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Quantification and comparison of metallothionein levels in crayfish (Orconectes propinquus) as a biomarker of metal pollution in the central Upper Peninsula of Michigan

Heather Anne Martin

QUANTIFICATION AND COMPARISON OF METALLOTHIONEIN LEVELS IN CRAYFISH (*Orconectes propinquus*) AS A BIOMARKER OF METAL POLLUTION IN THE CENTRAL UPPER PENINSULA OF MICHIGAN

By

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ABSTRACT

The health of aquatic ecosystems is continuously compromised by anthropogenic activities which introduce an excess of metals into the water. The harmful effects are magnified when these metals infiltrate terrestrial ecosystems through bioaccumulation. Sediment and water analyses are typically employed to monitor the impact of human activities on aquatic ecosystems, but these measurements do not account for an organism's response to potentially toxic metals. This problem is often addressed by identifying and measuring a biomarker related to the contaminant. Metallothionein is a low molecular weight, cysteine rich protein found in crayfish which functions in the binding of metals (primarily zinc, copper, cadmium, and mercury). Due to metallothionein's induction by heavy metals, quantification of this protein in organisms affected by metal pollution has been utilized as an early detection system in the maintenance of environmental integrity. In the past, methods employed to quantify metallothionein have been elaborate and expensive. In the present study, a cost effective and time efficient cadmium saturation method was developed to measure metallothionein in the gills of crayfish collected from four different locations in the Central Upper Peninsula of Michigan. Linear regression analysis comparing metallothionein concentrations in crayfish tissues to metals present in their environmental water revealed a weak correlation between zinc and metallothionein. The results suggested that ambient metal concentrations were too low to induce metallothionein and physiological variations overshadowed the effects of metal exposure. For metallothionein to be an effective biomarker, metal concentrations must be high enough to induce synthesis of the protein.

DEDICATION

With the utmost pride and deepest gratitude, I dedicate this thesis to my parents, Jack and Betty Martin, for teaching me the importance of maintaining integrity in my work while accomplishing my goals. From the both of you, I learned that success depends on not only the results, but also the method. To my brothers, Chris and Craig Martin, thank you for being inspiring examples of this principle throughout the years.

I extend this dedication to the Biology and Chemistry faculty members, fellow scientists, and friends who believed in my ability as a researcher to successfully accomplish this project. Your endless support and unwavering confidence in me will always be appreciated and remembered.

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I attribute the completion of my research to Dr. John Ejnik's involvement in this project. When Dr. Ejnik first arrived at NMU, he immediately proved his dedication to his profession by taking on the time-consuming task of educating and training me in the field of analytical chemistry. He graciously donated a significant amount of his time to help me develop methods, operate and repair instruments, analyze samples, and learn numerous biochemical techniques. He always made himself available to answer questions and troubleshoot any problems that I encountered. Dr. Ejnik's guidance proved

to be invaluable to this project and my growth as a researcher. I'm very fortunate to have worked with him and benefit from his expertise.

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LIST OF ABBREVIATIONS

Name	Abbreviation
Atomic Absorption Spectroscopy	AAS
5,5'-dithiobis(2-nitrobenzoic acid)	DTNB
Dead River	DR
Deer Lake	DL
East Branch of the Escanaba River	EBER
Inductively Coupled Plasma Mass Spectrometer	ICP-MS
Laughing Whitefish River	LWR
Metal ion	M
Metallothionein	MT
Metallothionein Isoform 1	MT-1
Metallothionein Isoform 2	MT-2
Nuclear Magnetic Resonance	NMR
Sulfhydryl	S
Ultraviolet	UV
Visible	Vis

LIST OF REAGENTS

Reagent	Source
Ammonium Phosphate matrix modifier	SCP Science
β-mercaptoethanol	Sigma Chemical Co.
Cadmium Standard	Sigma Aldrich Chemical Co.
Cadmium Chloride	Allied Chemical Co.
Copper Standard	JT Baker
Cuprous Chloride	Matheson Coleman & Bell
5,5'-dithiobis(2-nitrobenzoic acid)	Aldrich Chemical Co.
Gold Blank Standard 1	Claritas PPT®
Rabbit Metallothionein	Northern Michigan University
Sephadex G-15, G-25, G-50, G-75	Sigma Aldrich Chemical Co.
Trisma Base	Fisher Biotech
Zinc Standard	Aldrich Chemical Co.

Chapter One: Introduction

I. Metallothionein Overview

Metallothionein (MT) is a multifunctional protein which was first isolated from equine kidney cortex (Margoshes and Vallee, 1957). Since its initial isolation metallothionein has been found to be present throughout the animal and plant kingdoms. In vertebrates this protein is found in the intestines, retina, bone, and pineal gland, but is most predominant in the liver and kidney (Richards, 1989). In invertebrates, such as decapod crustaceans, metallothionein is most commonly extracted from the hepatopancreas, gills, and muscles.

Metallothionein not only exhibits diversity in its location, it also has a wide variety of functions. Metallothionein influences the absorption, uptake by tissues, transport, storage, and detoxification of metals (Vallee, 1991), such as zinc, copper, cadmium, and mercury, which are the most common in respect to its primary functions (Bremmer, 1991). In addition to metallothionein's role in metal regulation, it also serves as an antioxidant by protecting cells against electrophilic agents such as reactive oxygen species and free radicals (Kagi and Schaffer, 1988).

Since the discovery of cadmium-binding metallothionein in equine kidney cortex a number of other isoforms have been isolated which are classified according to their structural characteristics. The following scheme describes the criteria for the separation of metallothioneins into three groups (Kojima, 1991).

> "Class I: Polypeptides with locations of cysteine closely related to those in equine kidney metallothionein; cysteine content of approximately 30%, large proportion of serine residues (14%), and basic amino acids, such as arginine and lysine;
>
> Class II: Polypeptides with locations of cysteine only distantly related to those in equine kidney metallothionein, such as in yeast metallothionein; contain less cysteine than Class I and may contain phenylalanine, tyrosine, and histidine;
>
> Class III: Atypical, nontranslationally synthesized metal-thiolate polypeptides, such as cadystin, phytochelatin, and homophytochelatin; have restricted amino acid sequence limited mainly to cysteine, glutamic acid, and glycine."

II. Metallothionein Structure

Extensive structural analyses, including NMR, amino acid sequencing, and x-ray crystallography of metallothionein reveals that this protein has a low molecular weight (6000-6500 Da), is rich in cysteine, and consists of 57-62 amino acids (Brouwer et al., 1995). MT is unique in the fact that it lacks aromatic amino acids, histidine (except for Class II), and disulfide bonds (Munoz et al., 2002).

