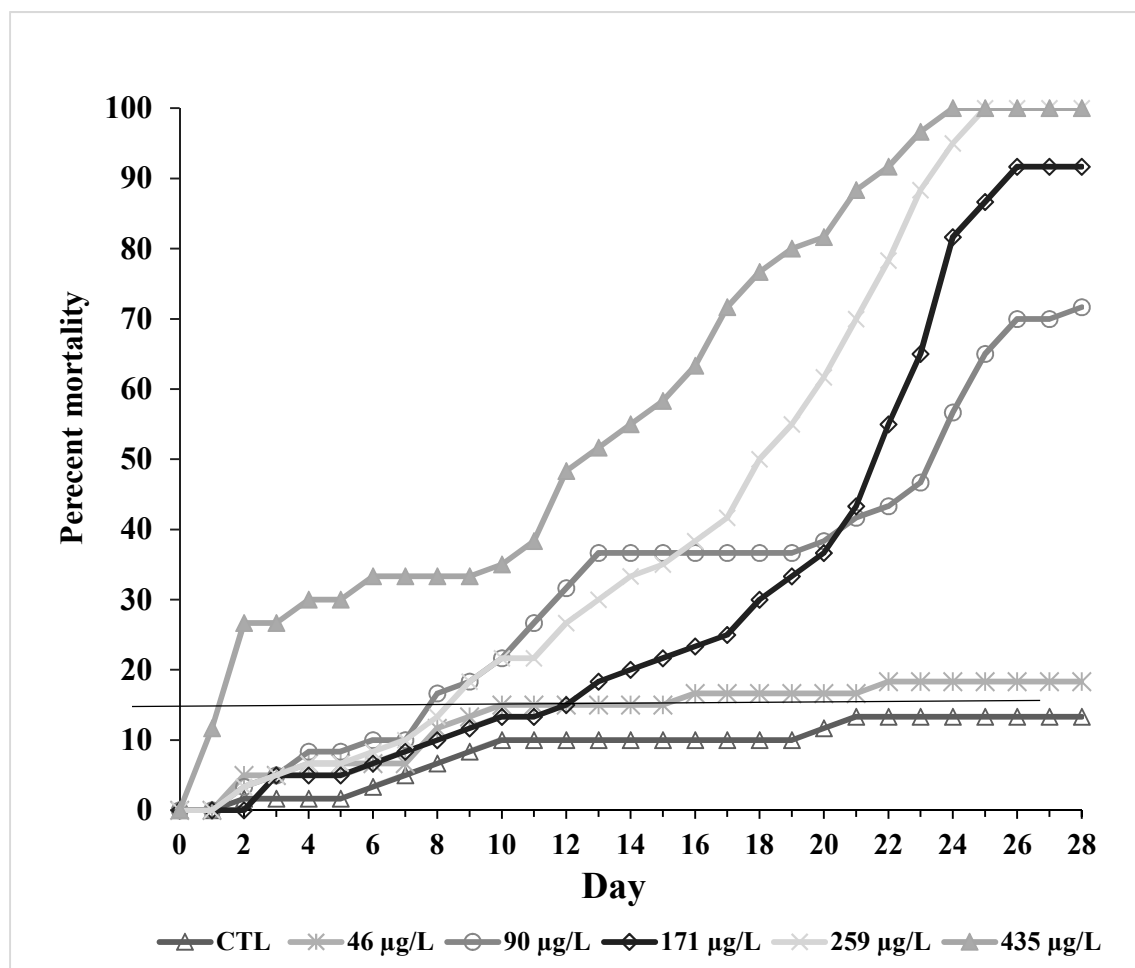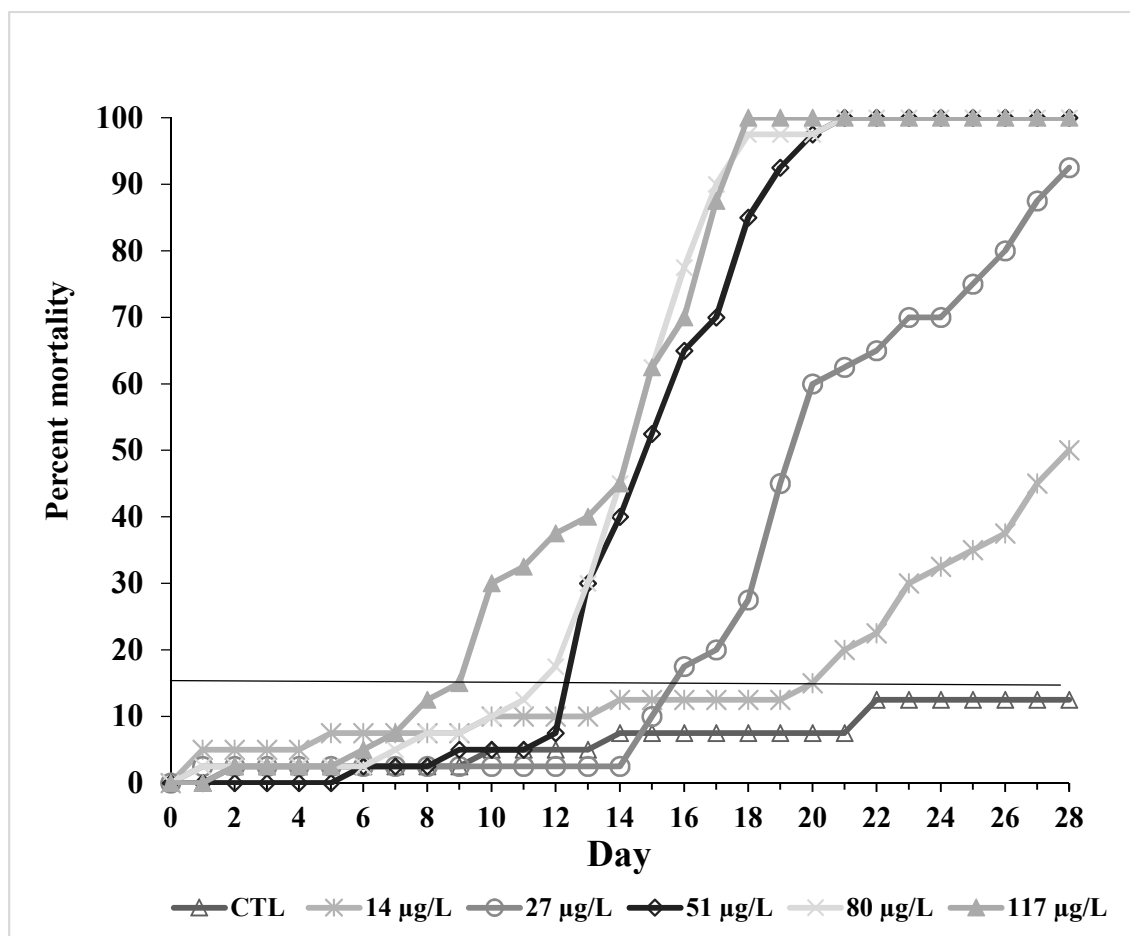
Figure 2. Cumulative mortality of *A. affinis* fry exposed to Pb at 14 ppt salinity. The horizontal line at 15% indicates the maximum percent mortality allowed in control groups per ASTM guidelines.
