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**SUB-LETHAL EFFECTS OF CADMIUM ON AUDITORY STRUCTURE AND FUNCTION IN THE
FATHEAD (*Pimephales promelas*) AND BLUNTNOSE (*Pimephales notatus*) MINNOWS**

By

JENNIFER SZE-CHEN LOW

A Thesis
Submitted to the Faculty of Graduate Studies
Through Biological Sciences
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The Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada

2009

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Sub-lethal effects of cadmium on auditory structure and function in the fathead
(*Pimephales promelas*) and bluntnose (*Pimephales notatus*) minnows

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DECLARATION OF CO-AUTHORSHIP

I hereby declare that this thesis incorporates material that is the result of joint research undertaken in collaboration with my supervisor, Dr. Dennis Higgs, who co-authored the second chapter of my thesis. While I was the primary researcher to develop the key ideas, experimental designs, data analysis and interpretation, the work could not have been completed without the contribution and dedication of Dr. Dennis Higgs. As my supervisor he was essential to the proper development of techniques and data acquisition and provided statistical advice.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from my co-author to include the above materials in my thesis.

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ABSTRACT

Aquatic ecosystems are threatened by environmental contaminants and many heavy metals can influence both the structure and function of vital sense organs in fishes. The current study examines the effects of cadmium on auditory structure and function in cyprinid fishes. In the lab, fish were exposed for 96 h to a range of cadmium concentrations and both hearing sensitivity and hair cell morphology were quantified. While hair cell numbers were unaffected, cadmium caused an increase in auditory threshold, with a critical range for toxic effects of cadmium estimated at 2.1-2.9 $\mu\text{g/L}$. In the field, fish were collected from sites along the Detroit River to assess if differences in cadmium effects exist from site to site. No differences in hair cell number or hearing sensitivity were observed between each field site. The current study demonstrates sub-lethal effects of cadmium on fish sensory function while also pointing to the need for more careful interpretation of cadmium impacts on aquatic populations.

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TABLE OF CONTENTS

| | |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| DECLARATION OF CO-AUTHORSHIP | III |
| ABSTRACT | V |
| ACKNOWLEDGMENTS | VI |
| LIST OF TABLES | IX |
| LIST OF FIGURES | X |
| CHAPTER 1: GENERAL INTRODUCTION | 1 |
| MECHANOSENSATION IN FISHES..... | 1 |
| CADMIUM TOXICITY | 4 |
| THE FATHEAD AND BLUNTNOSE MINNOWS | 10 |
| THESIS OBJECTIVES..... | 12 |
| REFERENCES | 14 |
| CHAPTER 2: SUB-LETHAL EFFECTS OF CADMIUM ON AUDITORY STRUCTURE AND FUNCTION IN THE FATHEAD (<i>PIMEPHALES PROMELAS</i>) AND BLUNTNOSE (<i>PIMEPHALES NOTATUS</i>) MINNOWS | 24 |
| INTRODUCTION..... | 24 |
| METHODS | 26 |
| <i>STUDY SPECIES</i> | 26 |
| <i>FLOW-THROUGH CADMIUM EXPOSURE</i> | 27 |
| <i>AUDITORY BRAINSTEM RESPONSE</i> | 28 |
| <i>HAIR CELL MORPHOLOGY</i> | 30 |
| <i>FIELD STUDIES</i> | 30 |
| <i>STATISTICAL ANALYSIS</i> | 31 |
| RESULTS..... | 32 |
| <i>CADMIUM EXPOSURE & WATER QUALITY</i> | 32 |
| <i>AUDITORY PHYSIOLOGY</i> | 33 |
| <i>HAIR CELL MORPHOLOGY</i> | 36 |
| <i>FIELD STUDIES</i> | 37 |
| DISCUSSION..... | 38 |
| REFERENCES | 44 |
| VITA AUCTORIS | 80 |

LIST OF TABLES

| | | |
|------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Table 2.1 | Measured and targeted cadmium levels ($\mu\text{g/L}$). Cadmium levels in all treatment and control tanks were measured using graphite furnace atomic absorption spectrometry (GFAAS)..... | 51 |
| Table 2.2 | Measured water quality parameters in 96 hour lab studies at 12 h intervals (Cd) and 0, 48, & 96 h (Control). Parameters measured include pH, alkalinity (ppm), hardness (ppm), NO_2 (ppm), NO_3 (ppm), temperature ($^\circ\text{C}$), and Cl^- (mg/L)..... | 52 |
| Table 2.3 | Measured cadmium levels ($\mu\text{g/L}$) and water quality parameters (pH, alkalinity (ppm), hardness (ppm), NO_2 (ppm), NO_3 (ppm), temperature ($^\circ\text{C}$), and Cl^- (mg/L), at three field sites (Fighting Island = ‘contaminated’ site, Chewitt Bay = ‘intermediate’ site, and Peche Island = ‘clean’ site). Cadmium levels were measured using graphite furnace atomic absorption spectrometry (GFAAS)..... | 54 |

LIST OF FIGURES

- Figure 1.1** Generic representation of a fish inner ear. There are three inner ear end-organs: the saccule, lagena, and utricle. An otolith overlies the sensory epithelium containing hair cells of each end-organ.....22
- Figure 1.2** Diagram of sensory epithelium with individual hair cells. Each hair cell is composed of many stereocilia and one kinocilium. The otolith sits on top of the hair cells.....23
- Figure 2.1** Flow-through tank system set-up for 96 hour cadmium exposure and control trials. Dechlorinated tap water was continually added from a water reservoir to one of two mixing buckets (cadmium and control). Cadmium was continuously added to the cadmium mixing bucket from a $\text{Cd}(\text{NO}_3)_2$ stock standard solution. Water from each mixing bucket flowed into one of 6 treatment tanks (3 cadmium, 3 control).....55
- Figure 2.2** Diagram of auditory brainstem response (ABR) set-up. Both the speaker and fish are lowered under water. Recording and reference electrodes are connected to the TDT system 3, from which a computer receives information. The entire set-up is located in a sound-reducing chamber.....56
- Figure 2.3** Auditory brainstem response (ABR) traces. Series represents traces recorded in response to tone bursts played at increasing decibel levels. All traces were taken at 200 Hz.....57
- Figure 2.4** Pictures of fathead minnow sensory epithelium containing auditory hair cells: A) saccule endorgan. B) utricle endorgan. C) lagena endorgan stained with phalloidin.....58

Figure 2.5 Location of field sites along Detroit River. Bluntnose minnows were collected from each field site using a bag seine. A) Fighting Island = ‘contaminated’ site. B) Chewitt Bay = ‘intermediate’ site. C) Peche Island = ‘clean’ site.....**60**

Figure 2.6 a. Audiograms of hearing threshold responses (\pm standard error) in fish exposed to [Cd] = 6.49 $\mu\text{g/L}$ compared to control fish. Letters indicate groups in homogeneous subsets. Asterisks (*) indicate significant difference in hearing threshold between cadmium and control fish.....**61**

Figure 2.6 b. Audiograms of hearing threshold responses (\pm standard error) in fish exposed to [Cd] = 3.31 $\mu\text{g/L}$ compared to control fish. Letters indicate groups in homogeneous subsets. Asterisks (*) indicate significant difference in hearing threshold between cadmium and control fish.....**62**

Figure 2.6 c. Audiograms of hearing threshold responses (\pm standard error) in fish exposed to [Cd] = 1.698 $\mu\text{g/L}$ compared to control fish. Letters indicate groups in homogeneous subsets.....**63**

Figure 2.6 d. Audiograms of hearing threshold responses (\pm standard error) in fish exposed to [Cd] = 0.846 $\mu\text{g/L}$ compared to control fish. Letters indicate groups in homogeneous subsets.....**64**

Figure 2.6 e. Audiograms of hearing threshold responses (\pm standard error) in fish exposed to [Cd] = 0.593 $\mu\text{g/L}$ compared to control fish. Letters indicate groups in homogeneous subsets.....**65**

Figure 2.7 a. ‘Dose-response’-like curve created using threshold differences between fish exposed to a range of cadmium concentrations (from 0.593 to 6.49 $\mu\text{g/L}$) and control fish at low frequencies (100 & 200 Hz).....**66**

Figure 2.7 b. ‘Dose-response’-like curve created using threshold differences between fish exposed to a range of cadmium concentrations (from 0.593 to 6.49 $\mu\text{g/L}$) and control fish at best frequencies (400 & 600 Hz).....**67**

Figure 2.7 c. ‘Dose-response’-like curve created using threshold differences between fish exposed to a range of cadmium concentrations (from 0.593 to 6.49 $\mu\text{g/L}$) and control fish at high frequencies (800, 1000, & 2000 Hz).....**68**

Figure 2.8 a. Response latency (the amount of time it takes for the fish to respond to tone bursts being played) (\pm standard error) in fish exposed to $[\text{Cd}] = 6.49 \mu\text{g/L}$ and control fish.....**69**

Figure 2.8 b. Response latency (the amount of time it takes for the fish to respond to tone bursts being played) (\pm standard error) in fish exposed to $[\text{Cd}] = 3.31 \mu\text{g/L}$ and control fish. Letters represent groups in homogeneous subsets. Asterisks (*) indicate a significant difference in response latency between fish exposed to cadmium and control fish.....**70**

Figure 2.8 c. Response latency (the amount of time it takes for the fish to respond to tone bursts being played) (\pm standard error) in fish exposed to $[\text{Cd}] = 1.698 \mu\text{g/L}$ and control fish. Letters represent groups in homogeneous subsets.....**71**

Figure 2.8 d. Response latency (the amount of time it takes for the fish to respond to tone bursts being played) (\pm standard error) in fish exposed to $[\text{Cd}] = 0.846 \mu\text{g/L}$ and control fish. Letters represent groups in homogeneous subsets.....**72**

Figure 2.8 e. Response latency (the amount of time it takes for the fish to respond to tone bursts being played) (\pm standard error) in fish exposed to $[\text{Cd}] = 0.593 \mu\text{g/L}$ and control fish. Letters represent groups in homogeneous subsets.....**73**

| | |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Figure 2.9 a. Bar graph showing differences in hair cell numbers (+ standard error) in the saccule end-organ, between fish exposed to different cadmium levels and control fish..... | 74 |
| Figure 2.9 b. Bar graph showing differences in hair cell numbers (+ standard error) in the utricle end-organ, between fish exposed to different cadmium levels and control fish..... | 75 |
| Figure 2.9 c. Bar graph showing differences in hair cell numbers (+ standard error) in the lagena end-organ, between fish exposed to different cadmium levels and control fish..... | 76 |
| Figure 2.10 Audiograms of hearing threshold responses (\pm standard error) of bluntnose minnows collected from each field site along the Detroit River..... | 77 |
| Figure 2.11 Response latency (\pm standard error) of bluntnose minnows collected from each field site along the Detroit River..... | 78 |
| Figure 2.12 Hair cell differences (+ standard error) in each inner ear end-organ between bluntnose minnows collected from each field site along the Detroit River..... | 79 |

CHAPTER 1: GENERAL INTRODUCTION

Mechanosensation in Fishes

Mechanosensation in fishes is accomplished by both the auditory system and the mechanosensory lateral line. The fundamental physiological unit for both systems is the hair cell and the mechanism of activation of this hair cell is the same in each system; bending of apical hair cell extensions results in changes in membrane polarity and thus nervous activation. Because of this conserved receptor mechanism, external factors that affect the hair cell will affect both auditory and lateral line stimulation.

In fishes, the auditory system is used in many important contexts. For example, the auditory system plays a critical role in the mating process for many fish species (Ladich, 2004). Fishes, like the plainfin midshipman (*Porichthys notatus*) (Ibarra et al., 1983), rely on conspecific reproductive calls in order to attract fish of the opposite sex. Without the ability to hear, mating success would decrease and thus could have a serious impact on the fitness of the fish species. Acoustic communication is also important during predator avoidance. Predator avoidance can be accomplished by the acoustic detection of an approaching predator (Higgs, 2004). Predator avoidance is also attained as fish commonly vocalize for interspecific communication to warn others of an oncoming predator (Ladich, 2004). Thus, an inability to detect an approaching predator or an inability to detect a warning cue will result in a negative effect on survivorship. Any modification in biological functions of fishes that are associated with mating and reproduction, predator avoidance, foraging, and other social behaviours, can have a significant influence on fish populations (reviewed in Scott & Sloman, 2004).

'Sound' consists of both a pressure component and a displacement component (Rogers & Cox, 1988). As such, fishes require specializations in order to detect the pressure components of a sound wave (von Frisch, 1938). Fishes with specializations allowing pressure detection, such as the cyprinid fishes used in the current study, are able to detect both the pressure and displacement components of a sound wave through mechanical coupling between a gas filled chamber and the auditory hair cells (Hawkins & Myrberg, 1983; Chardon & Vanderwalle, 1997). Pressure impinging on the gaseous chamber sets off vibrations, which in turn stimulates the hair cells with a resulting increase in auditory bandwidth up to 4000 Hz or greater (Fay, 1988). Because of the differences in hearing specializations, the hearing range of fishes varies widely across species.

Regardless of specializations for pressure detection, the basic structure of the ear operates similarly in all teleosts. The inner ear of fishes is composed of three endorgans that are thought to be involved in sound detection (Figure 1.1). Each endorgan is a fluid-filled sac that contains a hard, calcareous structure called an otolith (Carlstrom, 1963; Popper & Platt, 1993). A sensory epithelium containing many sensory hair cells underlies each otolith (Platt & Popper, 1981). Each hair cell is a ciliary bundle composed of many stereocilia and one kinocilium (Platt & Popper, 1984) (Figure 1.2). It is the displacement of these hair cells that allows fishes to hear. Depending on the direction from which a sound is coming and the location of the kinocilium in the hair cell bundle, maximal displacement occurs when the ciliary bundle displaces towards the kinocilium. Because hair cells are the same density as water, as the water moves due to the presence of an incoming sound, the hair cells will also move, thus causing the otolith to lag behind.

This lag causes voltage-gated K^+ channels on the ciliary bundle to open, which initiates a cascade of events which allows the fish to hear. Thus, it is well known that the acoustic sensitivity of teleost fishes rely on their otolithic endorgans (Lowenstein, 1971).

It is important to study the structure and function of these hair cells and the various influences, anthropogenic and natural, that exist that may affect the auditory system. Evidence is accumulating that human actions can impact fish hearing. For example, ship noise has been found to impair the auditory sensitivity of fishes and decrease the ability to detect conspecific acoustic signals in the Lusitanian toadfish (*Halobatrachus didactylus*) (Scholik & Yan, 2002a; 2002b; Vasconcelos et al., 2007). Similarly, it has been found that natural toxins released by red tide can significantly reduce auditory sensitivity in the goldfish (*Carassius auratus*), showing that natural toxins can cause minor hearing loss in fishes (Lu & Tomchik, 2002).

The effects of heavy metal contaminants on the auditory system have not been studied; however it is clear that heavy metal toxicity can impact other important sensory systems, such as the olfactory and gustatory system (reviewed by Klapat et al., 1992). As detailed below, there is also evidence of heavy metal toxicity on the mechanosensory lateral line in fishes. This external sensory system aids in the interpretation of the surrounding environment through the detection of water motion (Coombs & Janssen, 1990; Coombs & Montgomery, 1994). Thus, because of the physiological similarities of the auditory hair cells to the mechanosensory neuromasts, results from studies on the mechanosensory system can have important implications for the auditory system.

Cadmium Toxicity

Aquatic ecosystems are threatened by environmental contaminants mainly because of their continued release through various anthropogenic sources. The accumulation of contaminants in local waters has been a concern with the Detroit River being officially listed as an Area of Concern (GLWQA & USEPA). According to the Remedial Action Plan for the Detroit River, cadmium is a parameter of concern (RAP Report, USEPA, 1996). Cadmium is a heavy metal that is non-essential and toxic to organisms at higher concentrations (Wright & Welbourn, 1994). It is found naturally at low concentrations in rocks and soils, and often contaminates sewage sludge and agricultural soils. Cadmium is also used in the production of numerous industrial products including alloys, batteries, and pigments (Hutton, 1983).

The manner in which a contaminant behaves in the aquatic environment depends on the qualities of the contaminant, the organisms involved and the environmental conditions. The level of effectiveness of a contaminant on an organism depends on bioavailability, which is simply defined as the portion of the total concentration of a chemical in the environment that is potentially available for biological action, such as uptake by an organism (Rand, 1995). This is a critical concept in ecotoxicology because if a contaminant is not bioavailable, it cannot produce an effect on an organism. According to the free-ion activity model (Morel, 1983), bioavailability is correlated with the free-metal concentration, and as such the free ion is often the most bioavailable form of a dissolved metal. This model is defined as “the universal importance of free metal ion activities in determining the uptake, nutrition and toxicity of all cationic trace metals” (Campbell & Tessier, 1996). A considerable amount of evidence suggests that metal

bioavailability is related to free ion activity, instead of total metal concentration (reviewed by Campbell, 1995). More recently with regards to cadmium availability, Li et al., (2009) found support for the free-ion activity model, as their results revealed that free Cd^{2+} activity was a better predictor for cadmium accumulation than total cadmium concentration in solution.

In the aquatic environment, metal bioavailability is affected by varying water chemistry conditions. Of particular importance in this regard is water hardness. Increasing water hardness results in the addition of hardness cations (i.e. Mg^{2+} and Ca^{2+}). Metal cations, such as Cd^{2+} and Pb^{2+} , will compete with hardness cations, to form complexes and compounds with anions, such as SO_4^{2-} & CO_3^{2-} , resulting in decreased metal availability compared to total metal concentration (Endovitskii et al., 2009). Hardness cations also compete with metal cations for uptake sites, and as such reduce metal bioavailability. For example, increases in water hardness cations, via CaCl_2 and MgSO_4 , resulted in a decrease in copper toxicity on the lateral line in zebrafish (Linbo et al., 2009). Studies have found that hardness cations like Mg^{2+} and Ca^{2+} also compete with free cadmium cations (Cd^{2+}), resulting in a decrease in cadmium toxicity with increasing water hardness (Li et al., 2009; Pascoe et al., 1986; Kinkade & Erdman, 1975).