A protein's function and reactivity are rooted in its structure. It is important to establish structural similarities among proteins (such as metallothionein) when making comparisons between species about the physiological behavior of a protein. Research conducted by Pedersen et al. (1996) provided invaluable structural information about

metallothioneins isolated from marine, semiterrestrial, and freshwater decapod crustaceans. Decapod crustacean metallothionein showed the strongest homology with Class I due to its cysteine content of approximately 30% along with large amounts of serine, lysine, and arginine residues (Pedersen et al., 1996). Comparing the amino acid sequences of the freshwater crayfish *A. astacus* and freshwater crab *P. potamios* with previously determined sequences from other decapod crustaceans such as crabs (*Scylla serrata*, *Callinectes sapidus*, and *Carcinus maenas*) and the lobster (*Homarus americanus*) revealed a high degree of sequence identity (Pedersen et al., 1996). Table1 highlights the conserved residues within the aforementioned decapod crustaceans' amino acid sequences (Pedersen et al., 1996).

Table 1: Amino acid sequences (conserved residues shown in bold face type) of metallothionein isoforms for decapod crustaceans (*Astacus astacus, Potamon potamios, Scylla serrata, Carcinus maenas, Callinectes sapidus,* and *Homarus americanus*).

	1 10 20 30
Carcinus MT I	**P**D**PCC**IDK **C**E**C**KE**G**G**CK**A**GC**K**CT**S**CRC**T**PC**EK**C**SSG
Astacus MT I	**P**G**PCC**NDV **C**E**C**AA**G**G**CK**T**GC**V**C**T**SCRC**S**PC**DK**C**TSG
Potamon MT I	**P**D**PCC**AEGT**C**E**C**EE**G**K**CK**A**GC**K**C**T**SCRC**S**PC**EK**C**TSE
Scylla MT I	**P**G**PCC**NDK **C**V**CK**E**G**G**CK**E**GC**Q**C**T**SCRC**S**PC**EK**C**SSG
Scylla MT II	**P**D**PCC**NDK **C**D**C**KE**G**E**CK**T**GC**K**C**T**SCRC**P**PC**EQ**C**SSG
Callinectes MT I	**P**G**PCC**NDK **C**V**C**QE**G**G**CK**A**GC**QCT**SCRC**G**PC**QK**C**TSG
Callinectes MT II	**P**D**PCC**NDK **C**E**C**KE**G**E**CK**T**GC**K**C**K**SCRC**P**PC**DK**C**SSG
Homarus MT I	**P**G**PCC**KDK **C**E**C**AE**G**G**CK**T**GC**K**C**T**SCRC**A**PC**EK**C**SSG
	40 50
Carcinus MT I	**C**K**C**TT**K**ED**C**C**KTCTKPCSCCP**
Astacus MT I	**C**K**C**PS**K**EE**C**A**K**T**C**S**K**P**CSCCP**
Potamon MT I	**C**E**C**KS**K**EE**C**A**K**N**C**T**K**P**CSCCP**
Scylla MT I	**C**KCAN**K**EE**C**S**K**T**C**S**K**A**CSCCP**T
Scylla MT II	**C**K**C**AN**K**ED**C**R**K**T**C**S**K**P**CSCCP**
Callinectes MT I	**C**K**C**AT**K**EE**C**S**K**T**C**T**K**P**CSCCP**K
Callinectes MT II	**C**K**C**TS**K**EE**C**S**K**T**C**S**K**P**CSCCP**
Homarus MT I	**C**K**C**PS**K**DE**C**A**K**T**C**S**K**P**C**?**CC**??

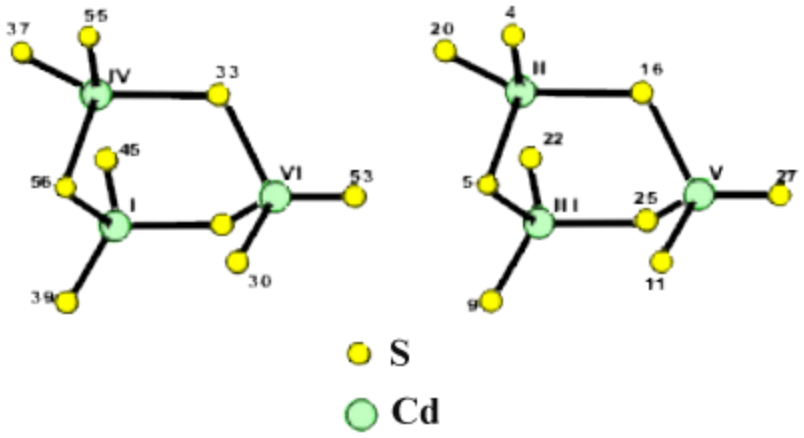
The amino acid sequences of metallothioneins from freshwater, marine, and semi-terrestrial decapod crustaceans share several structural features. All metallothioneins

analyzed contained 57 residues of which 18 are conserved cysteines (except for *Scylla serrata* MT I) (Pedersen et al., 1996). The conserved sequence motifs involving the cysteine residues include five Cys-X_{aa}-Cys-, two Cys-Cys-, and three Cys-X_{aa}-Y_{aa}-Cys- (Pedersen et al., 1996). The presence of 8 or 9 lysine residues satisfies the electrostatic requirements of Class I metallothioneins (with the exception of *A.astacus* which has only 6) (Pedersen et al., 1996). All of the metallothioneins sequenced showed sequence conservation between amino acids 24-27 and a Pro-X_{aa}-Pro-Cys-Cys- motif at the N-terminal end (Pedersen et al., 1996). Crustacean metallothioneins are valuable models when studying the structural and functional properties of metallothionein because of their limited variations of sequence and structure (Munoz et al., 2000).

The tertiary structure of metallothionein lends insight into not only the isolation of MT by gel filtration, but also into metal-thiolate geometry and stoichiometry. X-ray crystallography and nuclear magnetic resonance indicate that mammalian metallothionein consists of both an alpha domain and beta domain at the C-terminus and N-terminus, respectively (Robbins et al., 1991). The number of metals and cysteine residues contained in MT vary among organisms. Therefore, the metals (M) and sulfur containing cysteine residues (S) are often followed by a subscript to depict the amount of these components present in MT. The binding stoichiometry of various metals within each domain and the geometric configurations in which they are coordinated are dependent on the type of metal. Most metals exhibit saturation binding at 7 metal atoms per molecule of protein (Nielsen et al., 1985). These metals include Bi(III), Cd(II), Co(II), Hg(II), In(III), Ni(II), Pb(II), Sb(III), and Zn(II) (Nielsen et al., 1985). The distribution of metals in the two domains of M_7-MT is $M_4S_{11}\alpha$ and $M_3S_9\beta$ (Munoz et al., 1999). Metals in both

domains are tetrahedrally liganded by sulfur atoms from cysteine residues (Robbins et al., 1991). Monovalent cations, such as Ag(I), Au(I) and Cu(I), display not only higher, but also variable, metal to protein stoichiometries (Kagi and Schaffer, 1988). For example, stoichiometric experiments conducted by Chen et al. (1996) revealed that MT-2 from rabbit liver can bind 7-20 moles of copper.