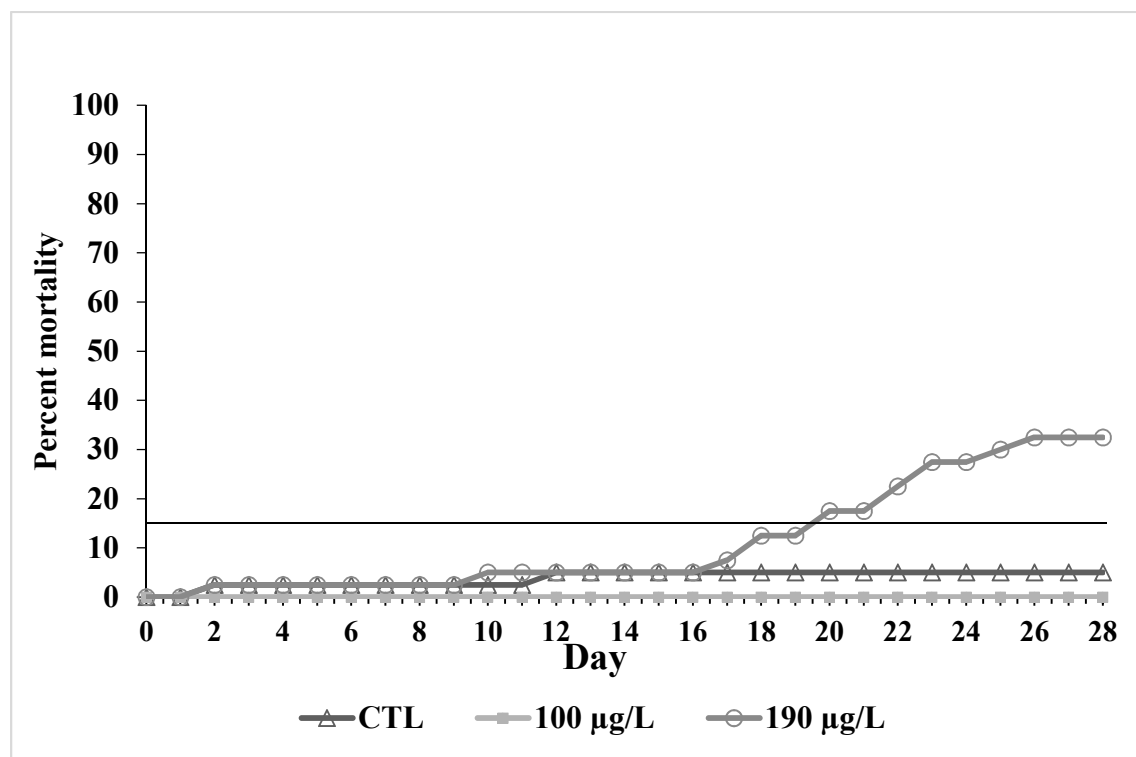
Figure 3. Cumulative mortality of *A. affinis* juveniles exposed to Pb at 28 ppt salinity. The horizontal line at 15% indicates the maximum percent mortality allowed in control groups per ASTM guidelines.