Metal toxicity can also be affected by metal speciation. Divalent cations (such as Mg^{2+} and Ca^{2+}) have been shown to inhibit cadmium uptake, indicating these ions can share the same uptake sites (Benaissa & Benguella, 2004). Free sodium (Na^+) and chloride (Cl^-) ions have been shown to have no effect on cadmium uptake, and as such do not share the same uptake sites (Benaissa & Benguella, 2004). The differences in behaviour of these ions and those that do share uptake sites are thought to involve the

metal species. Divalent metal cations (i.e. Mg^{2+} & Ca^{2+}) can bind to the same binding sites (Dransfield, 1992; Wolff et al., 1977), and therefore can affect metal uptake of other divalent metal cations (i.e. Cd^{2+}) via competition for these binding sites (Di Toro et al., 2001). This is evident in the chemical and physical similarities of cadmium to zinc (Zn^{2+}) and to a lesser degree, mercury (Hg^{2+}). Due to their similarities, as reflected in their close proximity in the periodic table, it is well known that cadmium and zinc interact and cadmium can often substitute for zinc (reviewed in Brozka & Moniuszko-Jakoniuk, 2001).

Another important parameter known to effect metal availability is pH. Sherman et al., (1987) compared cadmium toxicity in laboratory and field settings and found that LC_{50} values of cadmium differed in each setting. These variations were due to differences in water chemistry. Specifically, cadmium toxicity in laboratory and field assays was found to be affected by varying pH and water hardness (Sherman et al., 1987). Changes in pH affect the number of H^+ ions. These H^+ ions also compete with metal cations for binding sites and as such, affect metal toxicity. For example, increases in pH have resulted in increased toxicity of zinc (Zn^{2+}) and copper (Cu^{2+}), which indicates a reduced competition of these metal cations with H^+ (Wilde et al., 2006).

A caveat to the free-ion activity model is that it implies constant biological effects with constant free ion activity of divalent cations in the aquatic environment. As noted above, water chemistry parameters frequently change and can greatly influence metal toxicity. Thus, an extended free-ion activity model (Pagenkopf, 1983) was proposed, (and evolved to what is known as the biotic ligand model (Di Toro et al., 2001)) and considers multiple factors affecting bioavailability of metals, including differences in

water quality. This model accounts for modifications in metal speciation, which would result in a decrease in the potential for a metal to bind to a receptor site, as well as, changes in the concentrations of competing cations, which would decrease the amount of metal bound to receptor sites (as observed with increases in water hardness cations).

Support for this model was observed by Meyer et al., (1999), who examined the concentrations of nickel and copper at gill binding sites in the fathead minnow at varying water hardness levels. These measures were found to be a more accurate measure of acute Cu and Ni toxicity, consistently over a range of water hardness levels, compared to measures of free-ion activity. Such studies of metal binding to fish gills incorporates the equilibrium between metal cations and metals accumulated at binding sites as well as the competition with other cations for those binding sites. The biotic ligand model has also been supported by Brown & Markich (2000), who further concluded that this model can directly provide fundamental information through concentration-response experiments in a range of varying water chemistry conditions and that it may be a more useful tool in metal-organism interactions.

Metal bioavailability in sediments is more difficult to estimate. The concentration of metals in solid-phases influences bioavailability by governing the concentration of metals in the interstitial surrounding water and by direct ingestion of solids by benthic species. Total sediment-associated metal concentration therefore can be a poor indicator of available metal (Tessier et al, 1984). For example, Szalinska et al., (2006), examined the distribution of heavy metals in sediments of the Detroit River. In this study, metal distribution was fairly homogenous with the exception of elevated concentrations in the middle and lower reach of the river. Hydrological conditions such as sorting and

deposition, and flow rates are thought to be responsible for this observed difference in metal concentration. Thus, because of these constant variations, total sediment loads alone are often inaccurate in assessing bioavailable levels. Determining bioavailable contaminant loads may require more analysis in the aquatic environment, as seen by Arain et al., (2008), who examined total dissolved and bioavailable elements (i.e. cadmium) in water and sediment in lake water. They found high levels of cadmium in the water column itself, as well as high accumulation of cadmium in fish tissues, indicating the ability of cadmium to enter the food chain. In addition to these measures, they also examined sediment loads to predict bioavailability (Arain et al., 2008).

Metal toxicity in fishes can also be examined through behavioural assays. Studies in the past have shifted from lethal effects of metal contaminants to acute sub-lethal effects of metals on biological functions. Studies have shown that heavy metal contaminants found in the environment are known to induce sensory deficits in fishes (Baker & Montgomery, 2001; Faucher et al., 2006). While these deficits are not lethal, they do affect many vital functions in fishes. Cadmium is one such heavy metal that has been shown to alter behaviours in fishes by inhibiting sensory systems. Anthropogenic and natural processes continuously release cadmium from its natural settings and disperse it to different areas within the aquatic environment. Effects vary from the sub-organismic level to changes in the ecosystem as a whole (Wendelaar Bonga, 1997; Wright & Welbourn, 1994). Metal contaminants, such as cadmium, can result in serious changes to biological, chemical, physiological and behavioural functions in fishes (Atchison et al., 1987; de la Torre et al., 2000; Espina et al., 2000; Shedd et al., 2001). Cadmium has been shown to impair olfactory function in the banded kokopu (*Galaxias fasciatus*) by

eliminating their attraction to adult pheromones after exposure to low cadmium levels (Baker & Montgomery, 2001). Similarly, other metal contaminants have been shown to have detrimental effects on the mechanosensory system. Previous work has shown that cobalt can ablate neuromasts in the blind Mexican cavefish, resulting in alterations in swimming behaviours (Janssen, 2000). Change in swimming behaviour is known to be a characteristic response to heavy metal toxicity (Sorensen, 1991). Exposure to cadmium at high concentrations has also been found to alter swimming behaviour in the sea bass (*Dicentrarchus labrax*). The same high concentration cadmium exposure also resulted in full deprivation of hair cell bundles in neuromasts (Faucher et al., 2006). Interestingly, lower levels of cadmium have also been shown to inhibit lateral line function in fishes, also causing alterations in important swimming behaviours (Baker & Montgomery, 2001). These studies show the potential for metal contaminants found in the environment to have detrimental effects on vital fish sensory systems, and in turn, is likely to affect the overall health and fitness of a fish population.

Acute toxicity assays are quick and relatively inexpensive and important in risk assessment and establishing environmental standards. However, to increase ecological realism, chronic sub-lethal effects should be considered. Faucher et al., (2007), examined the chronic effects of a low-concentration cadmium level that represented cadmium levels observed in the field. Cadmium was found to cause damage to lateral line neuromasts after chronic exposure to low-concentrations. This is in contrast to an earlier study by Faucher et al., (2006), in which they found no effect of the same low-concentrations of cadmium after acute exposure. This leads to important implications regarding possible effects of long-term cadmium exposure similar to that seen in the

aquatic ecosystem. Relating these studies back to the field would provide a better understanding of cadmium effects as it applies in natural aquatic settings.

Because previous work has shown cadmium to be an effective contaminant on fish sensory structures and behaviours (Faucher et al., 2006; Baker & Montgomery, 2001), it is important to study the effects cadmium may have on local fishes and their ecosystems. Also, because the auditory system can be vital to fish survival, it is equally important to study the potential effects of this common metal contaminant on the structure and function of this system, as it relates to the overall health and fitness of fish populations and the Great Lakes ecosystem.

The Fathead and Bluntnose Minnows

The fathead minnow (*Pimephales promelas*) is a small freshwater fish belonging to the cyprinid family. Its distribution spans much of North America and is also found in some parts of Asia, and Europe (Page & Burr, 1991). The fathead minnow is an important model in aquatic toxicology due to its relative hardiness and reproductive capabilities. Fathead minnows are able to survive in conditions that are otherwise intolerable for other fish species (i.e. murky, low oxygenated, hot/cold, low/high pH, turbid waters). Fathead minnows are able to spawn all year round, with their peak reproductive season between May and September. The maximum size of a fathead minnow is around 10 cm, with a lifespan of approximately 2 years if they have spawned, 4 if they have not (Page & Burr, 1991). Fathead minnows in the wild are a grey-olive colour, while a rosey-red strain of fathead minnow can often be found in pet stores.

The bluntnose minnow (*Pimephales notatus*) is a small, freshwater cyprinid fish that is closely related to the fathead minnow. Its distribution is widespread over most of North America (Page & Burr, 1991), and is possibly the most abundant freshwater fish in the eastern United States. Bluntnose minnows have a maximum length of 11.0 cm and a life span of approximately 5 years. These fish inhabit a variety of aquatic environments, ranging from clear rocky streams to large rivers to glacial lakes (Page & Burr, 1991).

The fathead and bluntnose minnows are characterized as benthic species. These fish have sub-terminal mouths and as such are bottom-feeders. These fish will feed on many types of food including algae, detritus, and insects. More importantly, these fish feed off the bottom sediments where contaminants are deposited. This specific type of feeding facilitates the uptake of contaminants by providing a mode of availability to organisms and allows contaminants to accumulate and enter the food chain. Thus, these species are good models in aquatic toxicology as contaminants may be more available to them through their feeding behaviours compared to more pelagic fish species.

Fishes belonging to the cyprinid family are hearing specialists and therefore have a high frequency range (up to 4000 Hz) and a low hearing threshold. This enhanced auditory sensitivity is accomplished through the use of a swimbladder and Weberian ossicles (von Frisch, 1938). Weberian ossicles are modified vertebrae that connect the swim bladder to the inner ear (Evans, 1925), and convert the pressure component of sound (vibrations of the gasbladder) to displacement of the inner ear hair cells (Finneran & Hastings, 2000). Because fish in the *Pimephales* genus have a diverse habitat range, they are exposed to a wide range of aquatic and acoustic environments. As such, it

makes this genus of fish an ideal model to use in the current study, looking at the effects of a common pollutant on hearing ability over a wide auditory range.

Thesis Objectives

Fishes are linked to their aquatic environments through their sensory systems, and many pollutants are known to influence both the structure and function of their sense organs (reviewed in Blaxter & Hallers-Tjabbes, 1992; Klaprat et al., 1992). The auditory system plays a prominent role in fish communication and survival, thus it is important to examine the effects that metal contaminants may have on this vital sensory system. Because cadmium is known to have detrimental effects on some sensory systems in fishes, the primary objective of my thesis was to determine the structural and functional effects of cadmium exposure on the auditory system in the fathead and bluntnose minnows.

My data chapter consists of three objectives. The first objective examined the hearing ability of the fathead minnow in response to a range of acute cadmium exposure treatments. More specifically, auditory thresholds (the minimum sound level a fish is able to detect) and response latencies (the time it takes for the brain to respond to a sound) were determined using the auditory brainstem response technique. The second objective of my research examined the morphology of the inner ear hair cells of the fathead minnow in response to a range of acute cadmium exposure treatments. This was examined through the quantification of hair cell numbers using fluorescence microscopy. The third objective of my thesis examined the sub-lethal effects of cadmium on auditory structure and function in the bluntnose minnow. Field sites along the Detroit River were

selected to assess if differences in effects were observed between field sites. Bluntnose minnows were collected from each field site, from which I examined sub-lethal effects of cadmium on the auditory system. Using the same techniques from the first two objectives of my research, I compared the hair cell morphology and hearing abilities of bluntnose minnows from the different field sites.

To date, the effects of heavy metal contaminants on the auditory system in fishes have not been studied. This is partly due to the fact that the auditory system is an internal sensory system, and is not as easily accessible to waterborne contaminants in comparison to other sensory systems that would be at first risk to such contaminants. However, because the auditory system plays a vital role in fish survival, it is critical to study the effects of contaminants on this system. Thus, my thesis serves an important function in broadening our current knowledge on the effects of metal contaminants on sensory structure and function. It provides a new outlook by assessing the effects of a common heavy metal – cadmium – on the auditory system in fishes, a combination that has previously not been studied.

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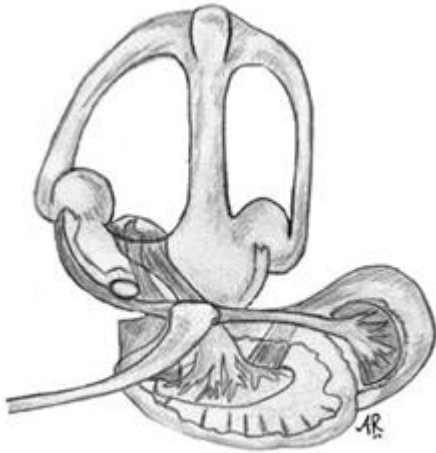


Figure 1.1 Generic representation of a fish inner ear. There are three inner ear end-organs: the saccule, lagena, and utricle. An otolith overlies the sensory epithelium containing hair cells of each end-organ. Artwork by Audrey Rollo.

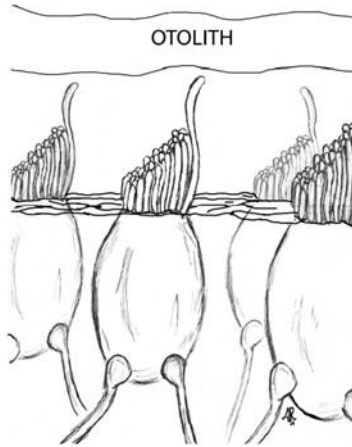


Figure 1.2 Diagram of sensory epithelium with individual hair cells. Each hair cell is composed of many stereocilia and one kinocilium. The otolith sits on top of the hair cells. Artwork by Audrey Rollo.

CHAPTER 2 – SUBLETHAL EFFECTS OF CADMIUM ON AUDITORY STRUCTURE AND FUNCTION IN FATHEAD (*PIMEPHALES PROMELAS*) AND BLUNTNOSE (*PIMEPHALES NOTATUS*) MINNOWS

INTRODUCTION

Aquatic ecosystems are threatened by environmental pollutants mainly because of their continued release through various anthropogenic sources. With industrialization occurring near water bodies, toxicity levels of contaminants in the water are of concern. Heavy metals are of particular concern due to their toxicity and ability to bioaccumulate in the aquatic ecosystem. Because of the pertinent role fishes play in the aquatic environment, it is important to study the potential effects heavy metals may have on fish survival. Studies in the past have shifted from lethal effects of metal contaminants to acute sub-lethal effects of metals on biological functions. These studies have been important for the purposes of risk assessment and the establishment of water quality criteria (Pickering & Henderson, 1966; Benoit, 1975; Bishop & McIntosh, 1981; Khangarot, 1981; Sherman et al., 1987). Measured lethal contaminant levels are seldom found in the field and as such may provide minimal insight regarding the need for remediation efforts (Kimball & Levin, 1985). Such contaminant levels are often an order of magnitude higher than the measured effective concentrations (EC₅₀) seen in a variety of teleost fish species (reviewed in Atchison et al., 1987). These effective concentrations are known to induce biological deficits in fishes (Shedd et al., 2001; de la Torre et al., 2000; Espina et al., 2000; reviewed in Atchison et al., 1987). While these deficits are not lethal, they do affect many vital functions in fishes. Thus, evaluating EC₅₀ values in relation to behavioural toxicity tests results in changes that are often sensitive

indicators of sub-lethal effects; therefore making them a more applicable toxicity test to the aquatic environment (reviewed in Atchison et al., 1987).

Sub-lethal effects of metal contaminants are important to study as fishes are often exposed to levels lower than the lethal concentration. Reproductive and non-reproductive interactions, predator avoidance, feeding, locomotion, and respiration are important fish behaviours that have been found to be affected by heavy metal toxicity (Klinck et al., 2007; Sellin & Kolok, 2006; Hansen et al., 2002; reviewed in Atchinson et al., 1987). More specifically, heavy metals are known to induce these behavioural deficits by targeting both the structure and function of vital sense organs (reviewed in Blaxter & Hallers-Tjabbes, 1992; reviewed in Klaprat et al. 1992). Increases in contaminant levels are a pressing issue as fishes are linked to their aquatic environments through their sensory systems. Cadmium is a heavy metal contaminant that has been shown to alter behaviours in fishes by inhibiting sensory systems. For example, exposure to cadmium at high concentrations has been found to alter swimming behaviour in the sea bass (*Dicentrarchus labrax*) due to the full deprivation of hair cell bundles in mechanosensory neuromasts (Faucher et al., 2006). Interestingly, lower levels of cadmium have also been shown to inhibit lateral line function in the banded kokopu (*Galaxias fasciatus*), also causing alterations in important swimming behaviours (Baker & Montgomery, 2001). Changes in swimming behaviour are known to be a characteristic response to heavy metal toxicity (Sorensen, 1991). These studies show that metal contaminants found in the environment can clearly have detrimental effects on vital fish sensory systems, and therefore overall fitness.

The current study examines the sub-lethal effects of cadmium on the structure and function of the auditory system in the fathead (*Pimephales promelas*) and bluntnose (*Pimephales notatus*) minnows. The auditory system is an important sensory system in fishes. Several previous studies have examined the structural effects and morphological damage to inner ear hair cells in response to noise exposure (Enger 1981; Hastings et al., 1996). These studies have found that mechanical damage to the inner ear hair cells will occur after intense noise exposure. Other studies have examined the functional effects of anthropogenic noise on hearing ability in fishes (Popper & Clarke, 1976; Scholik & Yan, 2001; 2002a;b; Vasconcelos et al., 2007), as well as the effects of natural pollutants, like red-tide, on hearing ability in fishes (Lu & Tomchik, 2002). However, the current study combines a functional and structural approach by examining both the auditory sensitivity and inner ear morphology of cyprinid fishes in response to heavy metal (cadmium) exposure. The current study attempts to link structural and functional deficits in the auditory system that may be caused by cadmium exposure and aid in the assessment of potential effects of a common metal contaminant on a sensory system vital to fish survival. A field study was also conducted to examine if any sub-lethal effects of cadmium exists between fishes from different field sties along the Detroit River. Extrapolation of these results back to the Great Lakes ecosystem will then allow for more accurate assessment of habitat quality for prioritization of remediation efforts.