Like mammalian metallothionein, invertebrate MT has two peptide domains that give the protein a dumbbell shape (Robbins et al., 1991). Both domains of crustacean metallothionein are analogous to the β domain of mammalian MT in that they contain three divalent metals tetrahedrally coordinated to nine cysteine residues (Munoz et al., 2002). The two M_3S_9 domains of invertebrate metallothionein are designated β_N and β_C because of their resemblance to the beta domain of mammalian MT (Munoz et al., 2002). In respect to divalent cations, saturation is reached at 6 metals per molecule of MT resulting in a cysteine to metal ratio of 18:6. In respect to univalent metals, such as Cu(I), crustacean metallothioneins also exhibit a deviation from the M_6-MT stoichiometry typical of divalent cations. Figure 1 illustrates the divalent metal-thiolate geometry and stoichiometry of the β_N and β_C domains of lobster metallothionein (Munoz et al., 2000).



Cd_3S_9 *beta*$_C$ domain **Cd_3S_9 *beta*$_N$ domain**

Figure 1: Positions of cysteine residues (numbers) and cadmium atoms (roman numerals) in the β_N and β_C domains of lobster metallothionein (Munoz et al., 2000).

III. Factors Affecting Metallothionein Concentration in Crustaceans

In polluted areas metal exposure is the main determinant of metallothionein concentration in crustaceans (Serafim et al., 2002). Metals such as zinc, copper, cadmium and mercury, have been shown to induce metallothionein synthesis (Waalkes and Goering, 1990). However, biotic variations such as size, gender, molting, and reproductive state, and environmental factors, including water temperature, salinity, and hardness, can also cause variability in MT and tissue metal accumulation. Therefore it is important to consider the effects of both endogenous and exogenous factors when employing metallothionein as a biomarker.

Studies have been conducted to demonstrate the effects of water temperature and size of the animal on metal uptake and MT concentration. Sarafim et al. (2002) demonstrated that cadmium and metallothionein concentrations in gill tissue are dependent on temperature and size. Two different sizes (shell length 35 ± 0.5 and 52 ± 0.7) of mussels,

Mytilus galloprovincialis, were exposed to clean water and water containing Cd (100 µg/L) at temperatures representing the mean and extreme values measured in their natural environment. The correlation between metallothionein and temperature was demonstrated when three groups of mussels were maintained in clean seawater at 5, 18, and 25 °C for 34 days. The basal amounts of MT and cadmium concentration measured in the gills of mussels kept in the clean water positively correlated with an increase in temperature. Not only was the same temperature dependent increase in MT and cadmium accumulation exhibited in mussels exposed to Cd, but also the levels of metallothionein at the respective temperatures were significantly higher than those measured in the mussels kept in the clean water showing the effects of cadmium exposure on MT synthesis (Serafim et al., 2002). The initial rates of metallothionein synthesis were also positively correlated with temperature which translates into a decreased capacity for metal sequestration at lower temperatures due to reduction in MT levels (Serafim et al., 2002). At 18 and 25 °C, cadmium induced metallothionein synthesis in the gills of both small and large mussels, but the large mussels exhibited a significant 2 fold increase in metallothionein (Serafim et al., 2002). In a review examining the endogenous and exogenous factors influencing metal uptake and, consequently, metallothionien levels, Pourang et al. (2004) attributed higher metal and MT concentrations to increases in metal speciation and in metabolic rates caused by elevated ambient temperatures.

Howard and Hacker (1990) investigated the effects of salinity, temperature, and cadmium on a metallothionein-like Cd binding protein in cadmium treated and untreated grass shrimp (*Palaemonectes pugio*) subjected to variations in temperature and salinity ranging from 20-30 °C and 15%-35%, respectively. Howard and Hacker (1990) found

that elevations in cadmium binding protein levels in whole shrimp after exposure corresponded to increases in temperature and cadmium exposure and to decreases in salinity. The highest Cd binding protein concentrations were found in cadmium-treated shrimp subjected to high temperatures and low salinities (Howard and Hacker, 1990). These workers provided the same explanation as Serafim et al. (2002) for the corresponding increases in metallothionein levels and temperature. There was an indirect correlation between salinity and metallothionein that could be attributed to the uptake of cadmium. In increased salinities cadmium complexes with chloride and decreases the bioavailability of the metal (Howard and Hacker, 1990). As a result cadmium uptake is decreased and the synthesis of metallothionein reduced. The increased uptake of cadmium at lower salinities was also discussed in regards to physiological adaptations of the osmoregulating grass shrimp. These shrimp normally maintain a hyperosmotic balance with their environment by increasing the uptake of salts in low salinities which results in a raised net uptake of cadmium ions (Howard and Hacker, 1990).

Legras et al. (2000) investigated the effects of salinity on metallothionein levels and metal bioaccumulation in the hepatopancreas and gills of the crabs *Pachygrapsus marmoratus* and *Carcinus maenas*. Intermolt female and male crabs were collected from three different sites located in an estuary heavily contaminated with cadmium, zinc, and copper (Legras et al., 2000). The three collection sites were chosen due to their differences in salinity and total protein, metallothionein, and metal concentrations were measured in both the hepatopancreas and gills (Legras et al., 2000). The results indicated an inverse relationship between metal uptake and salinity in these species whereby

soluble cadmium and copper concentrations were inversely related to salinity (Legras et al., 2000). These same observations were found for soluble zinc in *Carcinus maenas*, but not in *Pachygrapsus marmoratus* (Legras et al., 2000). Although these findings substantiated the findings of Howard and Hacker (1990) regarding the effects of salinity on metal bioavailability, Legras et al. (2000) found that metallothionein levels in the organs of *P. marmoratus* were positively correlated to salinity. Because total protein concentrations in the hepatopancreas of both species were elevated when crabs were exposed to higher salinities Legras et al. (2000) suggested that the elevated hepatopancreatic metallothionein levels exhibited by *P. marmoratus* were linked to alterations in general protein metabolism rather than to metal concentrations per se. In similar studies conducted by Mouneyrac et al. (2001) the relationship between MT concentration and protein metabolism was attributed to hypo-osmotic stress experienced by marine crabs exposed to low salinities, where increased blood serum protein levels correlated to decreased protein tissue levels and lower metallothionein concentrations.