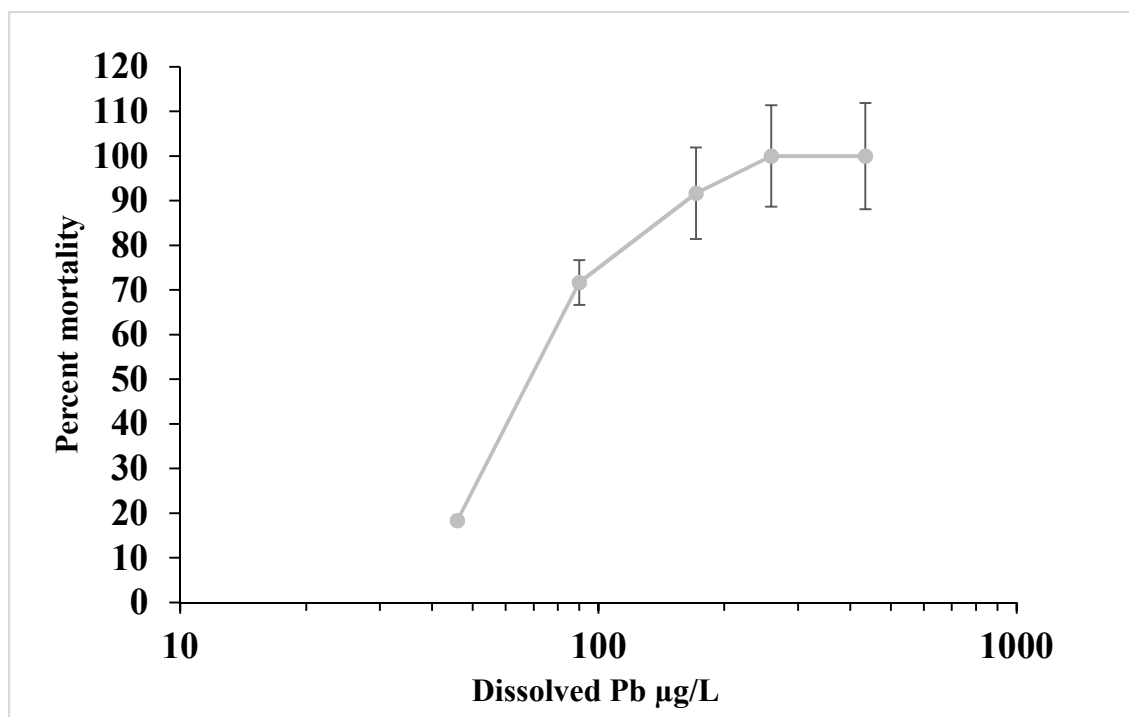Figure 4. Pb dose response curve for *A. affinis* fry in high salinity (28 ppt). Error bars represent ± one standard deviation.
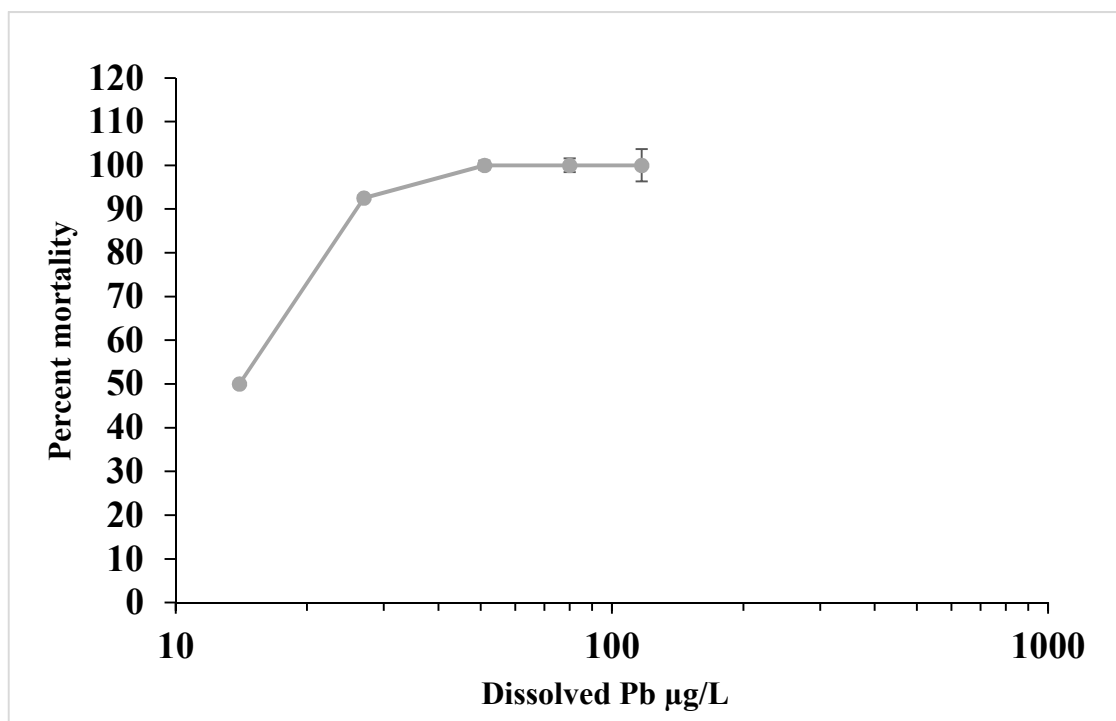
Figure 5. Pb dose response curve for *A. affinis* fry in low salinity (14 ppt). Error bars represent ± one standard deviation.
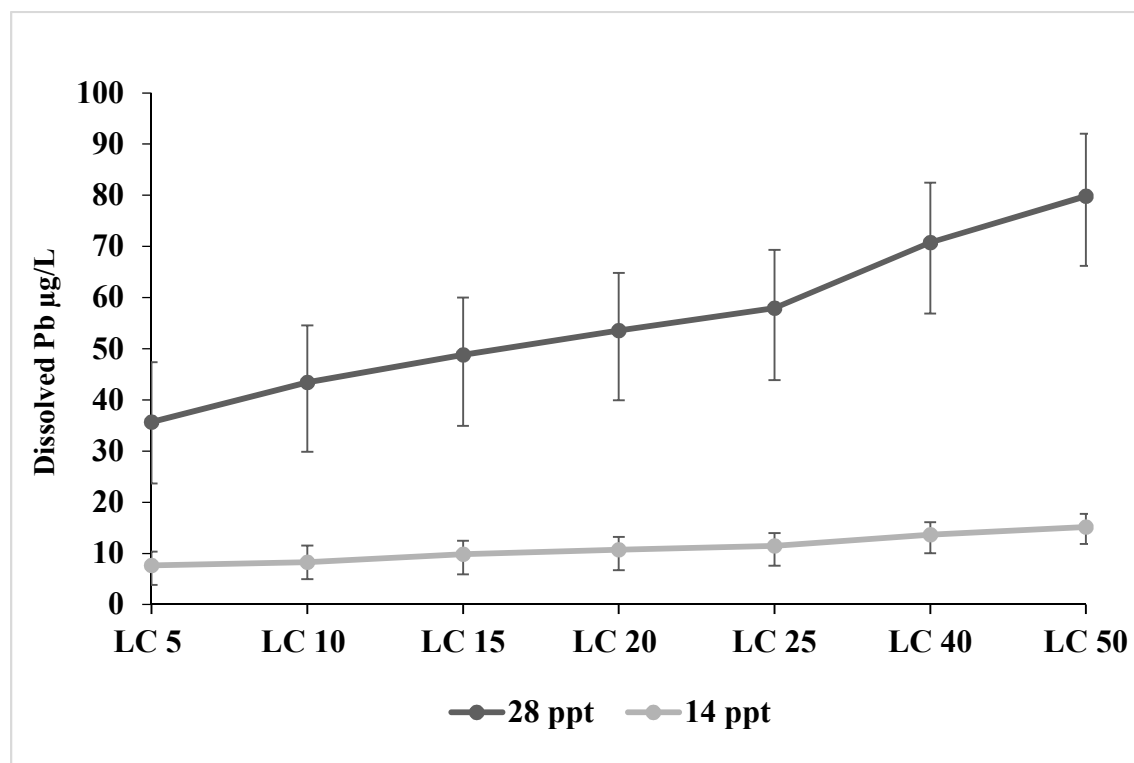
Figure 6. Pb toxicity lethal effect (LC) concentrations for *A. affinis* fry at 28 ppt and *A. affinis* fry at 14 ppt salinity experiments. Error bars represent 95% confidence intervals.