METHODS

Study species

Fathead minnows used were obtained from a local fish supplier (Pro-Fish Centre, Windsor, ON). Fish were kept in the University of Windsor animal facilities. Prior to

exposure trials, fish were housed in tanks containing aerated, 18-20°C dechlorinated tap water. Fish were fed Nutrafin® fish flakes (Tetramin, Inc.), once per day, and were maintained at a 16:8 light/dark cycle. Fish used had total lengths ranging from 29 mm to 55 mm. Procedures used in this study were approved by the University of Windsor Animal Care Committee, in compliance with guidelines established by the Canadian Council of Animal Care.

Flow-through cadmium exposure

Cadmium exposure and control trials took place in a ‘dose-response bioassay’ fashion, involving 5 cadmium treatments and corresponding controls. Trials took place in a flow-through tank system (Figure 2.1). Dechlorinated tap water was continually added from a water reservoir to one of two mixing buckets (cadmium and control). Water flow-through from each mixing bucket continued to three 12L tanks (3 cadmium, 3 control). Cadmium was continuously added to the cadmium mixing bucket from a Cd(NO₃)₂ stock standard solution (Merck, Cd standard solution 1000 mg⁻¹ in nitric acid 0.5M) in a 19L carboy with spigot. This flow-through system was used to ensure cadmium exposure was constant throughout the duration of each trial.

Cadmium exposure and control trials took place for 96 hours. Ten fathead minnows were placed in each 12L tank. Temperature was maintained at 18-20°C. Target cadmium concentrations that were tested were 0.5, 0.625, 1.25, 2.5, and 5 µg/L. After 96 hours of exposure in the flow-through system, 3 fish from each cadmium tank (total n = 9) and 2 fish from each control tank (total n = 6) were haphazardly selected for physiological auditory assays.

Water samples (30 mL H₂O in 1% HNO₃) were taken every 12 hours throughout the duration of each 96 hour trial from cadmium tanks, and at 0, 48, and 96 hours for control tanks. Water samples were filtered using a 0.45 µm syringe tip filter (PALL Acrodisc™ 25mm syringe filter) and acidified to 1% with concentrated trace-metal grade nitric acid (Fisher Scientific, Canada) (Klinck et al., 2007). Water samples were then analyzed for concentration of cadmium using graphite furnace atomic absorption spectrometry (GFAAS) housed at McMaster University. A Varian GTA 120 graphite tube atomizer coupled to a Varian AA220FS spectrometer (Mulgrave, Australia) was used. A certified reference material for trace metals (TM-15) originating from the National Water Research Institute of Canada was measured to ensure accuracy of metal measurement. Water quality measurements of pH, alkalinity, hardness, nitrite, nitrate, temperature, and chlorine levels were also taken at 0, 48, and 96 hours.

Auditory brainstem response

To obtain auditory thresholds (the minimum sound level that can be detected), sound was presented through an underwater speaker connected to a Tucker-Davis Technologies (TDT, Gainesville FL) physiological recording system. Physiological hearing assays were conducted in a sound reducing chamber inside of a PVC cylindrical tube (112cm long, 26.4 cm in diameter) filled with dechlorinated tap water (Figure 2.2). The hearing ability of each fathead minnow was tested physiologically using an auditory brainstem response (ABR) technique (Kenyon et al., 1998; Higgs et al., 2001). Fish were placed into a net holder and secured with mesh netting placed behind the opercular edge, ensuring minimal respiratory obstruction. The net holder was then lowered underwater. Small stainless steel electrodes (Rochester Electromedical Inc.) were used to detect

brainstem activity. A recording electrode was placed under the skin just over the brainstem (using the pre-operculum as a position marker) and a reference electrode was placed under the skin just in front of the eyes of the fish. Brainstem activity in response to sound played from an underwater speaker was recorded as a differential response between the reference and recording electrodes and sent via a fibre optic cable to a computer using TDT BioSig program software. Tone bursts ranging from 100 to 2000 Hz were played, and thresholds were analysed using TDT SigGen and BioSig software on a computer connected to the recording system. Any responses to sound stimuli were seen as definite peaks above background levels, typically occurring at 7-10 ms after sound presentation began (Figure 2.3). Sounds were presented in increasing decibel levels until a clear response was observed. This first response was defined as the threshold for auditory detection. The visual method of threshold detection is commonly used for ABR studies and shows no difference in threshold estimate from more statistical approaches (Mann et al., 2001; Brittan-Powell et al., 2002). Responses were continued at least 10dB above threshold to examine possible differences in latency of the response. Using BioSig software (TDT), response latency was determined as the amount of time it takes for the brain to respond to a sound being played. That is, the time from when the sound is played to the peak of the first clear response that was observed in each ABR trace. This method was applied to fish from all cadmium and control treatment groups. The threshold difference between control and cadmium-exposed fish was quantified at each frequency tested to produce a 'dose-response'-like curve in order to estimate an effective concentration. After ABR testing, fish were euthanized with an overdose of

0.1M clove oil, measured for total length (mm) and fixed in 4% paraformaldehyde (PFA) for no longer than 1 week for examinations of hair cell morphology.

Hair cell morphology

Fish fixed in 4% PFA, previously used for ABR assays, were used for hair cell morphology analysis. The right saccule, lagena, and utricles were removed via dissections. Actin fibres of the hair cells were stained with an Oregon green phalloidin (Molecular Probes Inc., Eugene OR) and phosphate buffer (PB) mixture (12.5 μ L phalloidin : 200 μ L PB). Each epithelium was mounted on a glass slide and imaged under a fluorescence microscope at 200X magnification (Figure 2.4). Hair cells were counted manually from saved images using Northern Eclipse imaging software (Empix Imaging Inc., Mississauga, ON) and hair cell numbers for each endorgan were calculated. This procedure was performed on fish from all cadmium and control treatment trials.

Field studies

Bluntnose minnows were collected, using a bag seine, from each of three sites along the Detroit River in the Spring of 2009 (Figure 2.5). Site A (Fighting Island) was denoted as the 'contaminated' site due to its location downstream of the heavily industrialized Zug Island. Site B (Chewitt Bay) was denoted as the 'intermediate' site and Site C (Peché Island) was denoted as the 'clean' site because of its location near Lake St. Clair. Water samples (30 mL H₂O in 1% HNO₃) were also collected from each site. Field water samples were analyzed for cadmium using graphite furnace atomic absorption spectrometry. Water quality measurements of pH, alkalinity, hardness, nitrite, nitrate, temperature, and chlorine levels were also taken at each site. After collection,

bluntnose minnows were brought back to the University of Windsor Animal Quarters and kept overnight in aerated river water.

To assess hearing ability in field-caught fish, the auditory brainstem response described above was used. Response latency was also measured to assess any differences between fish from different field sites. Five fish from each field site were tested within 24 hours of capture. Fish were then sacrificed with an overdose of 1M clove oil and fixed in 4% paraformaldehyde for no longer than 1 week. The same dissection procedure used in the lab portion for hair cell morphology analysis was used for the field portion (see above). Endorgans from each ear were stained with phalloidin and viewed using fluorescence microscopy. Hair cell numbers from each of the three endorgans were then quantified using Northern Eclipse Imaging software.

Statistical analysis

A mixed-model ANOVA was run with time and tank as independent variables and cadmium treatment as the dependent variable. This was done to determine if there was any variation in cadmium level at each 12 hour interval and if there was any variation between treatment tanks. For hearing threshold analysis for cadmium exposure trials, five separate two-way ANOVAs were run for each [Cd] and their corresponding control, with hearing threshold as the dependent variable, and treatment and frequency as the independent variables. Tukey's post-hoc was then run with threshold to see what the best frequency was in cadmium exposed fish. Independent t-tests were run between cadmium ([Cd] = 6.49 and 3.31 $\mu\text{g/L}$) and control fish at 400 and 600 Hz with subsequent Bonferroni adjustment of significance level. For response latency analysis for cadmium exposure trials, five separate two-way ANOVAs were run with each [Cd] and their

corresponding control, with response latency as the dependent variable and treatment and frequency as the independent variables. Independent t-tests were run between cadmium ([Cd] = 6.49 and 3.31 µg/L) and control fish at 400 and 600 Hz with subsequent Bonferroni adjustment of significance level. For hair cell morphology analysis, independent t-tests were used to determine if hair cell numbers in each endorgan differed significantly between cadmium exposed and control fish for each cadmium treatment level.

In our field study analysis, a two-way ANOVA was run with hearing threshold as the dependent variable, and field site and frequency as the independent variables. This was used to determine if hearing threshold differed significantly between fish from different field sites. Tukey's post-hoc was then run with threshold to see what the best frequency was in fish from each field site. A second two-way ANOVA was run with response latency as the dependent variable, and field site and frequency as the independent variables. This was run to see if response latencies differed between fish from different field sites. For hair cell morphology analysis from our field study, a two-way ANOVA was run with hair cell number as the dependent variable, and field site and endorgan as the independent variables. This was used to determine if hair cell numbers differed significantly between fish from different field sites.

RESULTS

Cadmium exposure & water quality

Using GFAAS, cadmium levels for each cadmium treatment tank were measured at 12 hour intervals (at 0, 24, and 96 h for control tanks) and compared to the targeted levels (Table 2.1). Cadmium levels that were measured were found to be close to

targeted cadmium levels. A mixed-model ANOVA revealed no significant variation between 12 hour measurements ($p > 0.05$) or tanks ($p > 0.05$) at any cadmium treatment level. Water quality measurements were also recorded at 0, 48, and 96 hours (Table 2.2). No obvious differences between water quality parameters were observed between tanks.

Auditory physiology

Auditory thresholds of fathead minnows exposed to $[Cd] = 6.49 \mu\text{g/L}$ ranged from 110 – 125 dB re 1 μPa and the audiogram bandwidth ranged from 100 – 2000 Hz (Figure 2.6a). By comparison, control fathead minnows had auditory thresholds ranging from 105 – 125 dB re 1 μPa and the audiogram bandwidth ranged from 100 -2000 Hz. At this exposure level, a significant effect of cadmium treatment and frequency level on hearing threshold was found ($p < 0.01$), with no interaction between treatment and frequency ($p > 0.05$). Tukey's post-hoc revealed that the best frequency in cadmium exposed fish occurred at 400 and 600 Hz. Independent t-tests between cadmium and control fish revealed a significant increase in hearing threshold (decreased sensitivity) at 400 Hz in cadmium exposed fish ($p < 0.025$), but not at 600 Hz ($p > 0.025$).

Auditory thresholds of fathead minnows exposed to $[Cd] = 3.31 \mu\text{g/L}$ ranged from 105 – 125 dB re 1 μPa (Figure 2.6b) and the audiogram bandwidth ranged from 100 – 2000 Hz. By comparison, control fathead minnows had auditory thresholds ranging from 100 – 120 dB re 1 μPa and the audiogram bandwidth ranged from 100 – 2000 Hz. Significant effects of cadmium treatment and frequency level on hearing threshold were found ($p < 0.01$), with no interaction between treatment and frequency ($p > 0.05$). Tukey's post-hoc revealed that the best frequency in cadmium exposed fish occurred at 400 and 600 Hz. Independent t-tests between cadmium and control fish revealed a

significant increase in hearing threshold at 400 and 600 Hz ($p < 0.025$) in cadmium exposed fish.

Auditory thresholds of fathead minnows exposed to $[Cd] = 1.698 \mu\text{g/L}$ ranged from 105 – 125 dB re 1 μPa and the audiogram bandwidth ranged from 100 – 2000 Hz (Figure 2.6c). By comparison, control fathead minnows had auditory thresholds ranging from 105 – 125 dB re 1 μPa and the audiogram bandwidth ranged from 100 – 2000 Hz. At this exposure level, no significant effect of cadmium treatment on hearing threshold was found ($p > 0.05$). The overall hearing threshold of fish exposed to 1.698 $\mu\text{g/L}$ of cadmium did not differ from control fish at any frequency tested. Significant effects of frequency level on hearing thresholds were found ($p < 0.01$), with no interaction between treatment and frequency ($p > 0.05$). Tukey's post-hoc revealed that the best frequency in cadmium exposed fish occurred at 400 and 600 Hz.

Auditory thresholds of fathead minnows exposed to $[Cd] = 0.846 \mu\text{g/L}$ ranged from 105 – 125 dB re 1 μPa and the audiogram bandwidth ranged from 100 – 2000 Hz (Figure 2.6d). By comparison, control fathead minnows had auditory thresholds ranging from 110 – 125 dB re 1 μPa and the audiogram bandwidth ranged from 100 – 2000 Hz. At this exposure level, no significant effect of cadmium treatment on hearing threshold was found ($p > 0.05$). The overall hearing threshold of fish exposed to 0.846 $\mu\text{g/L}$ of cadmium did not differ from control fish at any frequency tested. Significant effects of frequency level on hearing thresholds were found ($p < 0.01$), with no interaction between treatment and frequency ($p > 0.05$). Tukey's post-hoc revealed that the best frequency in cadmium exposed fish occurred at 400 and 600 Hz.

Auditory thresholds of fathead minnows exposed to $[Cd] = 0.593 \mu\text{g/L}$ ranged from 110 – 125 dB re 1 μPa and the audiogram bandwidth ranged from 100 – 2000 Hz (Figure 2.6e). By comparison, control fathead minnows had auditory thresholds ranging from 105 – 125 dB re 1 μPa and the audiogram bandwidth ranged from 100 – 2000 Hz. At this exposure level, no significant effect of cadmium treatment on hearing threshold was found ($p > 0.05$). The overall hearing threshold of fish exposed to $0.593 \mu\text{g/L}$ of cadmium did not differ from control fish at any frequency tested. Significant effects of frequency level on hearing thresholds were found ($p < 0.01$), with no interaction between treatment and frequency ($p > 0.05$). Tukey's post-hoc revealed that the best frequency in cadmium exposed fish occurred at 400 and 600 Hz.

Threshold differences between cadmium and control fish were measured to produce “dose-response”-like curves at low frequency (Figure 2.7 a), best frequency (Figure 2.7 b), and high frequency (Figure 2.7 c). Upon visual assessment, the best frequency curves revealed an estimated effective concentration of cadmium to be at approximately $2.1 \mu\text{g/L}$ at 400 Hz and $2.9 \mu\text{g/L}$ at 600 Hz. Threshold differences at high and low frequencies did not result in a typical “dose-response”-like curve and as such did not reveal an effective concentration of cadmium.

At $[Cd] = 6.49 \mu\text{g/L}$, a significant effect of cadmium treatment ($p < 0.01$) and frequency ($p < 0.01$) on response latency was found, with no interaction between treatment and frequency ($p > 0.05$). Overall response latency in control fish was significantly higher than in fish exposed to cadmium at this level (Figure 2.8a). Independent t-tests revealed no significant differences in response latency between

cadmium and control fish at 400 and 600 Hz ($p > 0.025$). This is in contrast to significant differences found in hearing threshold at these levels.

At $[Cd] = 3.31 \mu\text{g/L}$, a significant effect of cadmium treatment ($p < 0.01$) and frequency ($p < 0.01$) on response latency was found, with no interaction between treatment and frequency ($p > 0.05$). Overall response latency in control fish was significantly higher than in fish exposed to cadmium (Figure 2.8b). Consistent with hearing thresholds at these levels, independent t-tests revealed significant differences in response latency between cadmium and control fish specifically at 400 and 600 Hz ($p < 0.025$).

At $[Cd] = 1.698 \mu\text{g/L}$, no significant effect of cadmium treatment was found ($p > 0.05$). A significant effect of frequency on response latency was found ($p < 0.01$), with no interaction between treatment and frequency ($p > 0.05$) (Figure 2.8c). At $[Cd] = 0.846 \mu\text{g/L}$, no significant effect of cadmium treatment was found ($p > 0.05$). A significant effect of frequency on response latency was found ($p < 0.01$); with no interaction between treatment and frequency ($p > 0.05$) (Figure 2.8d). At $[Cd] = 0.593 \mu\text{g/L}$, no significant effect of cadmium treatment was found ($p > 0.05$). A significant effect of frequency on response latency was found ($p < 0.01$), with no interaction between treatment and frequency ($p > 0.05$) (Figure 2.8e).

Hair cell morphology

Independent t-tests revealed that fish exposed to cadmium at all 5 treatment levels (6.49, 3.31, 1.698, 0.846, 0.593) showed no significant differences in hair cell numbers compared to control fish ($p > 0.05$). This lack of difference in hair cell number was

consistent in all three inner ear end-organs (sacculle, utricle, lagena) (Figure 2.9 a, b, c, respectively).