Thawley et al. (2004) identified water hardness as one of the abiotic factors governing metal accumulation in the South African freshwater crab (*Potamonautes warreni*) collected from six sites that varied in both salinity and water hardness. These authors measured cadmium and zinc levels in the water, sediments, and crab tissues (gill, muscle, midgut gland, and carapace) and sodium and calcium in the water (as indicators of salinity and water hardness, respectively) (Thawley et al., 2004). Data from these experiments revealed an inverse relationship between metal uptake and both salinity and water hardness (Thawley et al., 2004). Hogstrand et al. (1996) examined zinc uptake mechanisms in freshwater rainbow trout and found that the uptake of zinc from water

occurred via the same pathway as calcium. Zinc and calcium moved across the apical membrane of gill epithelium through chloride cells (Hogstrand et al., 1996). Because these two elements share the same uptake mechanism, it was concluded that waterborne calcium competitively inhibited the influx of zinc (Hogstrand et al., 1996). As a result animals residing in hard water exhibited a lower accumulation of zinc in tissues than did animals collected from soft water. The results of similar studies conducted by Lucu and Obersnel (1996) in the marine crab, *Carcinus maenas*, suggested that waterborne cadmium was also transported across the apical membrane of the gills via calcium channels. In addition to the competition of cadmium and zinc with calcium for the same uptake sites, stimulation of calcium uptake mechanisms in soft water may cause incidental uptake of zinc and cadmium (Thawley et al., 2004) resulting in an increased tissue metal concentration that presumably caused elevated metallothionein concentrations in animals when in soft freshwater.

Gender related variations in MT levels have also been examined in studies investigating metallothionein's reliability as a biomarker, but the results were equivocal: either no significant differences in MT levels were detected between genders, or higher MT levels were found in females. Legras et al. (2000) found metallothionein levels were to be significantly higher in both the gills and hepatopancreas of females of *Pachygrapsus marmoratus* and *Carcinus maenas*. On the other hand, Canli et al. (1997) found no significant difference in the metallothionein concentrations of female and male lobsters (*Nephrops norvegicus*) in either the hepatopancreas or the gills. The absence of gender related differences in MT was observed not only in the control animals but also in

the lobsters that were simultaneously exposed to different concentrations of copper, zinc, and cadmium (Canli et al., 1997).

In respect to the effects of the molt cycle on metallothionein, the literature primarily focuses on the redistribution of metals bound to MT. Engel and Brouwer (1993) found that the concentrations of copper and zinc in the digestive glands, hemolymph, and metallothionein of the blue crab, *Callinectes sapidus*, changed significantly throughout the different stages of the molt cycle. During intermolt the main metal bound to metallothionein in the digestive gland was copper**,** but this changed to zinc once the crabs entered premolt, and after ecdysis copper once again became the predominant metal bound to metallothionein (Engel and Brouwer, 1993**).** A concomitant decrease in hemocyanin**,** the oxygen carrying protein in the crayfish's hemolymph, following ecdysis suggests that the displacement of metallothionein-bound zinc by copper may be linked to the catabolism of hemocyanin and subsequent release of Cu^{1+} (Engel and Brouwer, 1993**).** Because hemocyanin is structurally dependent upon copper, the increase in Cu-MT preceding resynthesis indicates that metallothionein functions to deliver the metal to hemocyanin (Engel and Brouwer, 1993**).**

While examining metallothionein's potential use as a biomarker of trace metal exposure researchers have discovered fluctuations in MT levels in response to physiological processes such as molting and reproduction. Mouneyrac et al. (2001) investigated the reproductive cycle's influence on metal storage (Cu, Cd, and Zn) and metallothionein content in the crab, *Pachygrapsus marmoratus*. To eliminate complications arising from additional physiological influences on metallothionein female and male intermolt crabs were collected at the same location from a metal rich-estuary

both during (June) and after (February) the summer reproductive season (Mouneyrac et al., 2001). Metallothionein and both soluble (supernatant including cytosol) and insoluble (pellet including mineral granules) metal concentrations were measured in the hepatopancreas and gills of blue crabs (*Callinectes sapidus*) (Mouneyrac et al., 2001). Specimens collected during the breeding season had the highest metallothionein concentrations in both organs, whereas concentrations of insoluble metals were higher in both the hepatopancreas and gills of crabs collected in February (Mouneyrac et al., 2001). These results suggest that the changes in metallothionein concentration were linked to alterations in general protein metabolism related to growth and reproductive status versus metal accumulation (Mouneyrac et al., 2001). The higher insoluble metal concentrations in specimens collected in February presents the possibility that essential metals are stored during the winter and then remobilized so they can be used in the summer during the breeding season (Mouneyrac et al., 2001). Regarding the soluble metal concentrations measured in the hepatopancreas and gills, there were positive correlations between metallothionein and both zinc and copper in June and February (Mouneyrac et al., 2001). The correlations between cytoplasmic cadmium and metallothionein were comparatively weaker than those determined for the essential metals which may be a result of the lower molar abundance of cadmium relative to zinc and copper (Mouneyrac et al., 2001).

Increased metallothionein levels during periods of oxidative stress have prompted investigations into its utility as a neutralizing ligand for reactive oxygen species. A dose-dependent upregulation of both metallothionein protein and MT mRNA was observed when two cell lines from rainbow trout and chinook salmon were exposed to H_2O_2 (Kling and Olsson, 2000). Reactive oxygen species such as H_2O_2 are generated as toxic by-

products of normal cellular processes (Livingstone, 2003). These reactive pro-oxidants have been shown to damage proteins, lipids, and DNA causing deleterious functional and pathological consequences for any organism (Livingstone, 2003). Metallothionein plays an integral role in the cell's defense against reactive pro-oxidants by acting as a neutralizing nucleophilic equivalent for damaging electrophilic agents (Dalton et al., 1996).

The induction of metallothionein by metals makes this protein an ideal biomarker for monitoring elevated influxes of metals into aquatic ecosystems. However, fluctuations in metallothioenin levels due to both endogenous and exogenous factors, such as gender, reproductive state, molting, salinity, water hardness, size, and temperature may compromise its effectiveness as a gauge for metal exposure exclusively. The influence of biotic and abiotic factors must therefore be eradicated when employing metallothionein as a monitoring tool for metal pollution in aquatic systems. In an attempt to achieve this objective species selected as biomonitors should be consistent in size, gender, and reproductive state and variations in exogenous factors, such as season, salinity, and water hardness, must be kept to a minimum.

IV. Metal Toxicity

Metal pollution in aquatic ecosystems is a concern due to the danger it poses to the health of inhabitants directly exposed to the toxicants. The threat of metal pollution is further magnified when the associated hazards infiltrate the terrestrial ecosystem through bioaccumulation. Although zinc is an essential metal for animals, physiological processes such as growth, survival, and reproduction can be significantly jeopardized when an organism is exposed to elevated Zn^{2+} concentrations in the water (Eisler, 1993).