Figure 7. Sublethal effect concentrations (EC) of Pb on *A. affinis* fry length at 14 ppt and 28 ppt salinities. Error bars represent 95% confidence intervals.
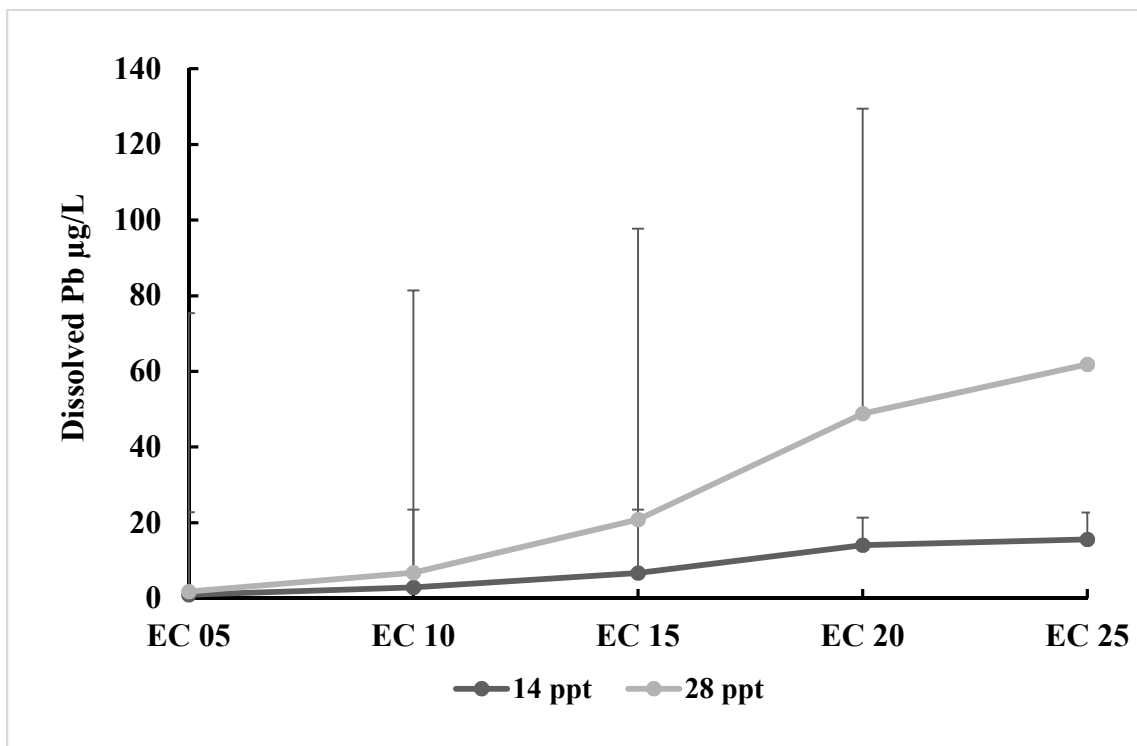
Figure 8. The influence of salinity on the effect of Pb on *A. affinis* dry weight at 14 ppt and 28 ppt salinities. Effect Concentrations (EC) concentrations for dry weight endpoint. Error bars represent 95% upper confidence intervals.
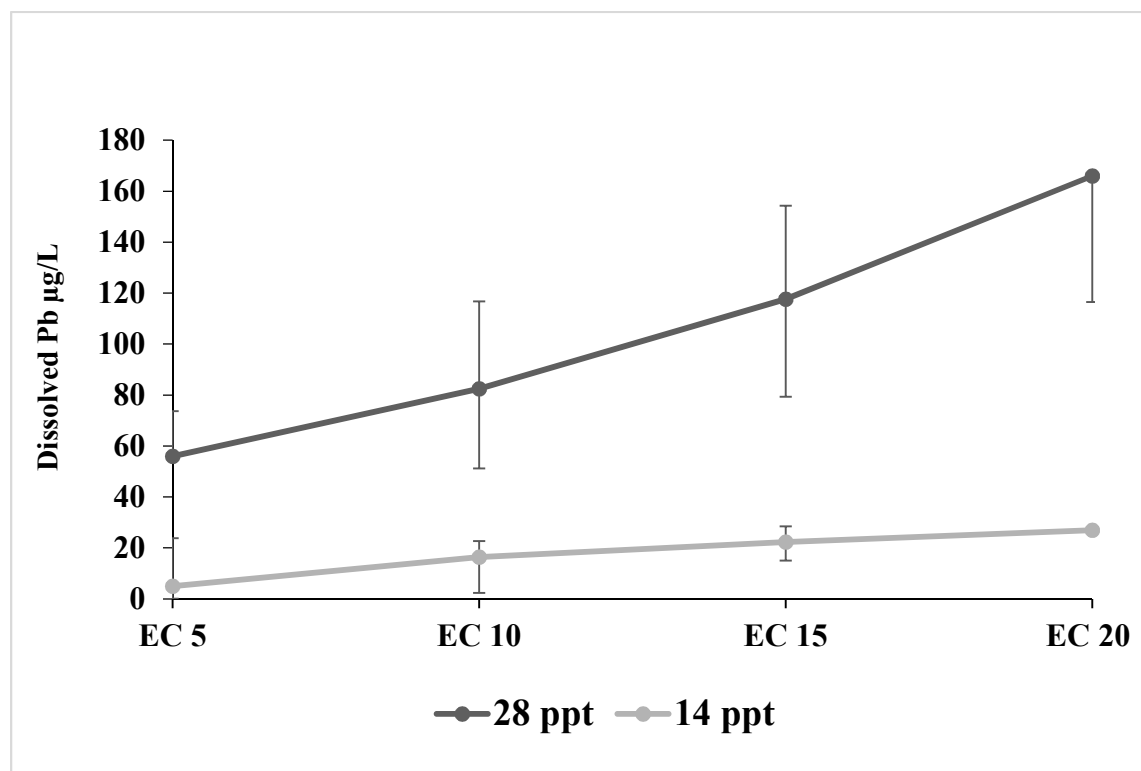
Table 6. Effect of Pb concentration on *A. affinis* growth at different salinities and life stages across Control (CTL) and Treatments (T1-T3).