Field studies

Cadmium levels measured at each field site did not differ significantly from one another (Table 2.3). Similarly, no obvious differences in water quality parameters measured were observed at each field site. Auditory thresholds of bluntnose minnows from Fighting Island ('contaminated' site), ranged from 105 to 125 dB re 1 μ Pa and the audiogram bandwidth ranged from 100 – 2000 Hz. Auditory thresholds of bluntnose minnows from Chewitt Bay ('middle' site), ranged from 105 to 125 dB re 1 μ Pa and the audiogram bandwidth ranged from 100 – 2000 Hz. Auditory thresholds of bluntnose minnows from Peche Island ('clean' site), ranged from 105 to 125 dB re 1 μ Pa and the audiogram bandwidth ranged from 100 – 2000 Hz. No significant effect of field site on hearing threshold was found ($p > 0.05$). The overall hearing threshold of fish from each field site did not differ from fish at other field sites at any frequency tested (Figure 2.10). Significant effects of frequency level on hearing thresholds were found ($p < 0.01$), with no interaction between treatment and frequency ($p > 0.05$). Tukey's post-hoc revealed that the best frequency in fish from all three field sites occurred at 200 and 400 Hz. Response latency analysis of fish from each field site revealed no significant effect of field site on response latency ($p > 0.05$) (Figure 2.11). A significant effect of frequency level on response latency was found ($p < 0.01$), with no interaction between site and frequency ($p > 0.05$). A two-way ANOVA revealed that fish caught at each field sight (Fighting Island, Chewitt Bay, Peche Island) showed no significant differences in hair cell numbers compared to the other field sites ($p > 0.05$). This lack of difference in hair

cell number was consistent for all three inner ear end-organs (sacculle, lagena, utricle) (Figure 2.12).

DISCUSSION

It is more than evident that the presence of heavy metals in the aquatic ecosystem is a concern due to their toxicity, bioaccumulation, and release through numerous anthropogenic sources. The auditory system is a sensory system in fishes that plays a vital role in communication and survival. Thus, it is surprising that the effects of heavy metal contaminants on this important sensory system in fishes have not been studied. Most studies involving anthropogenic influences on the auditory system in fishes have focused on either a structural damage or function deficits (Popper & Clarke, 1976; Scholik & Yan, 2001; 2002a; b; Vasconcelos et al., 2007). The current study, however, goes one step further and helps to provide a link between structure and function in the auditory system in response to acute heavy metal cadmium exposure.

Auditory brainstem response assays revealed that fishes exposed to higher concentrations of cadmium (6.49 and 3.31 $\mu\text{g/L}$) exhibited an overall significant increase in hearing threshold compared to control fish. This increase in hearing threshold indicates a decrease in hearing sensitivity. Thus, it is speculated that high levels of cadmium do have an effect on hearing ability in the fathead minnow. More specifically, significant differences in threshold were found at a frequency of 400 Hz at 6.49 $\mu\text{g/L}$ and at 400 and 600 Hz at 3.31 $\mu\text{g/L}$. As these frequencies represent the most acute hearing of this species, and others in the Cyprinidae (reviewed in Fay, 1988), cadmium effects here might be expected to be especially detrimental to auditory detection. Visual assessment of threshold difference curves at the levels of best frequency for these fish revealed an

effective concentration at approximately 2.1 µg/L at 400 Hz and 2.9 µg/L at 600 Hz, indicating that this might be the critical range in which cadmium has an effect on hearing ability in these fish. This estimated effective concentration is much lower than previously observed effective sub-lethal levels of cadmium in the fathead minnow (Pickering and Gast, 1972). This means that deficits to the auditory system may be a more sensitive indicator for cadmium toxicity effects in fishes.

Fishes exposed to higher cadmium concentrations (6.49 and 3.31 µg/L) also had an overall significant difference in response latency compared to control fish. However, response latency was longer in control fish than in cadmium exposed fish at these levels. Significant differences were found specifically at 400 and 600 Hz in fish exposed to 3.31 µg/L, although the same significant differences were not found at these frequencies in fish exposed to 6.49 µg/L of cadmium. These results are counter to what was expected, as it appears high levels of cadmium may cause fathead minnows to respond faster to a sound. Minimal research has been conducted on changes in response latency and as such offers little insight as to why this effect was observed. From work that has been conducted on latency, the current results showing a decrease in latency with increasing frequency is consistent with results found in another Cyprinidae species, the zebrafish (*Danio rerio*) (Higgs et al., 2003). More work must be done in this area in order for us to have a better understanding of effects seen on response latency.

Inner ear hair cell morphology assays following cadmium exposure trials revealed that no significant differences in hair cell numbers existed between cadmium-exposed and control fish. This result was consistent in all three inner ear endorgans for all five cadmium exposure levels. These results imply that while there appears to be a functional

deficit in hearing ability in response to high cadmium exposure, there are no morphological deficits with respect to hair cell numbers. It is speculated that although there may be no effect of cadmium on hair cell number, there may be an effect on hair cell ultrastructure. Several previous studies have examined the effects of intense noise exposure on the physical morphology of the inner ear in fishes (Enger, 1981; Hastings et al., 1996). In these studies, mechanical damage to auditory hair cells after intense noise exposure was found. Studies on the mechanosensory lateral line have shown that aminoglycoside antibiotics, such as gentamicin and streptomycin, are able to ablate lateral line neuromasts (Coombs et al., 2001; Song et al., 1995; Kaus, 1987). Similarly, studies have shown high cadmium exposure can also damage neuromasts (Faucher et al., 2006; Baker & Montgomery, 2001). Because the fundamental physiological unit of both the mechanosensory lateral line and auditory system is the hair cell, it is possible that cadmium is playing a similar role and causing mechanical damage to auditory hair cells. Damage to hair cell structure could result in hearing deficits as it could effect mechanical stimulation and thus effect hair cell displacement.

Other speculative mechanisms for the observed functional hearing deficits involve more specific steps in the auditory pathway and auditory nerves. Yan (1995) found that spontaneous and evoked action potentials in the lateral line nerve were extinguished in fathead minnows exposed to high levels of cadmium. This has some applicability to the auditory system due to the fact that the mechanism of activation of the hair cell is the same in each system; bending of apical hair cell extensions results in changes in membrane polarity and thus nervous activation. Because of this conserved receptor mechanism, external factors that affect the hair cell will affect both auditory and lateral

line stimulation. Thus, based on Yan's findings, it is possible that cadmium may be affecting action potentials firing at the auditory nerve and as a result is causing a functional deficit in hearing sensitivity.

A study by Lu and Tomchik (2002) on the effects of natural red-tide toxin also provides some insight to what may be causing these decreases in hearing sensitivity. In this study, the effects of brevetoxin-3, a neurotoxin purified from red tide, were examined on the hearing sensitivity of the goldfish (*Carassius auratus*). This study was the first of its kind to show that a natural toxin can cause hearing loss, specifically reduced auditory sensitivity and increased hearing thresholds, in fish. The mechanism speculated in this case involved toxic effects on voltage-gated Na⁺ channels that in turn could block the conduction of neural signals along the auditory pathway. A similar mechanistic breakdown in the auditory pathway can be speculated in regards to the current study. Studies have shown that cadmium can enter the cell through voltage-gated calcium channels (Hinkle et al., 1987; Gavazzo et al, 2005). Cadmium has also been shown to inhibit calcium channels and pumps (Kiss et al., 1994). Because calcium plays an important role in the auditory signalling pathway, any disruptions in its function can result in significant effects in hearing ability, and thus can be a possible explanation to the deficits in hearing sensitivity observed in the current study.

My field studies found no significant differences in hearing ability (threshold and response latency) of fish from the three field sites. Likewise, no significant differences were found in hair cell number in any of the three inner ear endorgans in fish from all three field sites. These results are not surprising, as GFAAS analysis revealed no obvious difference in waterborne cadmium between each site. Likewise, these measured

cadmium levels in the field were not as high as effective levels used in the lab. My field sites were selected based on their location along the Detroit River in order to assess if a gradient in cadmium levels exists and if so, would these differences be evidenced through auditory deficits. Szalinska et al., (2006), examined the distribution of heavy metals in sediments of the Detroit River. In this study, metal distribution was fairly homogenous with the exception of elevated concentrations in the middle and lower reach of the river. Hydrological conditions such as sorting and deposition, and flow rates are thought to be responsible for this observed difference in metal concentration. Thus, because of these constant variations, total sediment loads alone are often inaccurate in assessing bioavailable levels. There are many other variables in the field to consider that affect the bioavailability and toxicity of metals, in particular water quality parameters such as water hardness and pH (Borgmann, 1983). These parameters are important to consider when evaluating metal toxicity effects in fishes. For example, increasing water hardness increases the number of hardness cations, such as Ca^{2+} and Mg^{2+} . These hardness cations compete with free cadmium cations (Cd^{2+}) for compound/complex formations and uptake sites. This results in a decrease in metal availability, and thus a decrease in cadmium toxicity with increasing water hardness (Endovitskii et al., 2009; Li et al., 2009; Pascoe et al., 1986; Kinkade & Erdman, 1975). Studies on contaminant levels in the Detroit River have indicated that concentrations of cadmium exceeded the sediment quality objectives established for the Lowest Effect Level (LEL; Persaud et al., 1992) in most parts of the river (Szalinska et al., 2006). Such results could lead us to believe that there is a concern for cadmium toxicity effects in the river. However, my study shows that measured cadmium levels in the water column are not as high as previously measured sediment

loads (Szalinska et al., 2006). We also found no differences in sensory deficits of the auditory system in fish from different parts of the river. As such sediment loads may lead to overestimations in the likely effects of cadmium on fishes. As indicated previously, it is not enough to assess cadmium levels in the sediment alone to determine bioavailable metal contaminant effects. Hydrological conditions (i.e. hardness, pH, water flow) must also be considered, as well as characteristics of the organism itself (i.e. feeding behaviours). Thus, my study examines the structure and function of an important sensory system in a species that is exposed to all of the above mentioned parameters, making it a more accurate bioassay to use as an assessment tool for remediation efforts.

To my knowledge, this study is the first of its kind to examine the effects of a metal contaminant – cadmium – on auditory structure and function in fish. The deficits in hearing sensitivity in response to cadmium exposure that were found have important implications for effects on the fitness of these fish. The auditory system is a vital sensory system due to its role in important behaviours such as mating and predator avoidance (Ladich, 2004; Higgs, 2004). As such, studying the effects of a common heavy metal contaminant on the auditory system is important in the health assessment of fish populations, as well as for the overall health of the aquatic ecosystem.

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Table 2.1 Measured and targeted cadmium levels ($\mu\text{g/L}$). Cadmium levels in all treatment and control tanks were measured using graphite furnace atomic absorption spectrometry (GFAAS).

| Treatment | Tank/Site | [Cd] Target ($\mu\text{g/L}$) | [Cd] Actual ($\mu\text{g/L}$) |
|-----------|-----------|---------------------------------|---------------------------------|
| control | T1 | 0 | 0.031 |
| control | T2 | 0 | 0.0203 |
| control | T3 | 0 | 0.0167 |
| cadmium | T4 | 5 | 6.08 |
| cadmium | T5 | 5 | 6.80 |
| cadmium | T6 | 5 | 6.496 |
| control | T1 | 0 | 0.05 |
| control | T2 | 0 | 0.0423 |
| control | T3 | 0 | 0.0483 |
| cadmium | T4 | 2.5 | 3.20 |
| cadmium | T5 | 2.5 | 3.67 |
| cadmium | T6 | 2.5 | 3.05 |
| control | T1 | 0 | 0.024 |
| control | T2 | 0 | 0.014 |
| control | T3 | 0 | 0.029 |
| cadmium | T4 | 1.25 | 1.56 |
| cadmium | T5 | 1.25 | 1.81 |
| cadmium | T6 | 1.25 | 1.724 |
| control | T1 | 0 | 0.016 |
| control | T2 | 0 | 0.028 |
| control | T3 | 0 | 0.022 |
| cadmium | T4 | 0.625 | 0.811 |
| cadmium | T5 | 0.625 | 0.892 |
| cadmium | T6 | 0.625 | 0.834 |
| control | T1 | 0 | 0.0297 |
| control | T2 | 0 | 0.042 |
| control | T3 | 0 | 0.049 |
| cadmium | T4 | 0.5 | 0.584 |
| cadmium | T5 | 0.5 | 0.598 |
| cadmium | T6 | 0.5 | 0.597 |

Table 2.2 Measured water quality parameters in 96 hour lab studies at 12 h intervals (Cd) and 0, 48, & 96 h (Control). Parameters measured include pH, alkalinity (ppm), hardness (ppm), NO₂ (ppm), NO₃ (ppm), temperature (°C), and Cl⁻(mg/L).

Table 2.2

| Treatment | Hour | pH | Alkalinity (ppm) | Hardness (ppm) | NO ₂ (ppm) | NO ₃ (ppm) | Temp (°C) | Cl ⁻ (mg/L) |
|------------------------------|------|-----|------------------|----------------|-----------------------|-----------------------|-----------|------------------------|
| [Cd] = ~6.38 µg/L | 0 | 7 | 120 | 120 | 0 | 0 | 20 | 0 |
| | 48 | 7 | 180 | 120 | 0 | 20 | 20 | 0 |
| | 96 | 7 | 120-180 | 120 | 0 | 40 | 18 | 0 |
| Control | 0 | 7 | 120 | 120 | 0 | 0 | 20 | 0 |
| | 48 | 7 | 120 | 120 | 0 | 0-20 | 20 | 0 |
| | 96 | 7 | 120 | 120 | 0 | 0-20 | 18 | 0 |
| [Cd] = ~2.84 µg/L | 0 | 7.2 | 120-180 | 120 | 0 | 0-20 | 20 | 0 |
| | 48 | 7.2 | 120 | 120 | 0 | 20 | 20 | 0 |
| | 96 | 7.2 | 120 | 120 | 0 | 0-20 | 20 | 0 |
| Control | 0 | 7.2 | 120-180 | 120 | 0 | 0-20 | 20 | 0 |
| | 48 | 7.2 | 180 | 120 | 0 | 20 | 20 | 0 |
| | 96 | 7.2 | 120 | 120 | 0 | 0-20 | 20 | 0 |
| [Cd] = 1.629 µg/L | 0 | 7.2 | 120 | 120 | 0 | 0-20 | 22 | 0 |
| | 48 | 7.2 | 180 | 120 | 0 | 20 | 22 | 0 |
| | 96 | 7.2 | 120 | 120 | 0 | 20-40 | 20 | 0 |
| Control | 0 | 7.6 | 180 | 120-150 | 0 | 0-20 | 21 | 0 |
| | 48 | 7.2 | 180 | 120 | 0 | 20 | 22 | 0 |
| | 96 | 7.2 | 120 | 120 | 0 | 0 | 20 | 0 |
| [Cd] = 0.692 µg/L | 0 | 7.0 | 180 | 120 | 0 | 0-20 | 22 | 0 |
| | 48 | 7.4 | 120-180 | 120 | 0 | 0-20 | 22 | 0 |
| | 96 | 7.2 | 120 | 120 | 0 | 0-20 | 20 | 0 |
| Control | 0 | 7.4 | 120 | 120 | 0 | 0-20 | 22 | 0 |
| | 48 | 7.2 | 120-180 | 120 | 0 | 0-20 | 21 | 0 |
| | 96 | 7.2 | 120 | 120 | 0 | 0-20 | 20 | 0 |
| [Cd] = 0.305 µg/L | 0 | 7.0 | 120 | 120 | 0 | 0 | 16-18 | 0 |
| | 48 | 7.2 | 120 | 120 | 0 | 0-20 | 18 | 0 |
| | 96 | 7.4 | 180 | 120 | 0 | 0-20 | 18 | 0 |
| Control | 0 | 7.2 | 180 | 120 | 0 | 0 | 16-18 | 0 |
| | 48 | 7.4 | 120-180 | 120 | 0 | 0-20 | 18 | 0 |
| | 96 | 7.0 | 180 | 120 | 0 | 0-20 | 16 | 0 |

Table 2.3 Measured cadmium levels ($\mu\text{g/L}$) and water quality parameters (pH, alkalinity (ppm), hardness (ppm), NO_2 (ppm), NO_3 (ppm), temperature ($^\circ\text{C}$), and Cl^- (mg/L), at three field sites (Fighting Island = ‘contaminated’ site, Chewitt Bay = ‘intermediate’ site, and Peche Island = ‘clean’ site). Cadmium levels were measured using graphite furnace atomic absorption spectrometry (GFAAS).

| Tank/Site | [Cd] ($\mu\text{g/L}$) | pH | Alkalinity (ppm) | Hardness (ppm) | NO_2 (ppm) | NO_3 (ppm) | Temperature ($^\circ\text{C}$) |
|--------------------|-----------------------------|--------------|---------------------|-------------------|------------------------|------------------------|-------------------------------------|
| Fighting Island | 0.094 | 7.8 - 8.0 | 120 | 120 | 0 | 0 | 24 |
| Chewitt Bay | 0.078 | 7.8 | 120 | 120 | 0 | 0-20 | 22 |
| Peche Island | 0.062 | 8 | 120 | 120 | 0 | 20 | 24 |

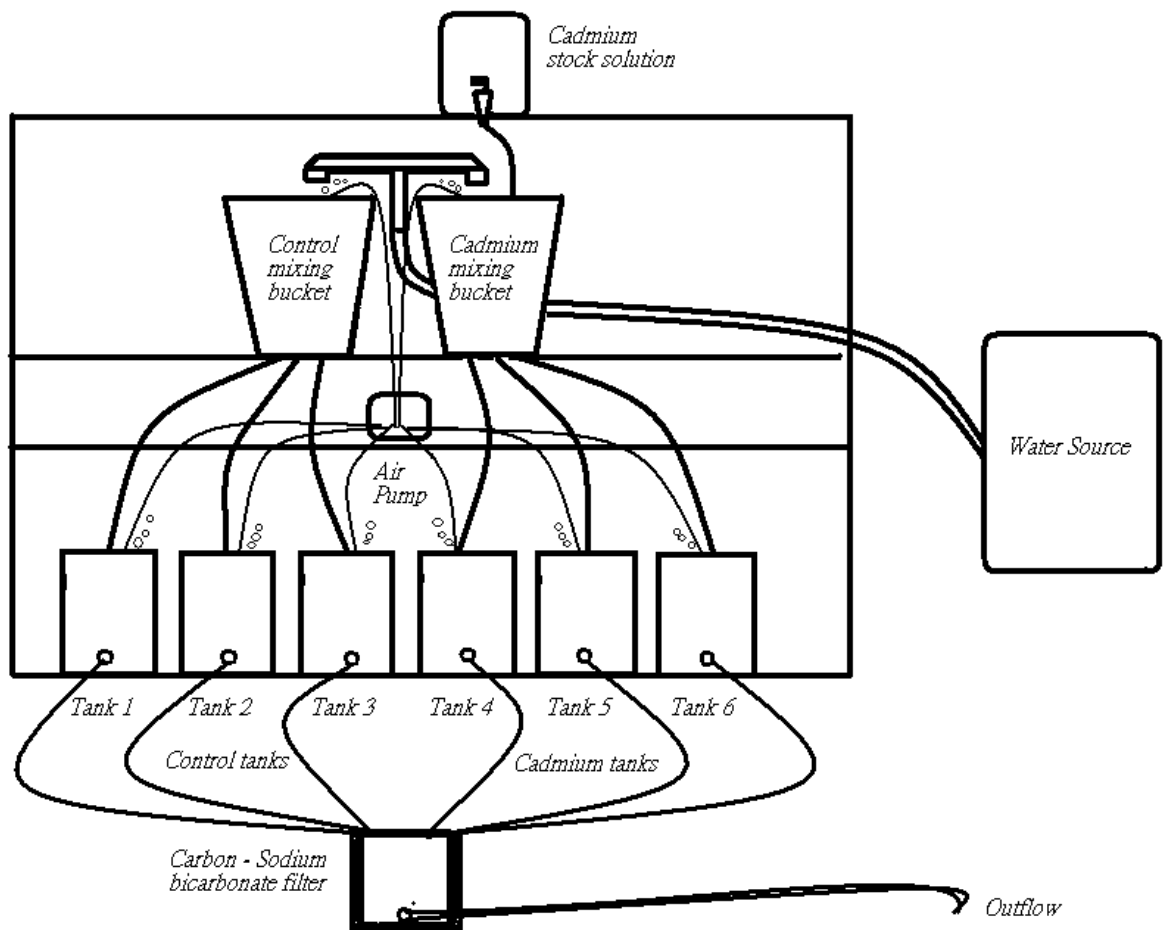


Figure 2.1 Flow-through tank system set-up for 96 hour cadmium exposure and control trials. Dechlorinated tap water was continually added from a water reservoir to one of two mixing buckets (cadmium and control). Cadmium was continuously added to the cadmium mixing bucket from a $\text{Cd}(\text{NO}_3)_2$ stock standard solution. Water from each mixing bucket flowed into one of 6 treatment tanks (3 cadmium, 3 control).