Although all living organisms require copper for normal growth and metabolism, high doses of copper can make it one of the most toxic heavy metals causing irreparable harm to freshwater and marine communities (Eisler, 1998). Shore crabs, *Carcinus maenas*, exposed to elevated copper levels suffered damage to their gill epithelias which causes tissue hypoxia at lethal concentrations (Eisler, 1998). Shore crabs exposed to elevated levels of waterborne copper exhibited decreased hemolymph sodium, gill Na^+/K^+ ATPase activity, hemolymph electrolytes, and hepatopancreatic enzyme activity (Eisler, 1998). High concentrations of copper have proven to be lethal to the rusty crayfish, *Orconectes rusticus*, due to this metal's ability to coagulate cellular proteins and obstruct respiratory function (Eisler, 1998). When rusty crayfish were exposed to sublethal copper concentrations animals exhibited tissue degeneration and disrupted glutathione equilibrium (Eisler, 1998). Mercury and its derivatives are nonessential to biological functions, but they are extremely poisonous to all living organisms. Mercury's toxicity resides in its ability to bind to the sulfhydryl groups in cysteine and to disrupt the disulfide bridges within the protein. As a result, thiol-containing proteins lose their shape and become nonfunctional. Freshwater and marine organisms exposed to low concentrations of mercury suffer detrimental aberrations in reproduction, growth, behavior, metabolism, blood chemistry, osmoregulation, and oxygen exchange (Eisler, 1987). Like mercury, cadmium is not physiologically essential or beneficial and organisms exposed to high concentrations of cadmium often die (Eisler, 1985). At sublethal concentrations, cadmium adversely affects growth, reproduction, mobilization, respiration, molting, and muscular contraction in aquatic organisms (Eisler, 1985).

The deleterious ramifications of metal contamination in aquatic ecosystems are not limited to the exposed inhabitants. Population declines of aquatic organisms affected by metal pollution can adversely affect the entire ecosystem, especially if the effected species plays an integral role in the cycling of metals. In the majority of freshwater systems, crayfish account for a large percentage of the aquatic biomass and sediment quality and environmental biogeochemical cycling of metals are both strongly dependent on crayfish populations for the cycling of metals (Horton, 2004). Consequently, a decline in the population of crayfish in an aquatic ecosystem would alter both terrestrial and aquatic environments.

In addition to population decline bioaccumulation necessitates strict monitoring of anthropogenic activities contributing to metal pollution of aquatic systems. Bioaccumulation facilitates the spread of contaminants into terrestrial environments and threatens the health of upper trophic level consumers. Cadmium and mercury are known carcinogens, teratogens, and mutagens (Eisler, 1985, 1987). Bioconcentration of mercury through the food chain has negative embryocidal, cytochemical, and histopathological consequences (Eisler, 1987). Due to an industrial plant's mercury discharge into a bay within Japan, people who ingested mercury-contaminated fish and shellfish suffered sensory impairment, constriction of visual fields, hearing loss, ataxia, and speech disturbances (Eisler, 1987). The major health risks of cadmium exposure in humans include kidney dysfunction, lung disease, osteoporosis, and cardiovascular hypertension (Jin et al., 1998; Staessen et al., 1996). Unlike cadmium and mercury, zinc is required for normal physiological processes, and Zn^{2+} concentrations are strictly regulated by homeostatic mechanisms. The risk of humans suffering deleterious effects from zinc

exposure is minimal. However, studies have demonstrated that exposure to high levels of zinc compounds caused respiratory and gastrointestinal toxicity in humans (Walsh et al., 1994). Even so, the ailments were reversible and limited to subjects suddenly exposed to high concentrations of zinc compounds under unlikely circumstances, such as smoke bombs and excessive consumption of zinc in therapeutic forms (Walsh et al., 1994). Like zinc, copper is also essential to normal physiological functions and homeostatically regulated. Although copper toxicity is infrequent in humans, the ingestion of food or water highly contaminated with copper does have adverse physiological repercussions (Pizarro et al., 1999). Symptoms from copper ingestion include excessive salivation, epigastric hepatic failure, nausea, vomiting, and diarrhea. Severe copper poisoning may result in intravascular hemolytic anemia, acute hepatic failure, acute tubular renal failure, shock, coma, and death (Pizarro et al., 1999).

V. Metal Detoxification Mechanisms

Organisms possess cellular, molecular, and physiological mechanisms that regulate and detoxify heavy metals circulating in the blood (Ahearn et al., 2004). These cellular detoxification pathways play an integral role in the prevention of physiological impairments caused by environmental exposure to high concentrations of heavy metals. The defense mechanisms used by crayfish to reduce excess heavy metals circulating in the hemolymph include: (1) physiological regulatory mechanisms used to balance metal uptake rates with excretion rates, (2) intracellular sequestration of heavy metals by high affinity metal binding ligands such as metallothionein, and (3) intracellular accumulation of metals into vacuoles containing concretions made up of insoluble metallic sulfur or phosphorous granules (Ahearn et al., 2004). Once a potentially toxic heavy metal

complexes with an anion the concretion is extruded from the cell by exocytosis and excreted back into the environment (Ahearn et al., 2004). Metallothionein-bound metals accumulate in lysosomes where the proteins are degraded and the freed metals are eliminated by exocytosis (Ahearn et al., 2004).

Crustaceans use the aforementioned detoxification mechanisms to counteract risks associated with sublethal metal exposure, and organisms don't show a preference for one detoxification pathway over another (Ahearn et al., 2004). However, it has been established that both essential and nonessential metals are stored in the body as granules for long periods of time, whereas metallothionein is the predominant regulatory mechanism employed for short term storage (Wallace et al., 2000).

VI. Metallothionein as a Biomarker for Metal Exposure

An aquatic ecosystem's status is often monitored by identifying and measuring the contaminants present in the environment. Although traditional sediment and water analysis methods are well developed, these measurements do not provide direct information about the biota's response to potentially toxic contaminants (Downs et al., 2001). As a result the fitness of the ecosystem already may be compromised and the damage may be irreparable by the time contaminant concentrations reach maximum accepted levels. To secure the health of the environment it is necessary to employ suitable early detection systems which monitor the physiological responses of organisms exposed to anthropogenic activities.