Organism Life Stage	Salinity (ppt)	Treatment µg/L	Length (mm)	Weight (mg)
Fry	14	CTL	14.8 ± 1.4	4.639 ± 0.77
		T1 (14 ± 1.2)*	13.7 ± 1.5	3.436 ± 0.56
		T2 (27 ± 2.1)	12.1 ± 0.8	2.225 ± 0.66
	28	CTL	12.2 ± 1.3	1.79 ± 0.19
		T1 (46 ± 10)	11.9 ± 1.5	1.60 ± 0.23
		T2 (90 ± 20)	10.8 ± 1.3	1.17 ± 0.00
		T3 (171 ± 22.7)	9.7 ± 0.5	0.80 ± 0.00
Juvenile	28	CTL	38.2 ± 4.0	617.0 ± 4.06
		T1 (100 ± 21.4)	34.1 ± 5.9	532.6 ± 160
		T2 (190 ± 30.4)	35.6 ± 2.5	727.5 ± 88.8

* Parentheses include measured dissolved Pb in µg/L ± one standard deviation.
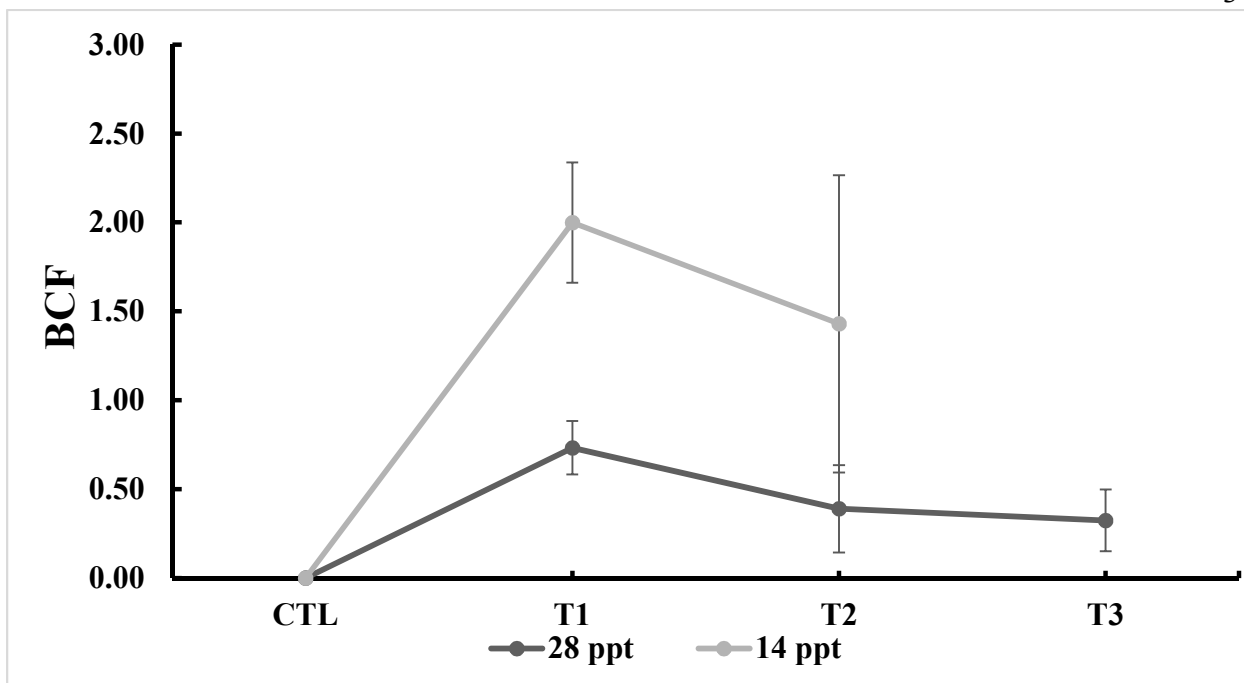
Figure 9. Effect of Pb concentration and salinity on Pb bioaccumulation of *A. affinis* fry represented by BCF (bioconcentration factor) values. Error bars represent ± one standard deviation.
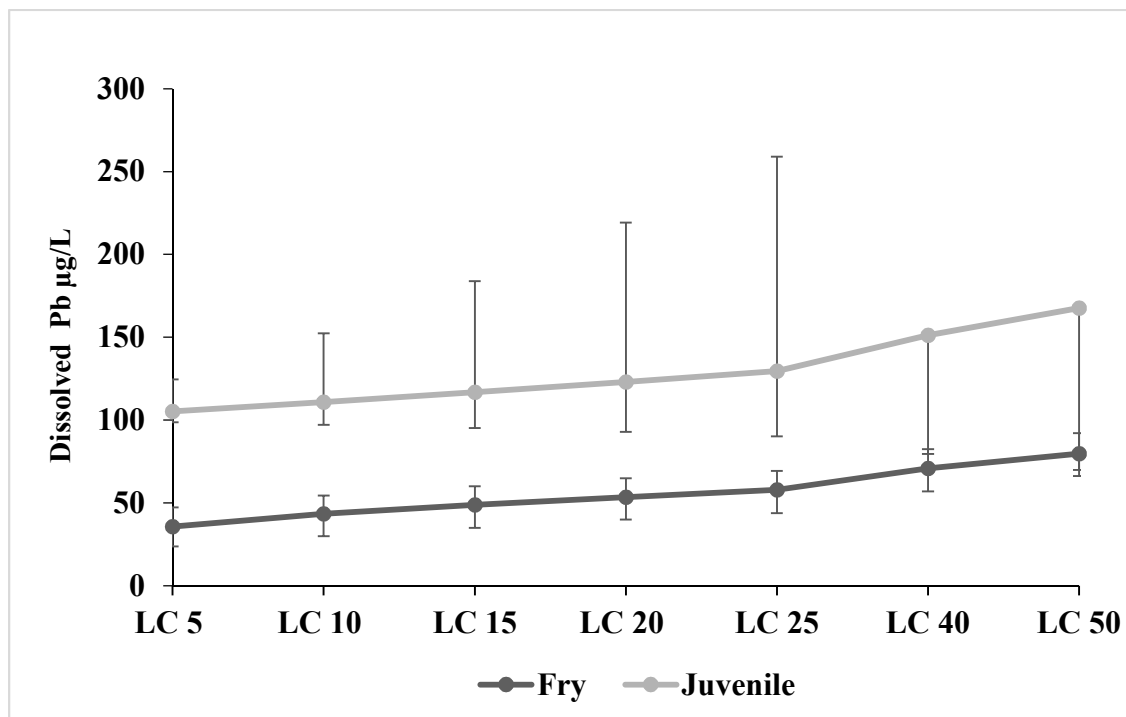
Figure 10. Pb induced mortality of fry and juvenile life stages of *A. affinis* at 28 ppt salinity LC concentrations. Error bars represent 95% confidence interval.