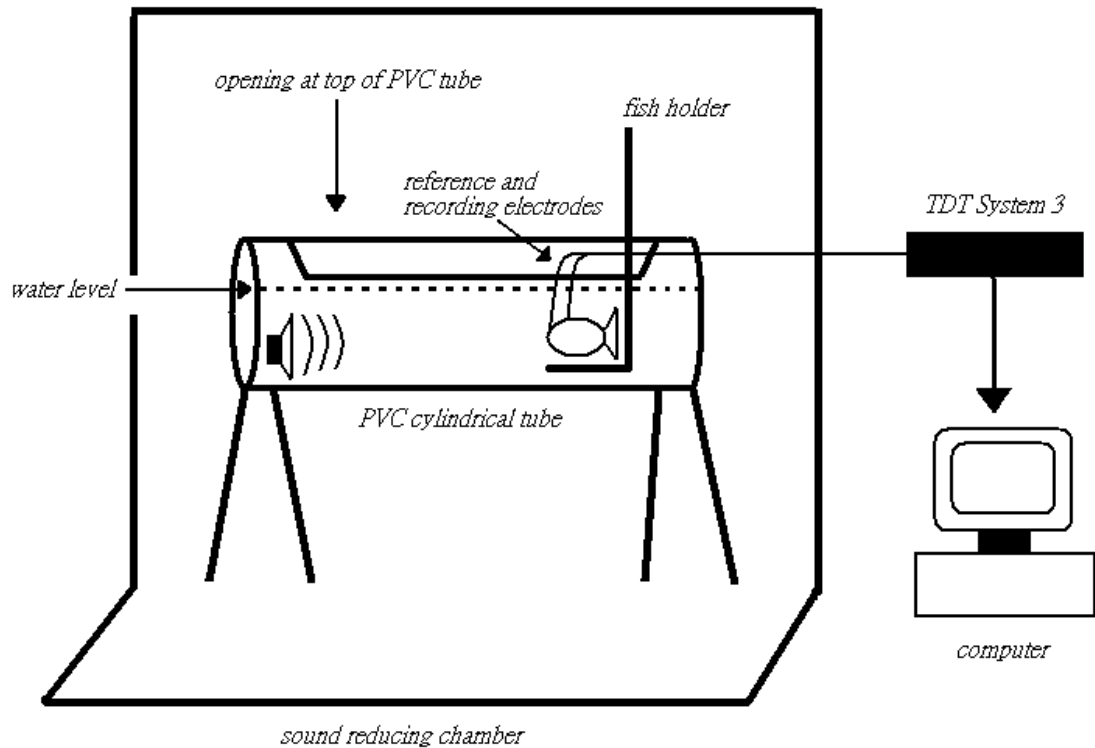


Figure 2.2 Diagram of auditory brainstem response (ABR) set-up. Both the speaker and fish are lowered under water. Recording and reference electrodes are connected to the TDT system 3, from which a computer receives information. The entire set-up is located in a sound-reducing chamber.

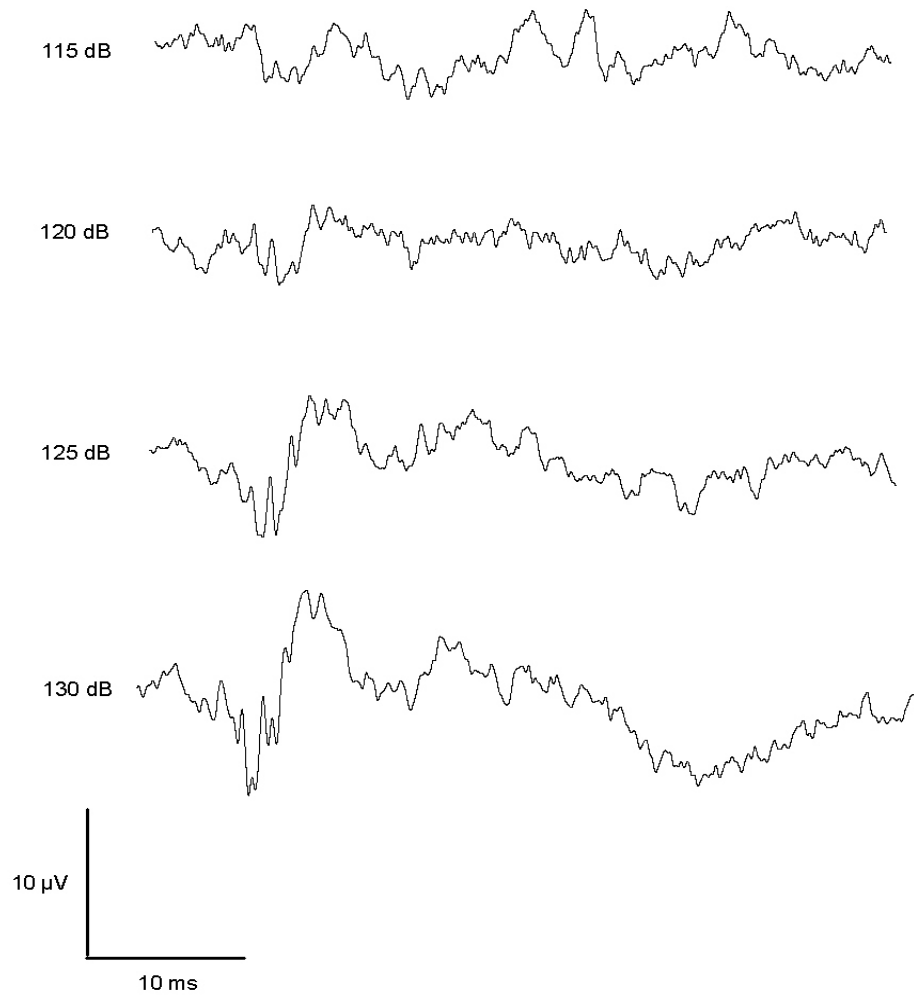
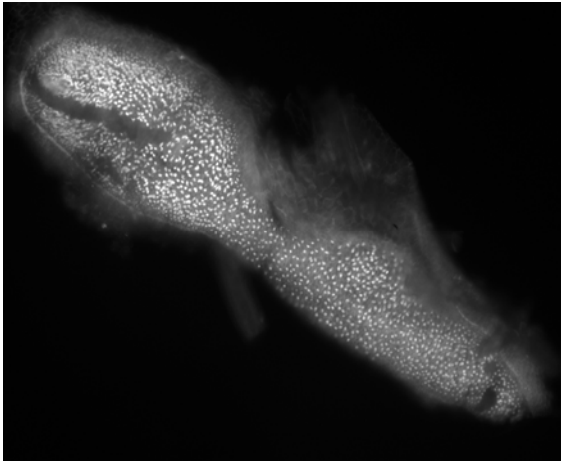


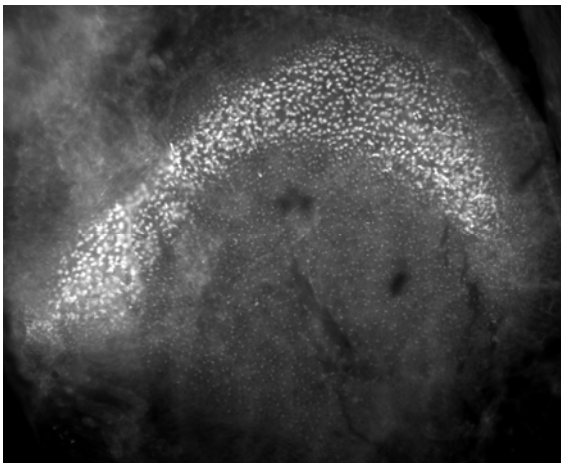
Figure 2.3 Auditory brainstem response (ABR) traces. Series represents traces recorded in response to tone bursts played at increasing decibel levels. All traces were taken at 200 Hz.

Figure 2.4 Pictures of fathead minnow sensory epithelium containing auditory hair cells: A) saccule endorgan. B) utricle endorgan. C) lagena endorgan stained with phalloidin.

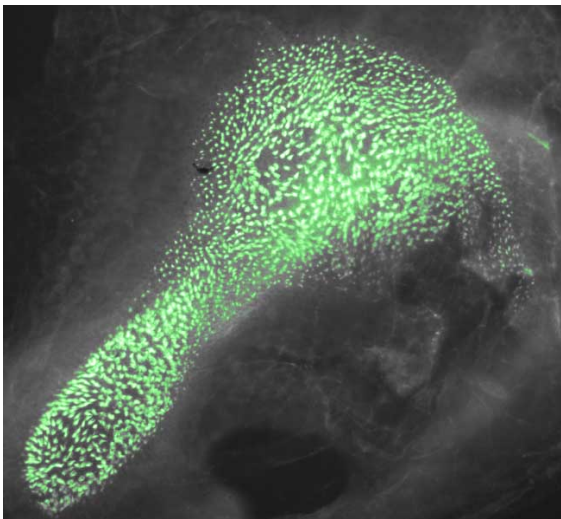
Figure 2.4



A



B



C

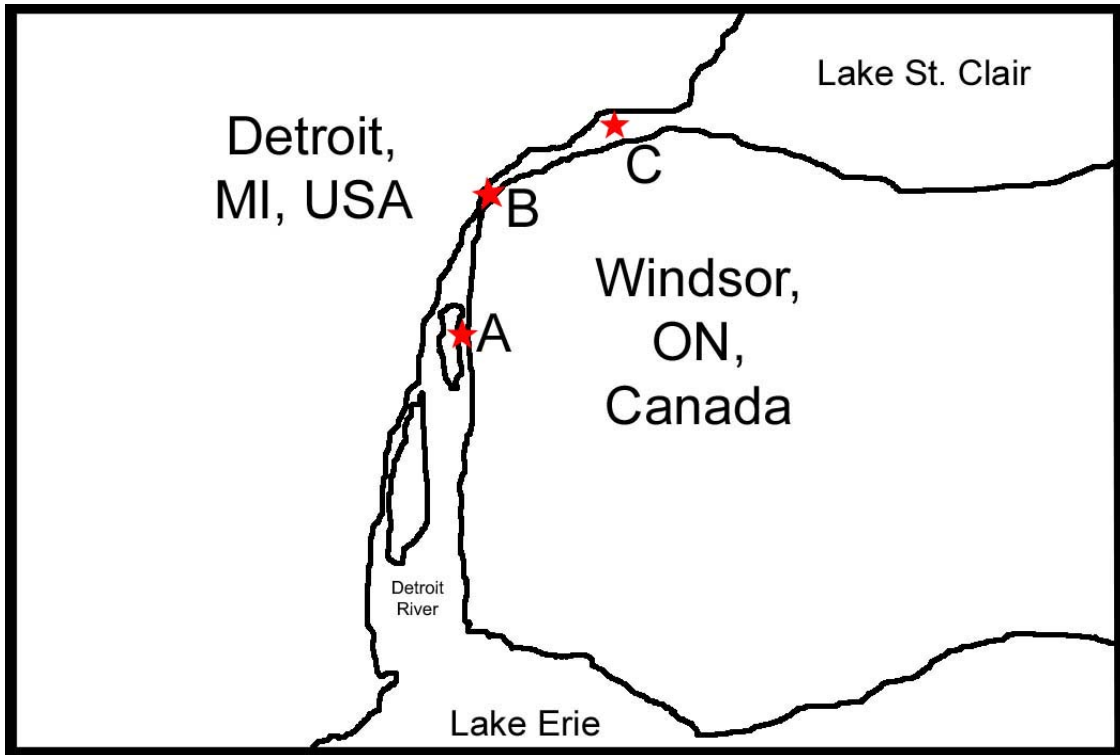


Figure 2.5 Location of field sites along Detroit River. Bluntnose minnows were collected from each field site using a bag seine. A) Fighting Island = ‘contaminated’ site. B) Chewitt Bay = ‘intermediate’ site. C) Peche Island = ‘clean’ site.

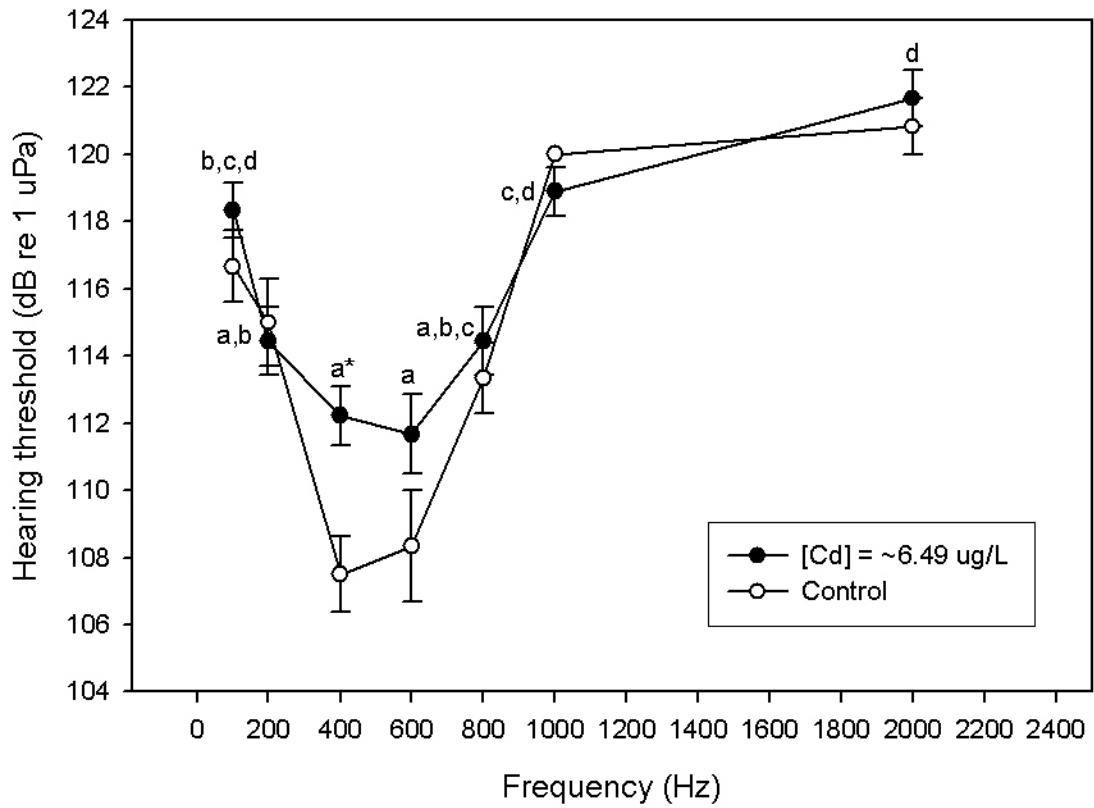


Figure 2.6 a. Audiograms of hearing threshold responses (\pm standard error) in fish exposed to $[Cd] = 6.49 \mu\text{g/L}$ compared to control fish. Letters indicate groups in homogeneous subsets. Asterisks (*) indicate significant difference in hearing threshold between cadmium and control fish.