In respect to metal contamination metallothionein has proven to be an invaluable tool for monitoring the effects of metal influxes into the environment. Several works, including both laboratory and field studies have established a dose dependent relationship

between metallothionein and trace metal exposure. For an indicator to qualify as a biomarker its levels must reflect the health status of the organism. Although studies addressing this criterion have been limited results have established a link between metallothionein levels and an animal's fitness. For example, research conducted by Brown and Parson (1978) revealed a correlation between reduced growth rates of fish and metallothionein saturation with mercury. In addition, oysters suffered diminished oxygen consumption upon saturation of metallothionein with cadmium (Engel and Fowler, 1979).

An organism's fitness becomes threatened when metal accumulation exceeds metallothionein production (Hyne and Maher, 2001). When the available metallothionein becomes saturated the excess accumulated metal is capable of interacting with susceptible enzymes (Hyne and Maher, 2001). As a result, cellular toxicities ensue and physiological processes such as reproduction and growth become compromised (Couillard and St-Cyr, 1997). These "spillover" effects caused by excess metal accumulation are preceded by increased metallothionein levels (Hyne and Maher, 2001). Therefore, MT measurements provide considerably more predictive information about health impairments than does tissue metal analysis.

Although the early warning capacity of metallothionein enhances its utility as a biomarker for metal pollution, most techniques employed to quantify metallothionein are both elaborate and expensive. The present study addresses the procedural disadvantages associated with metallothionein. The quantification method developed for this study is both time efficient and cost effective and further enhances metallothionein as a candidate for use as a biomarker of metal pollution.

For the present study crayfish were collected from four different aquatic ecosystems located in the Central Upper Peninsula of Michigan. Crayfish are ideal biomonitors for metal pollution because of the essential role they play in the biogeochemical cycling of metals (Horton, 2004). Population declines shown by crayfish may signal the presence of metal influxes into the environment due to anthropogenic activities such as mining or smelting that would threaten the health status of the entire ecosystem. The omnivorous predatory habits of crayfish at multiple trophic levels enhance their role in aquatic biomonitoring especially in respect to the bioaccumulation of metals. The spread of potentially toxic metals into the food chain of terrestrial ecosystems via the ingestion of crayfish further qualifies these organisms as ideal biomonitors of metal pollution. Possible variations in metallothionein concentrations due to gender, reproductive status, molt cycle, and size were eliminated by including only adult intermolt male crayfish with a similar mass. Variability resulting from the nutritional status of crayfish was eradicated by measuring MT levels in the gills. Although the hepatopancreas is the major storage organ for metals and contains the highest concentration of metallothionein (Ramo et al., 1989), the MT levels in this organ reflect metal intake via diet and sediment (Brouwer et al., 1995). When crayfish are exposed to metals in their water, metals accumulate in the gills, and the MT extracted from gill tissue is linked to metals in the water (Brouwer et al., 1995).

Chapter Two: Methods

I. Specimen Collection

Adult male intermolt specimens of the crayfish *Orconectes propinquus* (Girard, 1852) were collected from Dead River (Forestville Road), Deer Lake (Ishpeming), East Branch of the Escanaba River (Gwinn), and Laughing Whitefish River (Deerton) in Marquette County, Northern Michigan. Crayfish were collected from the rivers in the last two weeks of August using commercial fish tank nets. Crayfish were collected from Deer Lake using standard wire funnel Gee minnow traps made of 5 mm galvanized wire and steel mesh traps (41.5 cm long) baited with sausage.

Specimens were dried with a paper towel and immediately weighed on a field balance and the elapsed time between capture and sacrifice was recorded. Specimens were sacrificed on site by submerging the crayfish in a dewar of liquid nitrogen for 10-30 seconds or until the animals stopped moving. To maintain the integrity of the protein frozen specimens were transported on dry ice and stored at -70 ºC until the time of dissection. Specimens were identified using the keys provided by Hobbs and Jass (1988) and Gunderson (2002).

II. Water Collection and Analysis

The grab method was used to obtain water samples from each site on the last day of specimen collection. Water samples were stored in Nalgene HDPE bottles and acidified to a concentration of 1% HNO_3 to prevent metals from leaching out, precipitating, or adhering to the container. Because cadmium, copper, and zinc contents in the water samples were below the detection limits of the graphite furnace atomic absorption

spectrometer, 45 to 50 milliliters of sample were evaporated and subsequently reconstituted to 2 ml with 2% HNO_3. Three empty 50 ml disposable centrifuge tubes served as blanks and were run through the same process as the collected water samples to ensure that any metal present could be attributed solely to the collected water. Copper and zinc were measured by Flame Atomic Absorption Spectrometry (Perkin Elmer Model 5100 PC) and cadmium content was determined using Graphite Furnace Atomic Absorption Spectroscopy (Perkin Elmer Model AAnalyst 300 HGA 800). Mercury content of the unconcentrated water samples was determined using an Inductively Coupled Plasma Mass Spectrometer (Elan 6000 DRC) which had a detection limit of 0.2 ppt and a 0.6 ppt limit of quantitation. In order to prevent the Hg from sticking to the container, all samples and standards contained 1 ppm of Au^{1+} which acted as an amalgam by binding to the mercury and keeping it in solution.

III. Metallothionein Induction

Metallothionein was induced in crayfish purchased from Waubun Laboratories by injecting them on the ventral side of the abdomen at the third pleomere each day for twelve days with 10 µl of 11.59 mM $CdCl_2$•2 ½ H_2O. On the thirteenth day, the animals were sacrificed in liquid nitrogen and stored at -70 °C.

IV. Molecular Weight Comparison of Rabbit and Crayfish Metallothionein

Because purified crayfish metallothionein was not commercially available, purified Zn-MT from rabbit kidney was used as a reference during method development. The isoform 1 Zn-MT (~4.0 µM) was provided by Dr. John Ejnik (Northern Michigan University). Rabbit MT-1 and hepatopancreatic cytosol from an induced crayfish were applied to a Sephadex G-75 column to compare the volumes at which metallothionein