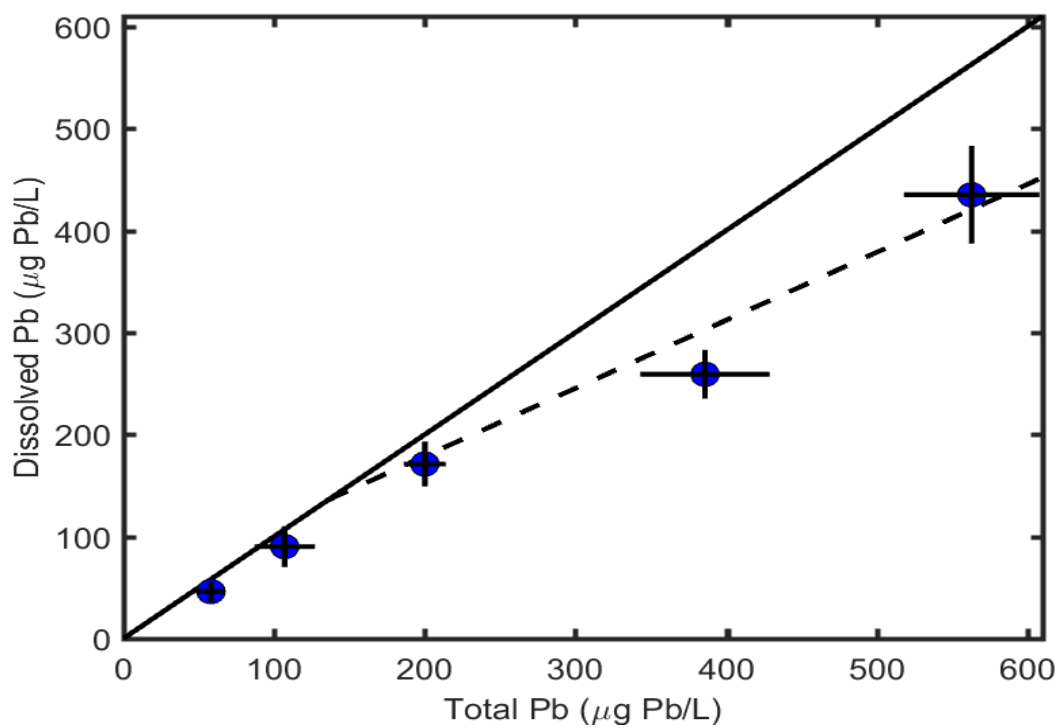
Figure 11: Measured (symbols) and modelled (dashed line) results for 28 ppt salinity exposure solution and the non-linear relationship between total Pb and dissolved Pb. Solid line represents point of reference for depicted non-linear relationship. Error bars are shown as ± one standard deviation around the measured results.
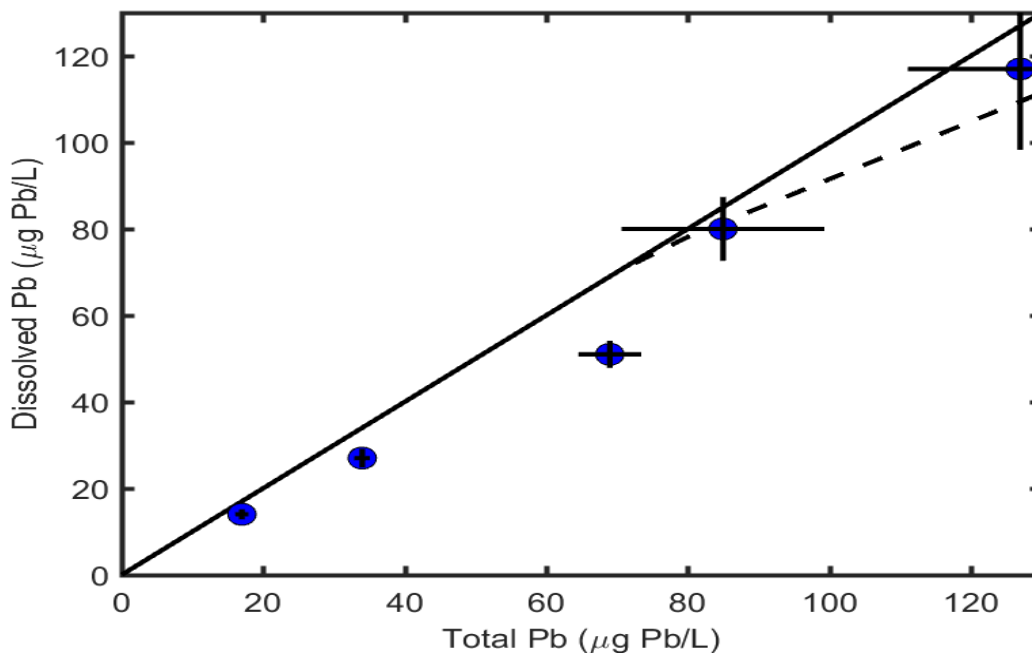
Figure 12: Measured (symbols) and modelled (dashed line) results for 14 ppt exposure solution. Solid line represents point of reference for depicted relationship. Calculations performed using measured anion concentrations and error bars are shown as one standard deviation about the measured results.
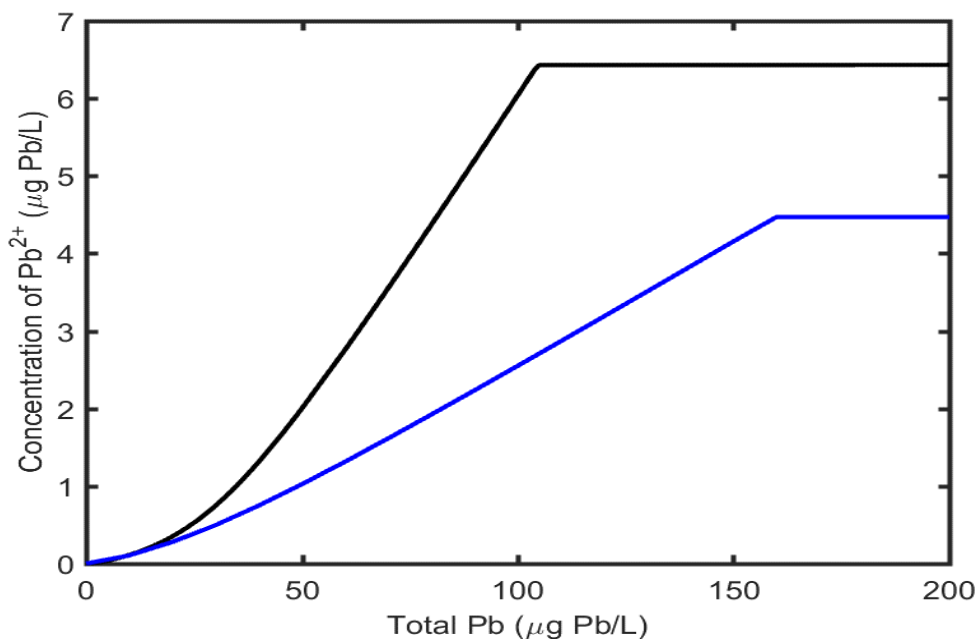
Figure 13. Pb saturation curves in 14 ppt (black) and 28 ppt (blue) salinity. Both experiment and modeled total Pb plateaus occur due to Pb saturation causing hydrocerrusite ($Pb_3(CO_3)_2(OH)_2$) to precipitate.