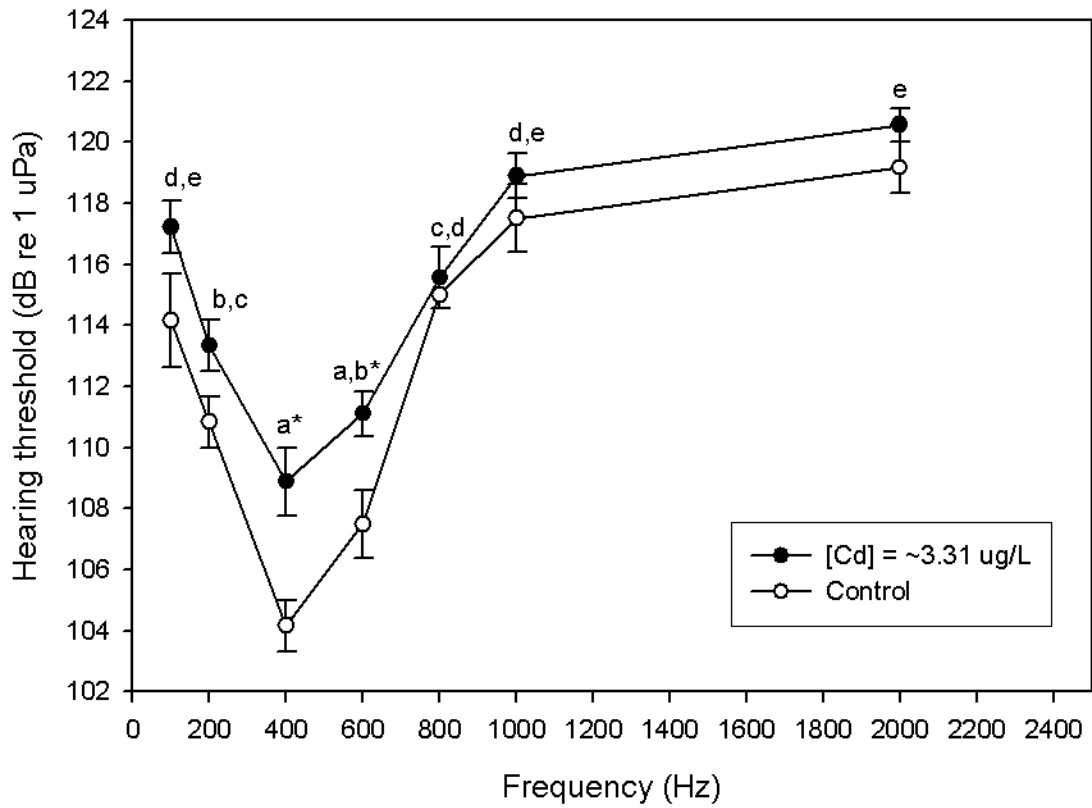


Figure 2.6 b. Audiograms of hearing threshold responses (\pm standard error) in fish exposed to $[Cd] = 3.31 \mu\text{g/L}$ compared to control fish. Letters indicate groups in homogeneous subsets. Asterisks (*) indicate significant difference in hearing threshold between cadmium and control fish.

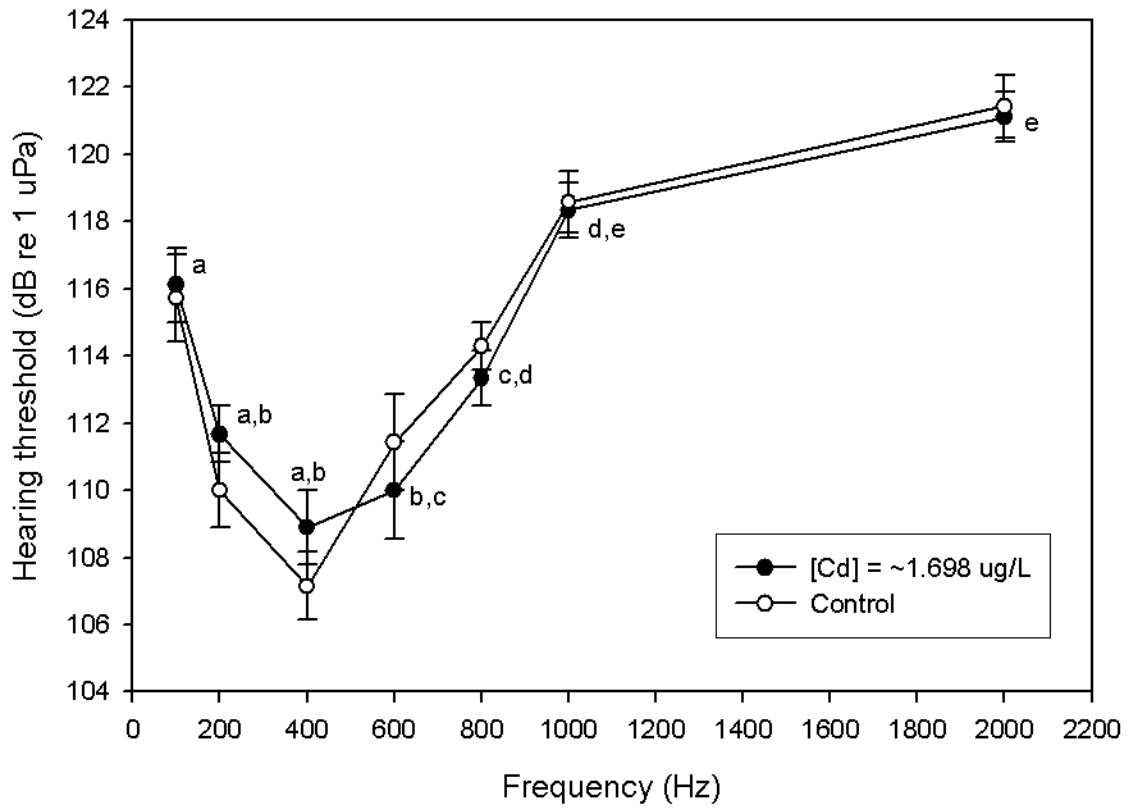


Figure 2.6 c. Audiograms of hearing threshold responses (\pm standard error) in fish exposed to [Cd] = 1.698 $\mu\text{g/L}$ compared to control fish. Letters indicate groups in homogeneous subsets.

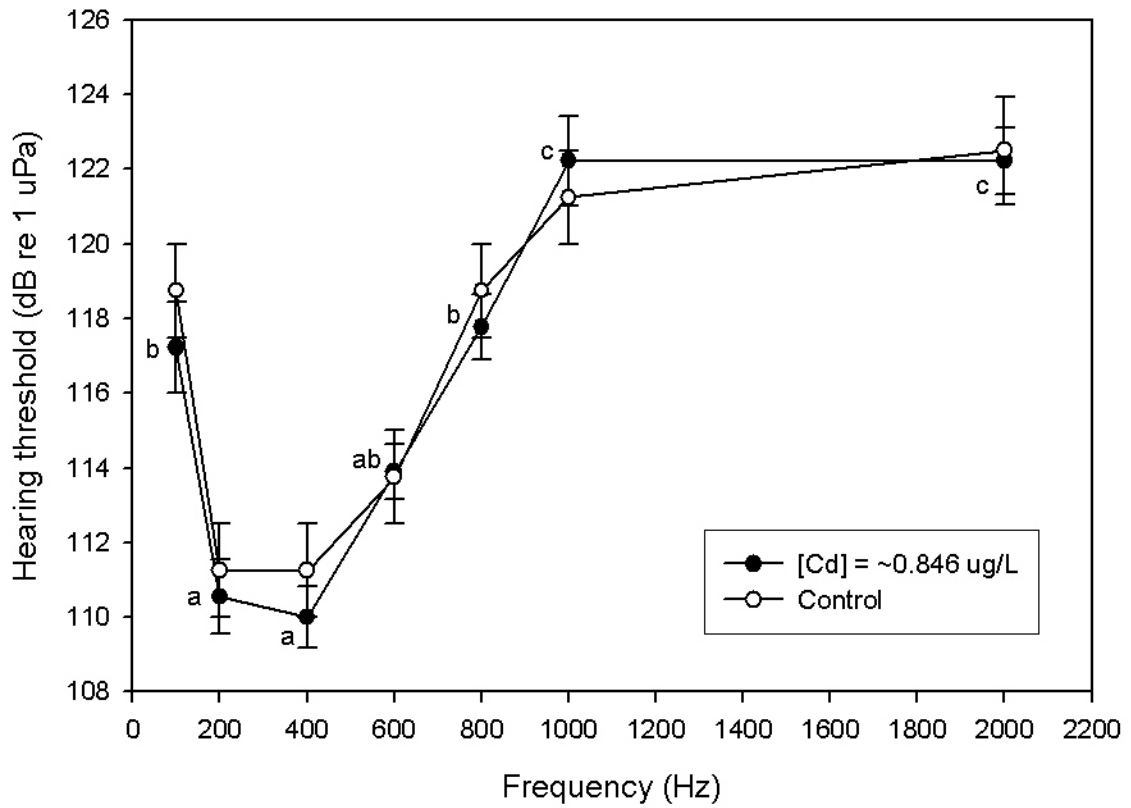


Figure 2.6 d. Audiograms of hearing threshold responses (\pm standard error) in fish exposed to $[Cd] = 0.846 \mu\text{g/L}$ compared to control fish. Letters indicate groups in homogeneous subsets.

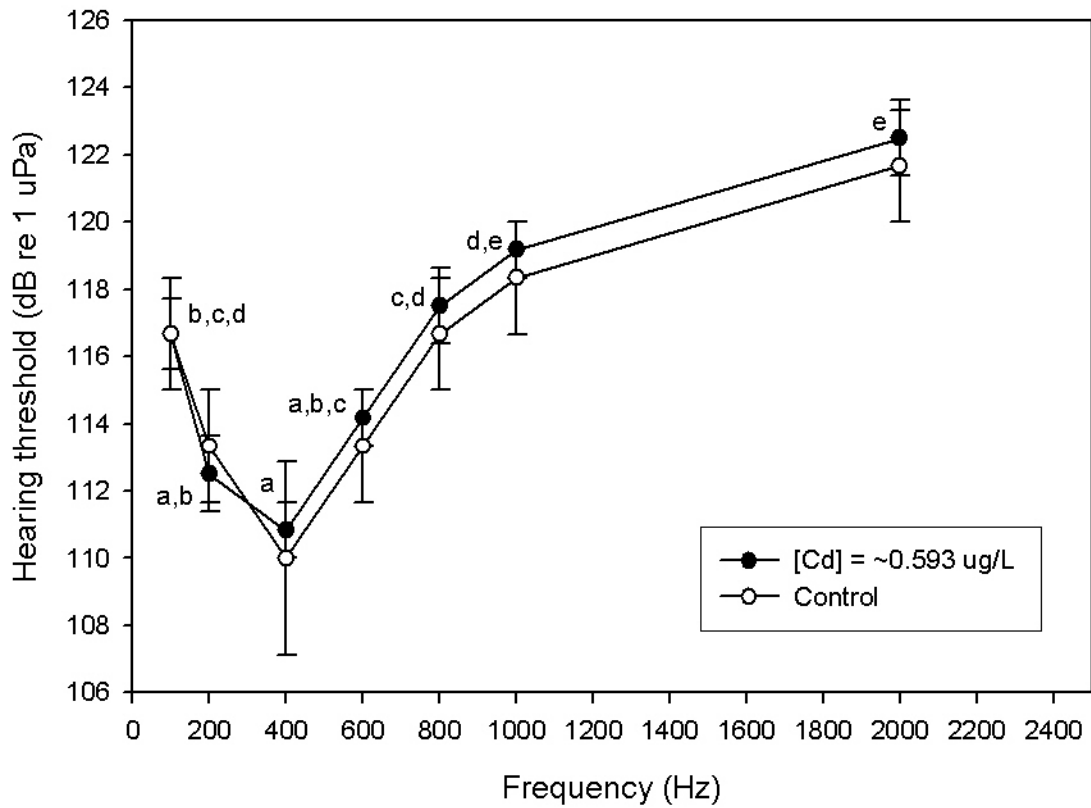


Figure 2.6 e. Audiograms of hearing threshold responses (\pm standard error) in fish exposed to [Cd] = 0.593 $\mu\text{g/L}$ compared to control fish. Letters indicate groups in homogeneous subsets.

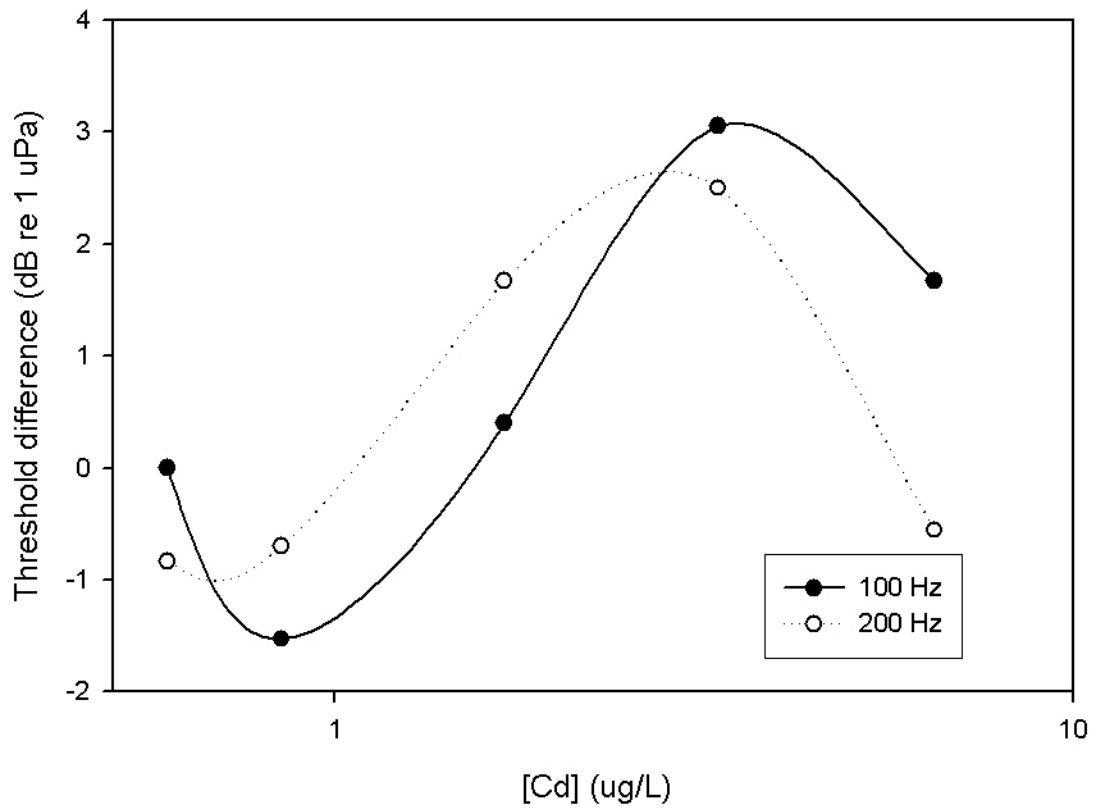


Figure 2.7 a. ‘Dose-response’-like curve created using threshold differences between fish exposed to a range of cadmium concentrations (from 0.593 to 6.49 $\mu\text{g/L}$) and control fish at low frequencies (100 & 200 Hz).

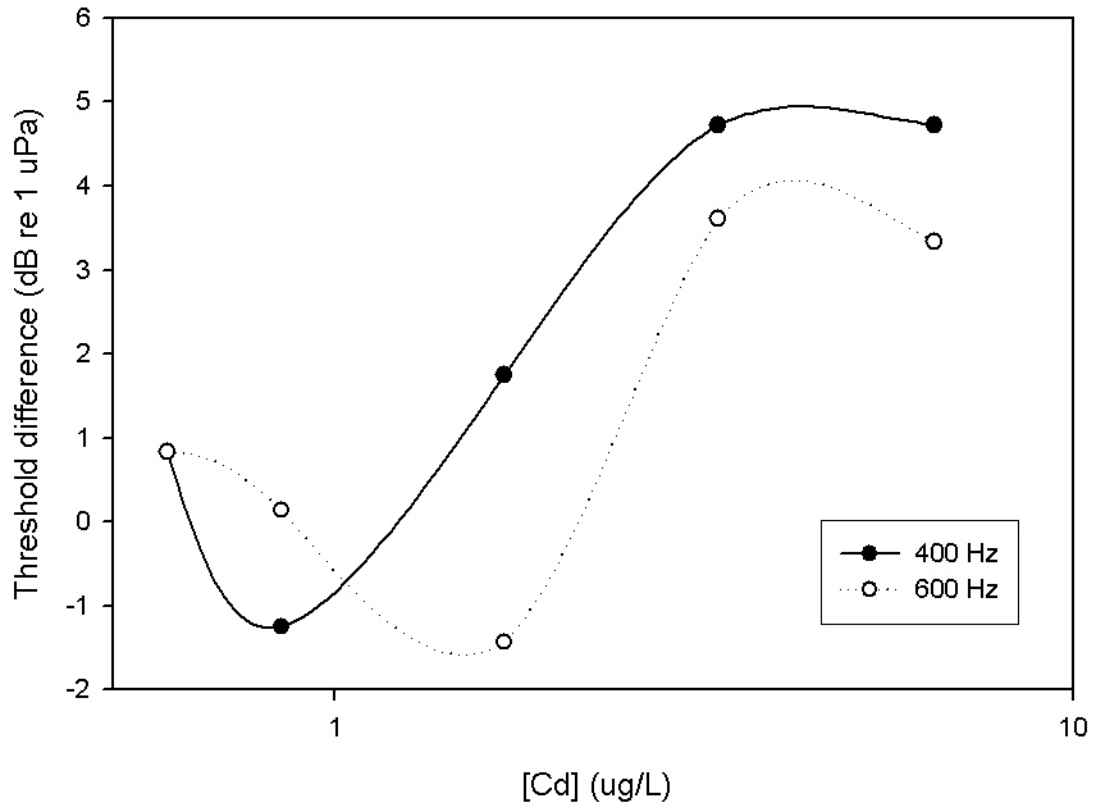


Figure 2.7 b. ‘Dose-response’-like curve created using threshold differences between fish exposed to a range of cadmium concentrations (from 0.593 to 6.49 $\mu\text{g/L}$) and control fish at best frequencies (400 & 600 Hz).