from each specimen would elute. Hydrated Sephadex G-75, which separates globular proteins between 1,000 and 50,000 MW, was packed into a Kontes Flex Column (1 cm X 120 cm) and subsequently equilibrated with elution buffer (5 mM Tris, pH 7.5, 2 mM β-mercaptoethanol) for 3 hours at 4 °C. To prevent oxidation of metallothionein, the elution buffer was degassed for 30 minutes and saturated with nitrogen gas for 15 minutes prior to equilibrating the column. Next, one ml of rabbit MT-1 (~4.0 µM) and 20 µl of Cd (1000 ppm) were mixed together for 1 minute and loaded onto the column. A total of one hundred 1 ml fractions were collected and cadmium content measured by Flame Atomic Absorption Spectroscopy (Perkin Elmer Model 5100 PC). The hepatopancreas (0.57424 g) from a crayfish injected with cadmium was excised, weighed, and crushed in liquid nitrogen using a mortar and pestle before transfer to a 1.5 ml microcentrifuge tube. Next, 1 ml of tissue prep buffer (10 mM Tris, pH7.5, 5 mM ß-mercaptoethanol) and 20 µl of Cd (1000 ppm) was added to the crushed tissue and the solution was vortexed for 1 minute (Fisher Scientific Model 232) and centrifuged for 1 hour (Sorvall RC5B Plus, F-20 Micro rotor) at 51,427 g and 4˚C. The supernatant (~0.5 ml) was transferred to a clean microcentrifuge tube and applied to a Sephadex G-75 Kontes Flex Column (120 cm X 1 cm) which had been washed for 3 hours with elution buffer to remove residual free metals and low molecular weight molecules. Forty nine fractions were eluted with 5 mM Tris, pH 7.5, 2 mM ß-mercaptoethanol at 4 °C. To minimize time and cost of analysis, the fraction volume was increased to two milliliters. Cadmium content in the fractions was determined using Flame Atomic Absorption Spectroscopy (Perkin Elmer Model 5100 PC).

V. Effects of Chelex

Hydrated Sephadex G-15 was packed into a Kontes Flex Column (50 cm X 1.5 cm) and equilibrated with elution buffer (5 mM Tris, pH 7.5, 2 mM β-mercaptoethanol) for 3 hours at 4 °C. To prevent oxidation of metallothionein, the elution buffer was degassed for 30 minutes and saturated with nitrogen for 15 minutes prior to equilibration and elution of the protein. After mixing 1ml of rabbit MT-1 (~4.0 µM) with 20 µl cadmium (1000 ppm), the protein was applied to the column and forty nine 2 ml fractions were collected. The column was washed for 3 hours with elution buffer to remove residual metal and low molecular weight molecules. Next, one ml of rabbit MT-1 (~4.0 µM) was combined with 20 µl of cadmium (1000 ppm) and ~0.1 g of Chelex 100. After vortexing the solution for 1 minute and centrifuging (Fisher Scientific Marathon MicroA) for 15 minutes at room temperature and 16,060 g, the supernatant (~0.8 ml) was decanted and applied to the Sephadex G-15 column. To achieve better peak resolution, the fraction volume was decreased to one milliliter. Cadmium content of all fifty one fractions was determined using Flame Atomic Absorption Spectroscopy (Perkin Elmer Model 5100 PC).

VI. Metal Displacement

The zinc-MT-2 (5.37 µM) used in the metal displacement experiment was provided by Dr. John Ejnik (Northern Michigan University). Rabbit MT-2 (12.5 ml), containing 10 mM β-mercaptoethanol, was combined with 37.5 ml of column buffer (5 mM Tris, pH 7.7, 2 mM β-mercaptoethanol). After adding Chelex 100 (0.1 g/ml solution), the mixture was centrifuged (International Equipment Co. Model HN) at room temperature and

1150 g for 15 minutes. Once the Chelex 100 settled, 10 ml of the supernatant was transferred to a clean 50 ml disposable centrifuge tube. After determining the zinc concentration by Flame-AAS, the zinc-MT was stored at 4 °C. The remaining 30 ml of supernatant was transferred to a clean tube and a twenty fold excess of Cu^{1+} was added to the zinc-MT. After vortexing the solution for 1 minute, 0.3 g of Chelex 100 was added to the solution. Once again, the solution was vortexed for 1 minute and centrifuged at room temperature and 1150 g for 15 minutes. Fifteen milliliters of the copper-MT was stored at 4 °C until analysis. Cadmium-MT was prepared by combining 1000 ppm Cd^{2+} (20 µl/ml solution) and 15 ml of copper-MT. After vortexing the solution for 1 minute, the protein was heat shocked at 95 °C for 4 minutes. Excess metal was removed by adding Chelex 100 (0.1g/ml solution), vortexing for 1 minute, and centrifuging (Fisher Scientific Marathon Micro A) for 15 minutes at room temperature and 16,060 g. Once the chelex settled, the supernatant was transferred to a 50 ml centrifuge tube. After determining the zinc, copper, and cadmium content of all three types of metallothionein, the copper metallothionein (7 ml) remaining after analysis was chelexed twice with 0.7 g of Chelex 100 and converted to cadmium-MT following the same procedure used earlier in the experiment. When the Chelex 100 settled, the supernatant was removed. Copper and cadmium content of the Cd-MT was measured by Flame-AAS.

VII. Tissue Preparation of Field Specimens

Gills were excised from crayfish and the tissue's wet weight was recorded. Tissue weighing between 0.0687 and 0.3169 grams was pulverized into a fine powder in a mortar with liquid nitrogen. Crushed tissue was transferred to a 1.5 ml microcentrifuge tube and combined with 500 µl of tissue prep buffer (10 mM Tris, pH 7.5, 5 mM

β-mercaptoethanol) and 10 µl of cadmium (1000 ppm). Solutions were vortexed (Fisher Scientific Model 232) for 1 minute and subjected to centrifugation (Sorvall RC5B Plus, F-20 Micro rotor) for 15 minutes at 51,427 g and 4 ˚C. The supernatant was transferred to a clean microcentrifuge tube and heat shocked for approximately 2 to 3 minutes at 95 ˚C in a dry bath (Thermodyne, Type 17600) to denature all heat unstable proteins. The heat-treated gill cytosol was centrifuged (Fisher Scientific Marathon Micro A) at room temperature and 16,060 g for 10 minutes to remove precipitated protein. The supernatant was then combined with approximately 50 to 70 mg Chelex 100, vortexed for 1 minute, and centrifuged (Fisher Scientific Marathon Micro A) for 15 minutes at room temperature and 16,060 g to remove excess free metal and loosely protein bound cadmium. Cytosol (~ 0.5 ml) was either immediately applied to a G-15 Sephadex column or stored at 4 ˚C until it could be loaded onto the desalting columns. Ten crayfish were processed per site with the exception of Dead River which had a sample size of 11.

VIII. Sephadex G-25/G-75 Chromatographic Isolation of Metallothionein

Different sizes and combinations of Sephadex (G-75, G-25/G-75, G-15) were used during method development to maximize peak separation and minimize run time. A combination of Sephadex G-25 and G-75 was used to isolate metallothionein from an induced crayfish. Hydrated Sephadex G-75 was poured into a Kontes Flex Column (120 cm x 1 cm) to a height of 62 cm. The top half of the column was filled with Sephadex G-25. The column buffer, 5 mM Tris, pH 7.5, 2 mM β –mercaptoethanol, was degassed for 30 minutes and saturated with nitrogen for 15 minutes to prevent oxidation of metallothionein. The Sephadex was washed for 3 hours with column buffer prior to application of the samples. Rabbit MT-1 (4.0 µM) was used to calibrate the column.