Discussion

Influence of salinity on Pb toxicity

The 14 ppt salinity experiment is representative of an estuary environment, whereas the 28 ppt salinity experiment is representative of a coastal marine environment. *A. affinis* Pb sensitivity increased in 14 ppt salinity waters in comparison to 28 ppt salinity waters. From a purely chemical perspective, an estuary represents a zone where seawater of high ionic strength is diluted by river water, resulting in a linear salinity gradient [31]. Results from my study indicate Pb is more toxic to *A. affinis* in estuarine than in marine environments. Explanations for the increased toxicity at lowered salinity include a combination of decreased competition of Pb and salt water cations at biotic ligand sites, *A. affinis* osmoregulatory stress due to lowered salinity coupled with Pb exposure results in a dual stressor effect [19], and increased Pb bioavailability in the lower salinity environment due to a reduction of Pb anionic complexation.

One component of the lower salinity/increased Pb toxicity effect is due to changes in ratios of major cations to Pb and the subsequent impact on organism uptake sites of cotransport and competition [31]. Pb causes ionoregulatory and osmoregulatory disruption to *A. affinis* [33]. Ionic mechanism of action for Pb mainly arises due to its ability to substitute other bivalent cations, e.g., Ca^{2+}, Mg^{2+}, Fe^{2+} and monovalent cations, such as Na^+ [16]. Lower salinity will decrease the total dissolved Pb to cation ratio within the water column. A decrease in total cations causes a decrease in competition at the biotic ligand; this allows Pb to substitute monovalent and divalent cations more readily [31] [1]. Once substituted, Pb stresses fundamental biological processes of fish physiology. These interactions occur at the cellular and

molecular levels and are a result of the ability of Pb to mimic or displace cations during specific physiologic processes [33].

Increased toxicity at a lower salinity could partly be due to a dual stressor effect- a contaminant presence combined with osmoregulatory stress. Salinity has been found to affect the physiology of marine organisms and the mechanisms they adopt to maintain an osmotic balance and thus their responses to metal exposure [1] [18]. *A. affinis* have been shown to be amenable to hyposaline water, e.g. 5 ppt, and hypersaline water, e.g. 60 ppt, in natural conditions within their respective habitats [19]. *A. affinis* is a euryhaline osmoregulator, tolerant of a wide range of salinities, and varies its behavior to satisfy their preferred isosmotic point [34].

This wide range of tolerable salinities presents an osmoregulatory-specific component of toxicity common in euryhaline fish. These mechanisms of dynamic control of osmoregulatory strategy include the ability to passively perceive changes in environmental salinity that disrupt body water and salt homeostasis by osmosensation. Once detected, a cascade of signaling networks then encode information about the direction and magnitude of salinity change, and epithelial transport and permeability effectors respond accordingly [35].

Salinity has a profound effect on *A. affinis* osmoregulatory and ionoregulatory physiology. In standard seawater, *A. affinis* lose water to their concentrated external environment and experience a net gain of ions. Consequently, marine fish drink ocean water to compensate for this water loss. While water is ingested, the increased ionic gradient across the gastrointestinal tract facilitates the absorption of water and simultaneously fish gills excrete

dissolved ionic salts gained [1] [36]. Euryhaline osmoregulation in dilute environments, where there is diffusive ion loss and osmotic water gain across the large surface area of the gill epithelium, causes *A. affinis* to produce large volumes of dilute urine in a kidney specialized for electrolyte absorption and gains via food and active ion uptake [36].

The isosmotic point of the organism and, in general, the relative cationic gradient between the internal fluids and the external environment can affect the osmoregulatory strategy of the organism and consequently its sensitivity to metals [31]. Reviewing eight studies on the influence of salinity to acute Cu toxicity, Grossel et al. found that none of the studies showed a linear increase in tolerance with increasing salinity, as competitive interactions among cations may have suggested [36]. The displayed sensitivities were not fully explained on the basis of Cu speciation. Therefore, a physiological stress combined with contaminant presence was hypothesized as the cause of observed increases in toxicity.

Although *A. affinis* tolerate lower salinities, the species is apparently physiologically better adapted to more marine conditions [19] [20]. In general, *A. affinis* spend most of their life in marine salinity. *A. affinis* spawn in harbors and estuaries in the summer months then migrate offshore in the fall. Studies have shown that one stress alone, e.g., salinity, may have no obvious effect on *A. affinis*, but in combination with another stress, e.g., chemical, it enhances the toxicity of the chemical. [21].

The aforementioned studies conclude that the physiology of the organism was a factor of toxicity and thus supports the observation that euryhaline species are more tolerant to metal

exposure at organism- specific salinities due to minimization of osmotic stress [12] [36]. I conclude that a factor of increased toxicity of *A. affinis* Pb exposure at lower salinity may be due to a dual stressor effect; Pb contaminant exposure combined with osmoregulatory stress of euryhaline *A. affinis*.