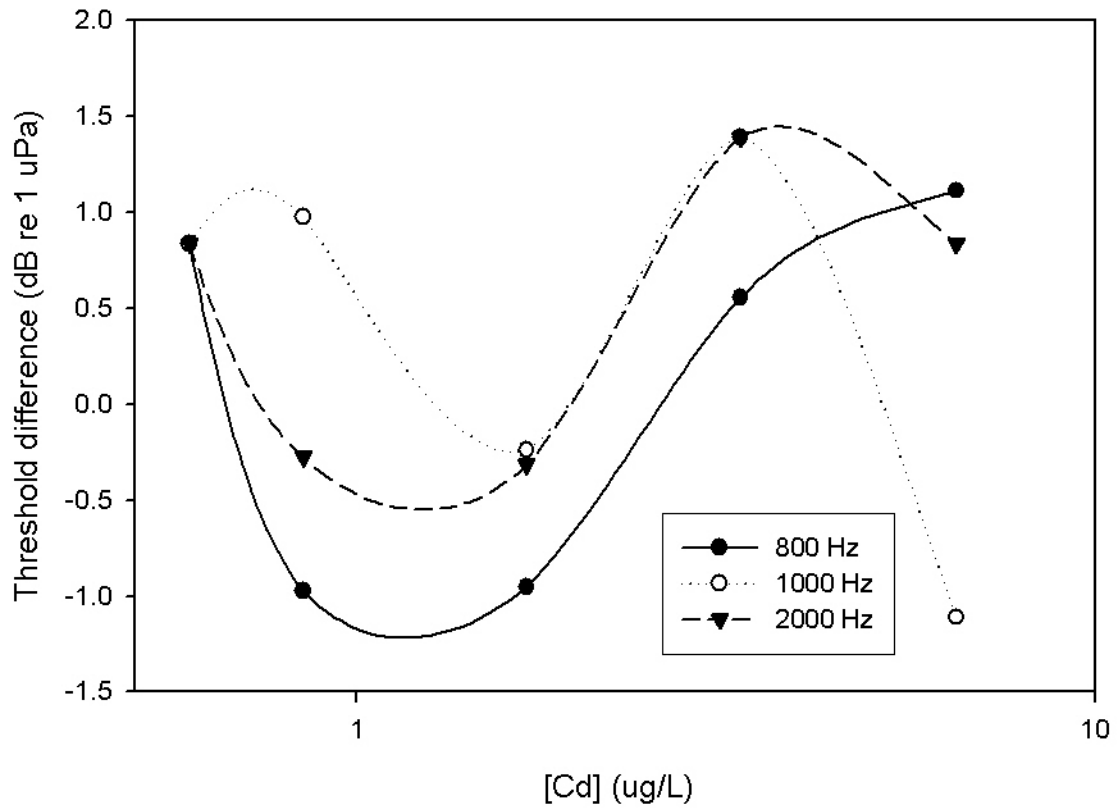


Figure 2.7 c. ‘Dose-response’-like curve created using threshold differences between fish exposed to a range of cadmium concentrations (from 0.593 to 6.49 $\mu\text{g/L}$) and control fish at high frequencies (800, 1000, & 2000 Hz).

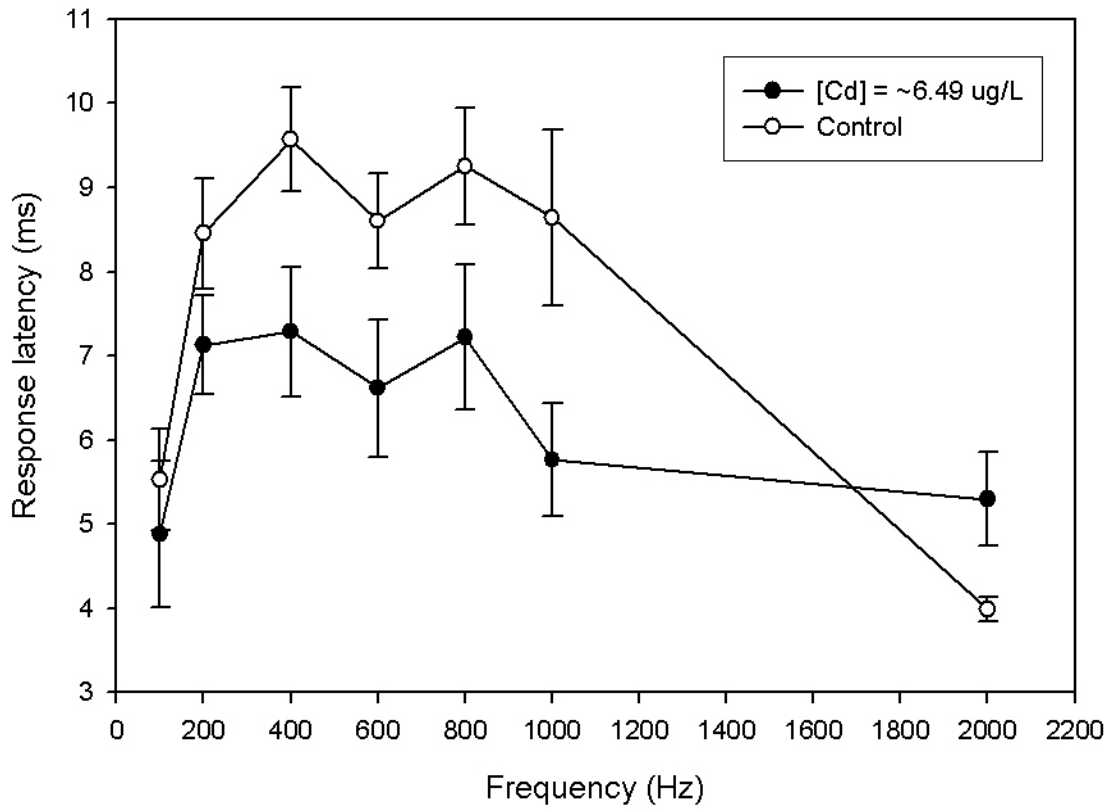


Figure 2.8 a. Response latency (the amount of time it takes for the fish to respond to tone bursts being played) (\pm standard error) in fish exposed to [Cd] = 6.49 $\mu\text{g/L}$ and control fish.

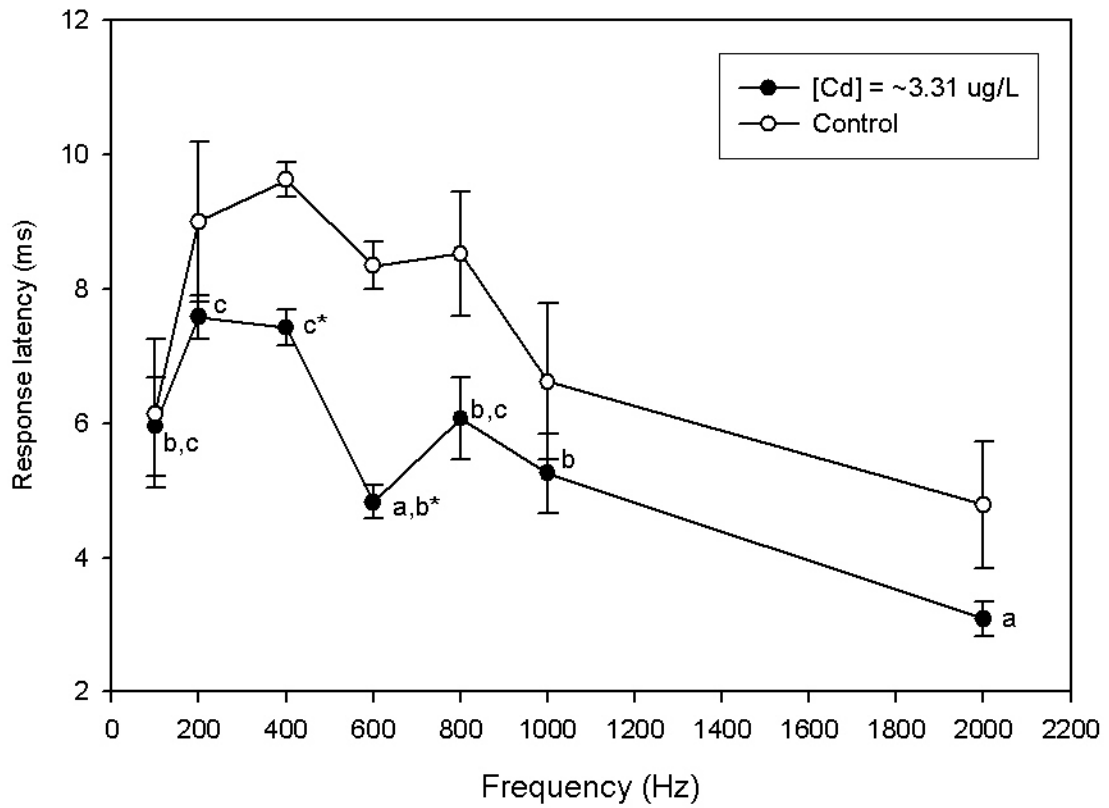


Figure 2.8 b. Response latency (the amount of time it takes for the fish to respond to tone bursts being played) (\pm standard error) in fish exposed to $[Cd] = 3.31 \mu\text{g/L}$ and control fish. Letters represent groups in homogeneous subsets. Asterisks (*) indicate a significant difference in response latency between fish exposed to cadmium and control fish.

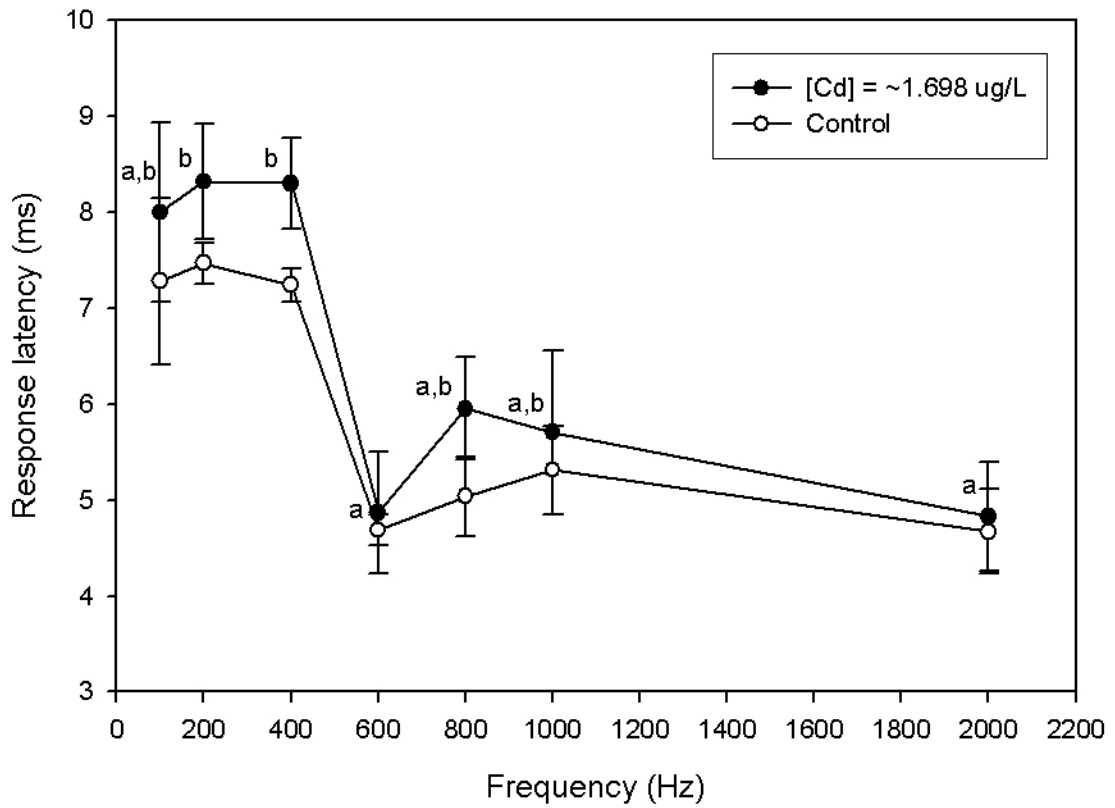


Figure 2.8 c. Response latency (the amount of time it takes for the fish to respond to tone bursts being played) (\pm standard error) in fish exposed to [Cd] = 1.698 $\mu\text{g/L}$ and control fish. Letters represent groups in homogeneous subsets.

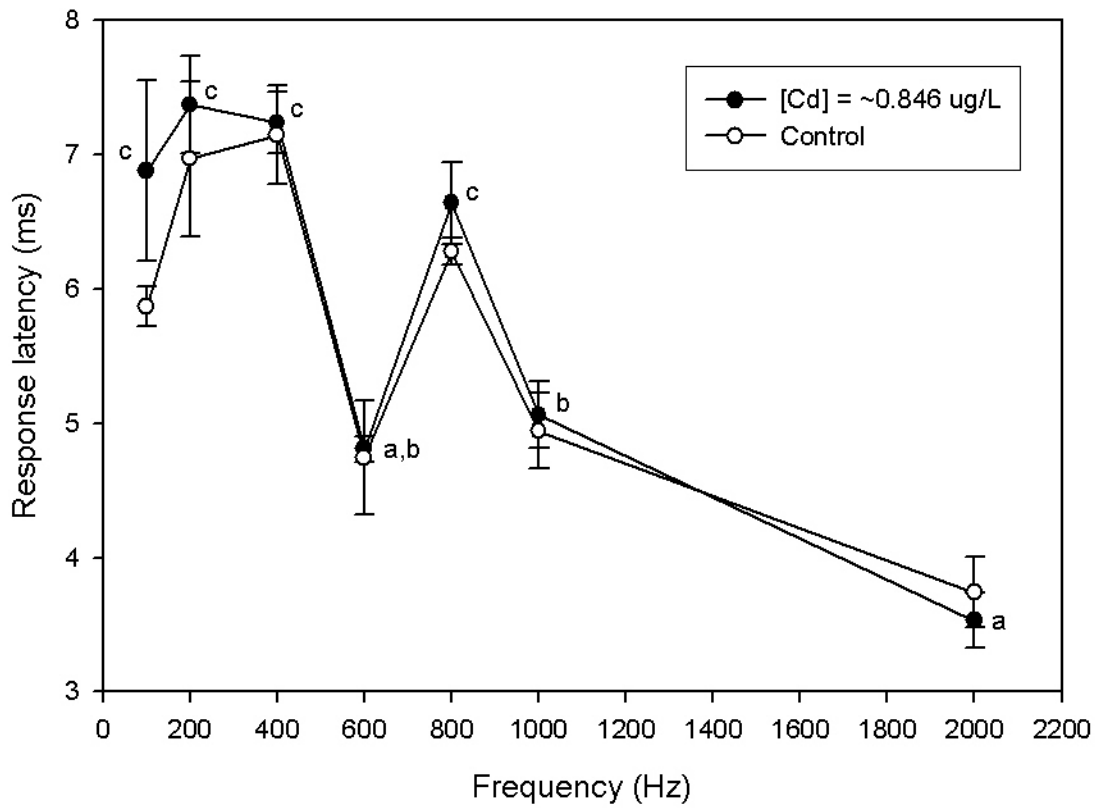


Figure 2.8 d. Response latency (the amount of time it takes for the fish to respond to tone bursts being played) (\pm standard error) in fish exposed to [Cd] = 0.846 $\mu\text{g/L}$ and control fish. Letters represent groups in homogeneous subsets.

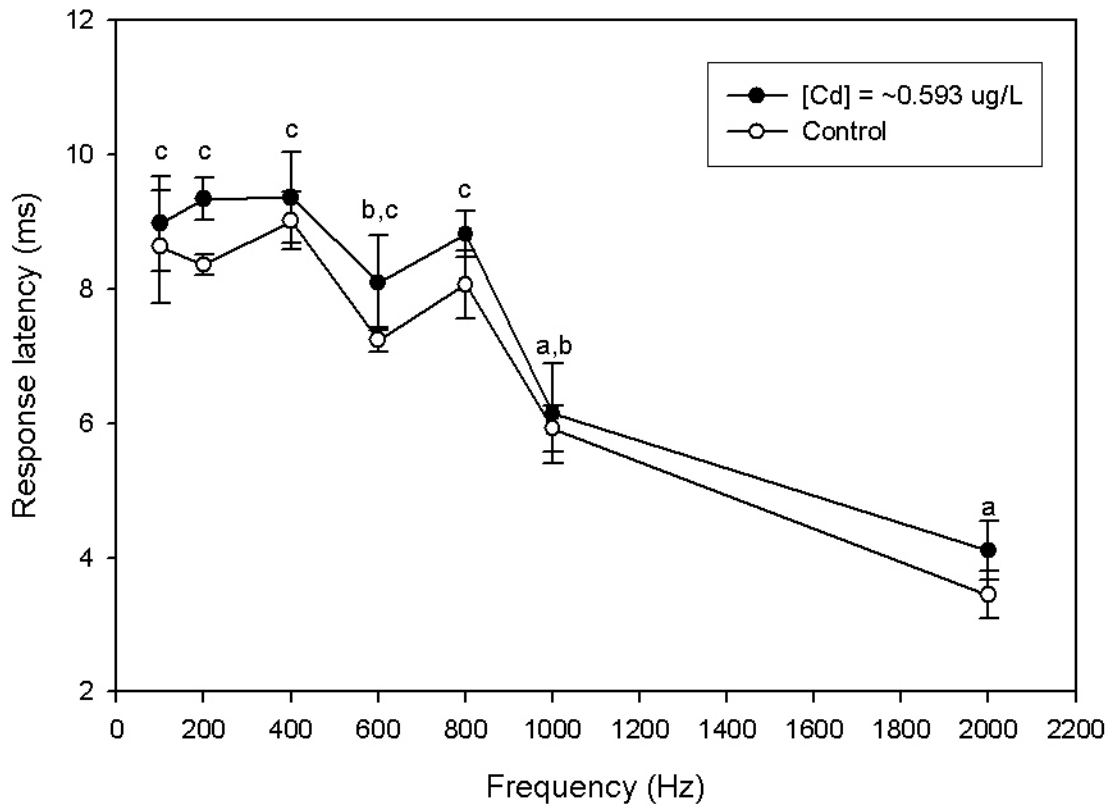


Figure 2.8 e. Response latency (the amount of time it takes for the fish to respond to tone bursts being played) (\pm standard error) in fish exposed to [Cd] = 0.593 μ g/L and control fish. Letters represent groups in homogeneous subsets.

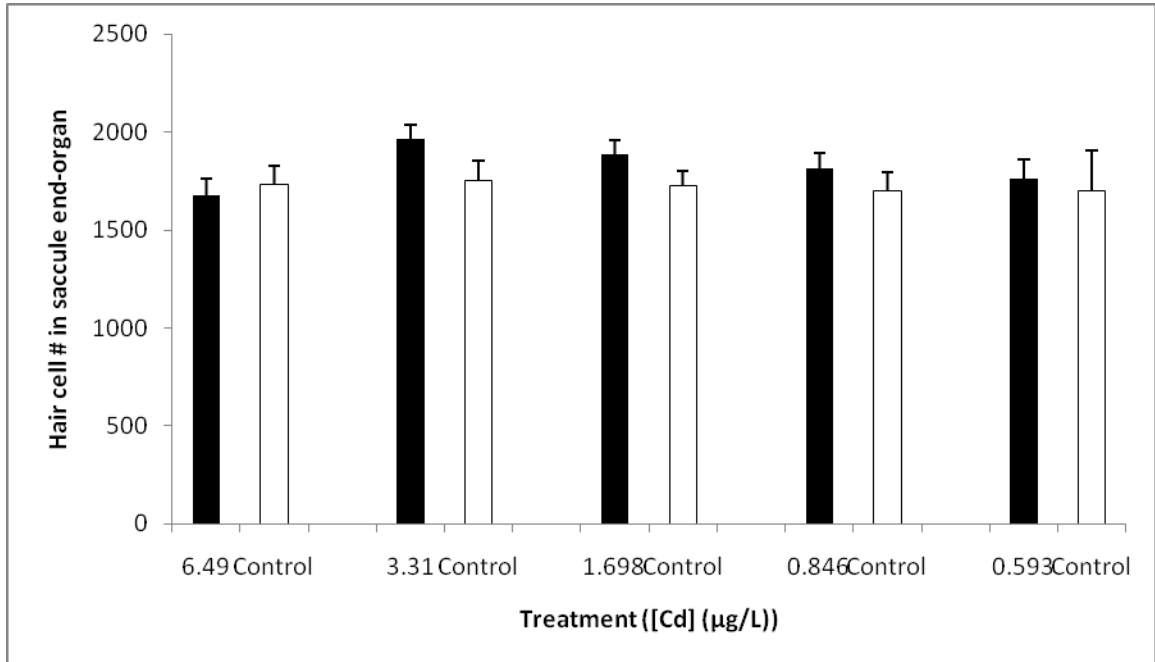


Figure 2.9 a. Bar graph showing differences in hair cell numbers (+ standard error) in the saccule end-organ, between fish exposed to different cadmium levels and control fish.

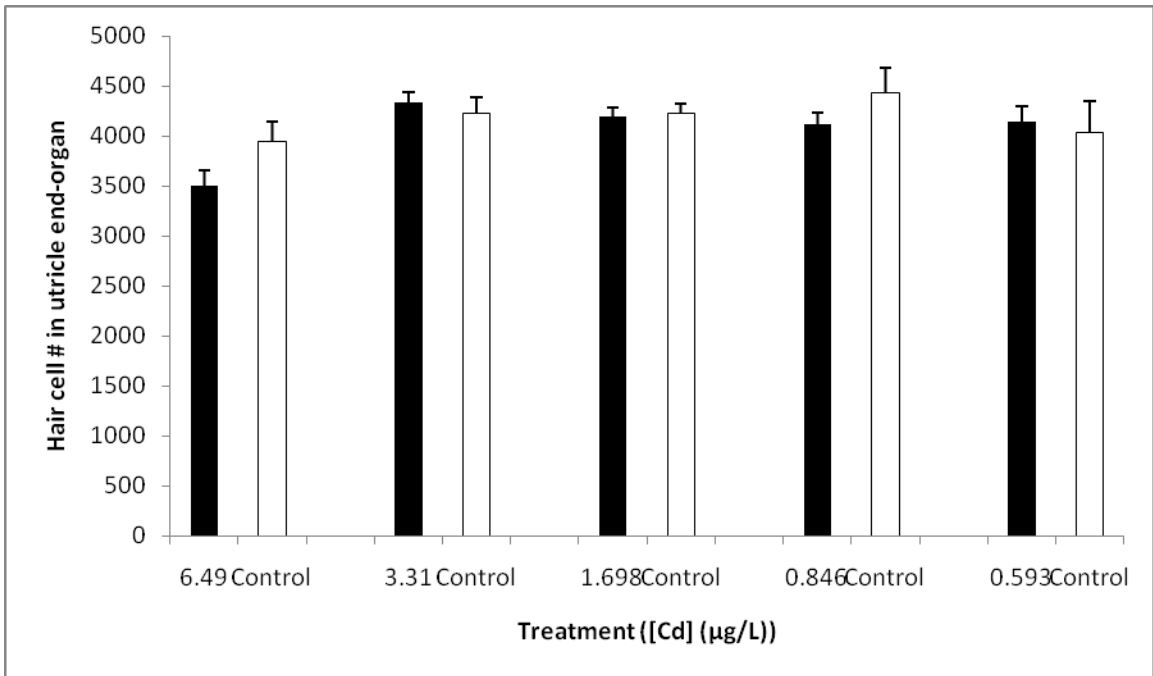


Figure 2.9 b. Bar graph showing differences in hair cell numbers (+ standard error) in the utricle end-organ, between fish exposed to different cadmium levels and control fish.

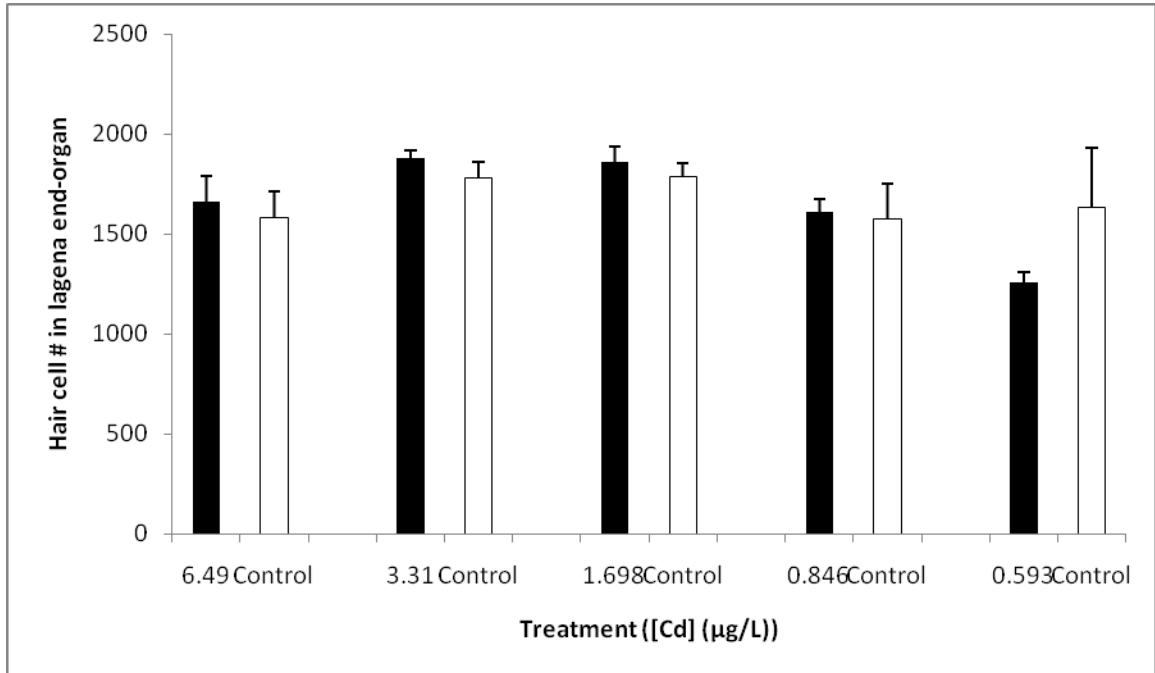


Figure 2.9 c. Bar graph showing differences in hair cell numbers (+ standard error) in the lagna end-organ, between fish exposed to different cadmium levels and control fish.

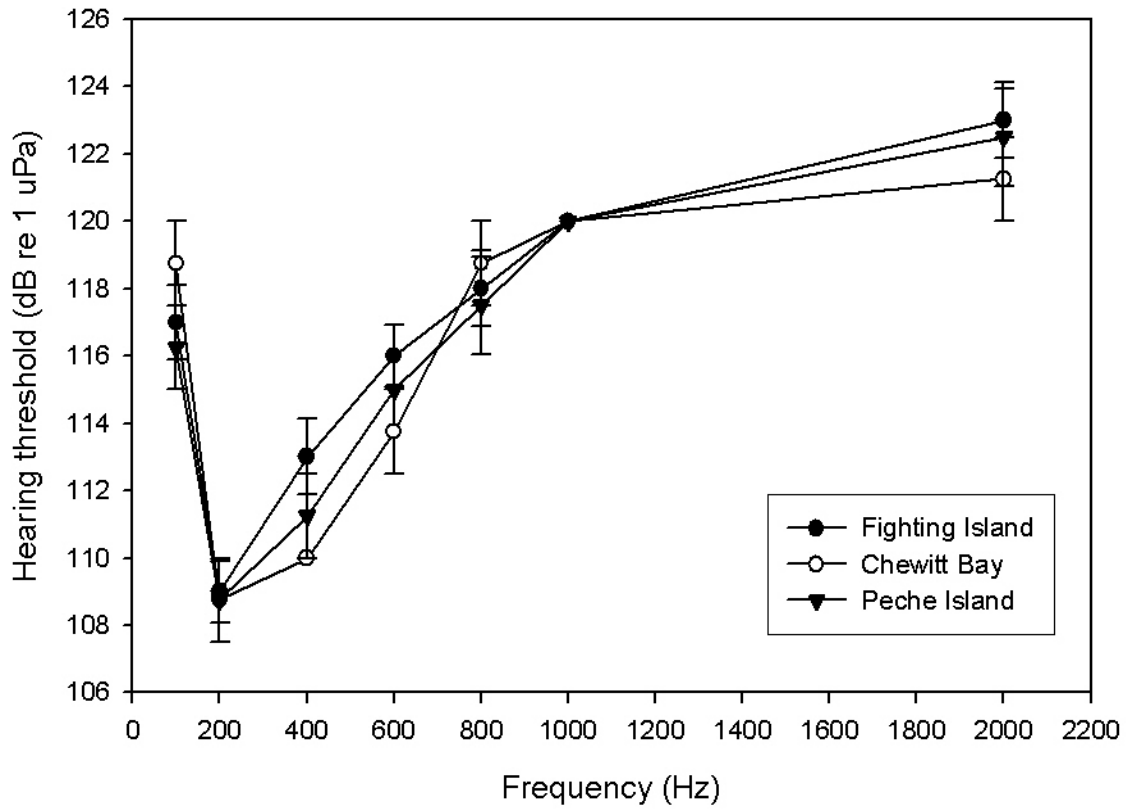


Figure 2.10 Audiograms of hearing threshold responses (\pm standard error) of bluntnose minnows collected from each field site along the Detroit River.

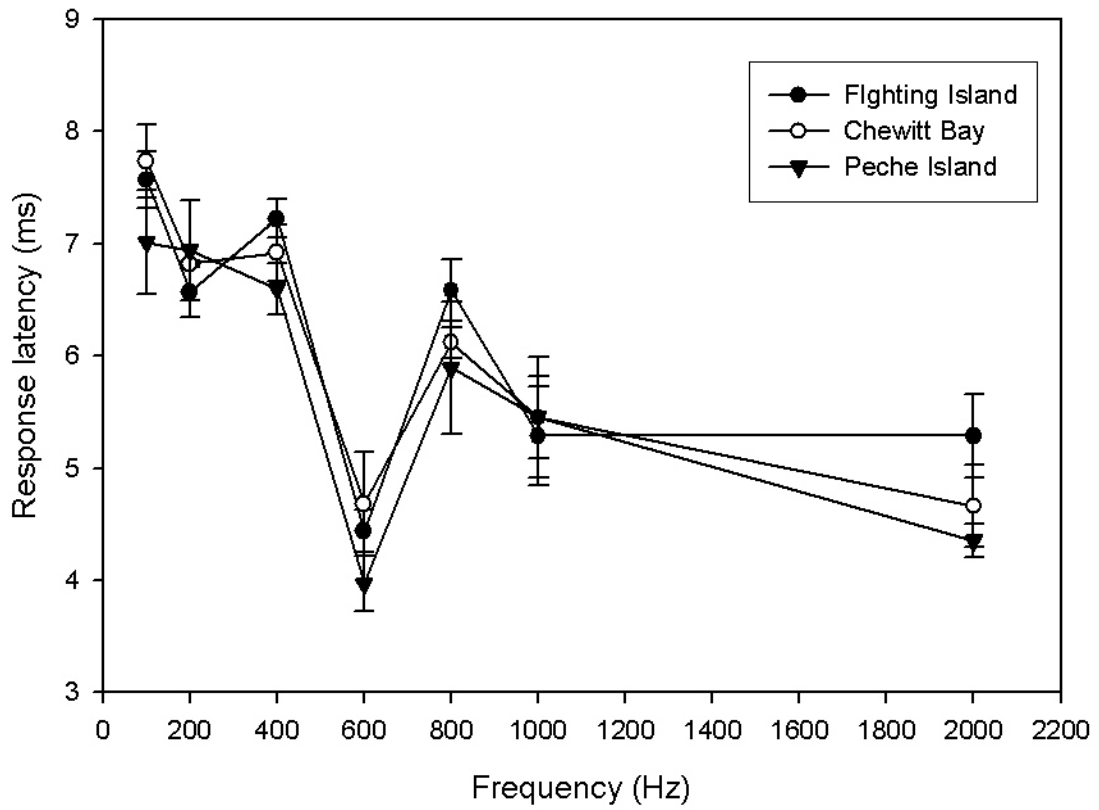


Figure 2.11 Response latency (\pm standard error) of bluntnose minnows collected from each field site along the Detroit River.

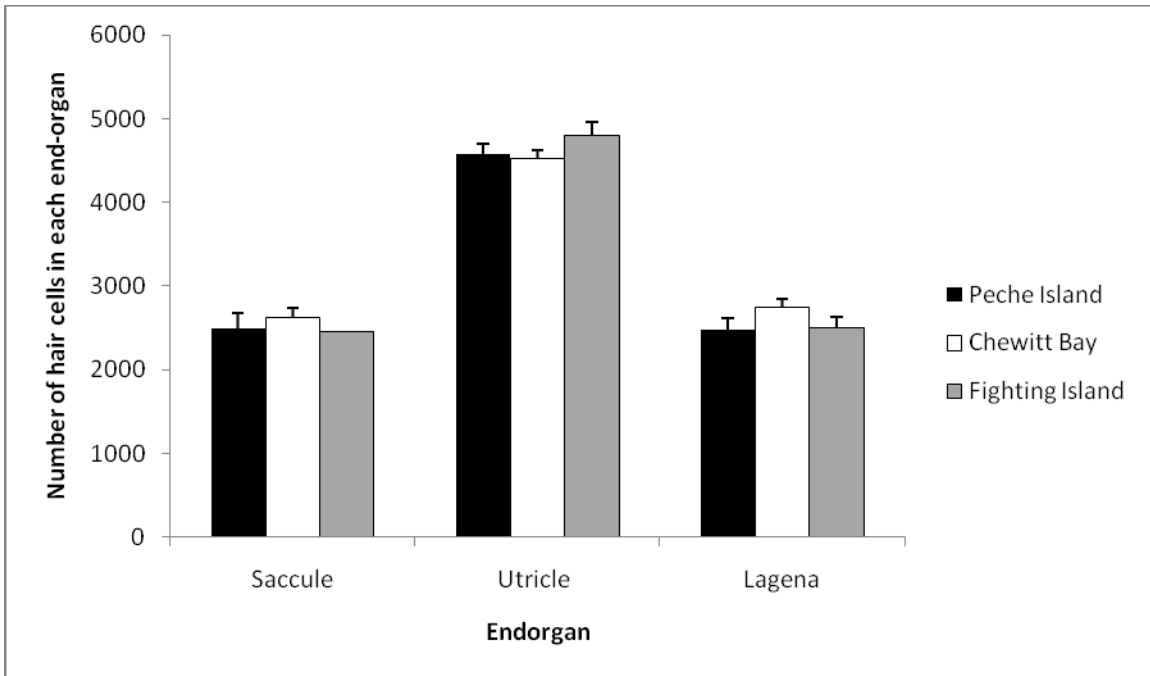


Figure 2.12 Hair cell differences (+ standard error) in each inner ear end-organ between bluntnose minnows collected from each field site along the Detroit River.

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