Several previously cited studies examined Cu toxicity and not Pb toxicity; however, Pb and Cu share a similar mechanism of toxicity and marine chemistry [31] [25] [36]. Both Cu and Pb cause ionoregulatory and osmoregulatory disruption at the biotic ligand, exhibit inverse relationships among toxicity and salinity, and speciate heavily to carbonate and chloride complexes in salt water [35]. Both metals are most bioavailable in the ionic Cu^{2+} and Pb^{2+} state. Water quality parameters such as pH, alkalinity, salinity and dissolved organic carbon (DOC) affect Pb and Cu bioavailability; therefore, an increase in the aforementioned water quality parameters generally decreases bioavailability of Cu and Pb [10].

Pb bioavailability increases in estuarine environments due to decreased speciation of Pb. Speciation of Pb is limited in estuary environments because less anionic solutes are present to react with Pb to form non-bioavailable Pb species. High salinity, i.e., 28 ppt, has a more protective effect against Pb toxicity than low salinity, i.e., 14 ppt, partly because it reduces the availability of the metal as a consequence of increased inorganic metal speciation.

In general, Pb^{2+} is more toxic than all other inorganic complexes of Pb [30]. Any factor, such as increased salinity, which increases complexation and decreases Pb^{2+} is likely to reduce Pb toxicity [19]. Salinity is frequently implicated as the strongest variable when one considers

bioaccumulation of Pb in fish [35] [39]. Bioaccumulation of Pb by marine organisms can be affected by its chemical forms [40]. In cases where Pb supply is limited, an increase in salinity may reduce the bioaccumulation because of Pb-chloro complex speciation [19]. Bourg et al. calculated Pb speciation as a function of salinity. Their calculations indicated that the prevalence of different forms and complexes of Pb^{2+} in standard seawater was: $PbCl^+$> $PbSO_4$> $PbCl_2$> $PbCl_3$-> $PbCO_3$> $PbOH^+$>Pb^{2+} [39].

For full strength seawater, Angel et al. present Visual Minteq model results and determine a solubility limit for seawater as 60 µg Pb/L with the precipitating phase being hydrocerussite- $(Pb_3(CO3)_2(OH)_2$ [25]. Nevertheless, the predicted solubility was significantly lower than the experimentally determined solubility limits measured in both natural and artificial seawater. Experimentally, Angel et al. determined solubility of 2000 µg Pb/L in natural seawater and 780 µg/L in artificial seawater [25]. Angel et al. attribute the differences between the measured natural and artificial seawater to organic complexation. This was attributed to the effects of natural organic matter, specifically weak Pb binding sites.

Solubility can be estimated from modelling and experimentally by comparing measured total lead, dissolved lead, and measured anion concentrations. The simultaneous equilibrium was presented in table notation and solved with a MATLAB program developed by Dr. Scott Smith at Wilfrid Laurier University. The MATLAB program was written based on the solution method of Carrayrou et al. (2002) [27]. The precipitated amounts match very closely between measured and modeled (Figure 11 and 12).

Ionic Pb^{2+} increased with increasing total Pb to approximately 105 µg/L for the low salinity (14 ppt) study until a saturation point occurred, whereas ionic Pb^{2+} increased with increasing total Pb to approximately 160 µg/L for the high salinity (28 ppt) study until a saturation point occurred (Figure 13). Water column saturation is limited because hydrocerrusite $(Pb_3(CO_3)_2(OH)_2)$ begins to precipitate from treatment solutions. Because pH and carbonate do not change, the free lead stays constant until carbonate starts to run out.

Lower carbonate concentrations (or levels) in the lower salinity experiment shows higher $[Pb^{2+}]$ is reached before precipitation. This occurs because solids buffer the free ion because the Ksp values must be satisfied. Because pH and carbonate do not change, the free lead remains until carbonate begins to run out. Lower carbonate in the lower salinity sample indicates that a higher Pb^{2+} concentration is reached before precipitation. Pb^{2+}, as with most ionic metals, is the most toxic species; therefore, a higher Pb^{+2} concentration in the lower salinity sample would be more bioavailable and more toxic to organisms [10]. The Ksp expression is $K=[OH][CO_3][Pb]$ and therefore $[Pb]=(K/([OH][CO_3])$. Because pH is constant we can write $[Pb]=K'/[CO_3]$. This means a higher $[CO_3]$ results in a lower $[Pb]$ because K' does not change.

Influence of organism age on Pb toxicity

This study found that *A. affinis* fry was more sensitive to chronic Pb exposure than juvenile *A. affinis* for the mortality, LC, endpoint. These conclusions are consistent with other studies. Mar and Bergman exposed brown trout, *Salmo trutta*, and rainbow trout, *Oncorhynchus mykiss*, fry and juveniles to a metals mixture (Zn, Cu, Pb, Cd) and found fry were more sensitive

than juveniles [41]. All fish life stages are sensitive to the toxic effects of Pb; however, embryos have been found to be more sensitive to Pb than later juvenile stages [42]. Pb reduces embryonic resistance with subsequent exposures. Earlier life stages of fish tend to be more sensitive to Pb than fish of later developmental stages [38].

CHAPTER FOUR

CONCLUSIONS AND SUGGESTIONS

Chronic metal exposures are more resource intensive; however, they are more indicative of metal contamination events. From this research, we have characterized the chronic toxicity of Pb to *A. affinis* and also the influence of salinity and organism age on Pb toxicity. Results of this study indicate that Pb toxicity is inversely related to both salinity and organism age. Implications of these results include an improved understanding of increased sensitivities that may occur at both estuary and other low salinity habitats that may guide protective policies for estuarine areas.

Results of this study will be used to develop a Pb chronic marine species sensitivity distribution and assist in developing guidelines for low saline environments. Results could be used to supplement the current marine Biotic Ligand Model, an important tool for ecological risk assessment. Additional metal studies need to be conducted, especially chronic exposures, to better understand and protect our water and wildlife resources.

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VITA

Erik's interest in biology, toxicology, and environmental science began during his undergraduate career at Loyola University Chicago. Upon completing his B.S. in environmental science, he began pursuing a master's in Biology. His research focus has primarily been metal toxicities to aquatic and marine species. In addition to conducting toxicity tests, he is also in charge of maintaining cultures of fathead minnows, Florida apple snails, and water fleas in Dr. Tham Hoang's laboratory at Loyola University Chicago.

Erik has presented his research at the 2015 Society of Environmental Toxicology and Chemistry national meeting regarding his research chronic Pb marine exposures. He plans to pursue a position in industry or academia upon finishing his degree.