Rabbit MT-1 (750 µl) was mixed with 750 µl of tissue prep buffer (10 mM Tris, pH 7.5, 5 mM β-mercaptoethanol) and 20 µl of cadmium (1000 ppm). After vortexing the solution for 1 minute, ~0.75 ml of the prep was applied to the column. One ml of eluate was collected per fraction. Flame Atomic Absorption Spectroscopy was used to determine cadmium concentration of the 79 fractions. The hepatopancreas was excised from an induced crayfish. The tissue, weighing 0.45835, was processed following the same procedure used for the field specimens. The resulting 1.0 ml of supernatant was applied to the Sephadex G-25/G-75 column after it had been washed for 3 hours with elution buffer. Thirty seven 2 ml fractions were collected. Cadmium concentration of the fractions was measured using Flame Atomic Absorption Spectroscopy. A UV Spectrophotometer (Shimadzu Model UV-160U**)** was used to measure absorbance of each fraction at 250 nm**.** The instrument was blanked with 5 mM Tris, pH 7.5, 2 mM β – mercaptoethanol prior to measuring the fractions.

IX. Sephadex G-15 Chromatographic Isolation of Metallothionein

Fine Sephadex G-15, which had been hydrated and heated on low for 15 minutes, was poured into four Kontes Flex Columns (50 cm X 1 cm) to equal heights. The Sephadex G-15 was washed with 5 mM Tris, pH 7.5, 2 mM β -mercaptoethanol to equilibrate the columns. To prevent oxidation of metallothionein the elution buffer was degassed for 30 minutes and saturated with nitrogen for 15 minutes.

The Sephadex G-15 (desalting) columns were calibrated with rabbit MT-1 (~4.0 µM) to determine which fractions would contain metallothionein from the gill cytosol of crayfish. The rabbit MT-1 sample was prepared in the same fashion as that previously used in the experiment investigating the effects of Chelex 100. After loading 0.8 ml of

rabbit MT-1 onto the column, one milliliter fractions were collected and cadmium content was measured using Flame Atomic Absorption Spectroscopy.

The gill cytosol (~ 0.5 ml) extracted from the field specimens was run through a desalting column to separate metallothionein from low molecular weight molecules and free metals. A minimum of eighteen fractions, approximately 2.0 ml in volume, were collected. In order to calculate moles of cadmium bound to metallothionein, the exact volume of the fractions was determined by transferring contents from a test tube into a 10 ml graduated cylinder. In between each application of cytosol, columns were washed for a minimum of 3 hours to remove any residual free metal and low molecular weight molecules. Analysis of the fractions coming off the column prior to metallothionein validated the absence of cadmium in the elution buffers and any remaining cadmium measured in the fractions containing metallothionein could be solely attributed to the samples applied to the column. Cadmium content of the collected fractions was measured using Graphite Furnace Atomic Absorption Spectroscopy (Perkin Elmer AAnalyst 300 HGA 800).

X. Determination of Sulfhydryl:Metal Ratio

Gills were excised from three of the crayfish injected with cadmium. The combined tissue weighing 5.1166 g was crushed in liquid nitrogen with a mortar and pestle. The pulverized tissue was divided into four portions and placed in 1.5 ml microcentrifuge tubes. After adding 1 ml of tissue prep buffer (10 mM Tris, pH 7.53, 5 mM β-mercaptoethanol) and 20 µl cadmium (1000 ppm), the tubes were vortexed for 1 minute and centrifuged (Sorvall RC5B Plus, F-20 Micro rotor) for 30 minutes at 51,427 g and 4 ˚C. The supernatant was transferred to clean tubes and heat shocked for 3 minutes at

95 °C in a dry bath (Thermodyne, Type 17600). The heat-treated gill cytosol was centrifuged (Fisher Scientific Model Marathon Micro A) at room temperature and 16,060 g for 10 minutes. The supernatant was then combined with approximately 90 to 110 mg Chelex 100, vortexed for 1 minute, and centrifuged (Fisher Scientific Marathon Micro A) for 15 minutes at room temperature and 16,060 g. The supernatant from each tube was transferred into a 10 ml amicon stirred ultrafiltration cell (Millipore Model 8200) containing a hydrated amicon regenerated cellulose ultrafiltration membrane (Millipore) and concentrated down to approximately 1.75 ml. The concentrated cytosol was stored overnight at 4 °C in a 1.5 ml microcentrifuge tube.

On the following day, the gill cytosol was applied to a Sephadex G-15 column which had been washed with elution buffer (5 mM Tris, pH 7.54) for three hours at 4 °C. Thirty drops of eluate were collected per fraction. The absorbance of the collected fractions at 250 nm and 220 nm was measured on an UV Spectrophotometer (Shimadzu Model UV-160U) which had been blanked with 5 mM Tris, pH 7.54. Fractions showing a high absorption at 250 nm and 220 nm were pooled, added to a 10 ml amicon stirred ultrafiltration cell (Millipore Model 8010) containing a hydrated amicon regenerated cellulose ultrafiltration membrane (Millipore), and concentrated down to 1.5 ml. The nominal molecular weight limit (NMWL) for this particular membrane was 1,000. Therefore, any molecules with a molecular weight greater than 1,000 were retained in the cell while those less than 1,000 filtered through the membrane as waste.

Concentrated gill cytosol (0.1 ml) was mixed with 0.8 ml of buffer (0.1 mM Tris, pH 7.49) and 0.1 ml of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The solution was mixed and immediately placed in a UV-Vis Spectrophotometer (Shimadzu, UV-

3101PC) which had been blanked with a solution consisting of 0.8 ml of 0.1 mM Tris, pH 7.49, 0.1 ml of column buffer (5 mM Tris, pH 7.54), and 0.1 ml of 10 mM DTNB. The reduction reaction was monitored by measuring absorption at 412 nm every 5 seconds for 60 minutes. After running the kinetic reaction ran 60 minutes an absorbance reading of the protein sample and blank was taken at 412 nm. The cadmium content of the concentrated cytosol was determined by Flame Atomic Absorption Spectroscopy (Perkin Elmer Model 5100 PC) after diluting the sample by a factor of ten with 2% nitric acid.

XI. Metal Analysis

Atomic Absorption Spectroscopy (AAS) was used to determine the metal concentration of the various samples. Whether the samples were analyzed by Graphite Furnace (Perkin Elmer AAnalyst 300 HGA-800) or Flame (Perkin Elmer Model 5100 PC) AAS was contingent upon the metal to be analyzed and its expected concentration. Graphite Furnace AAS has much lower detections limits for most metals so this method was chosen to measure the Cd^{2+} concentration in fractions from columns loaded with crayfish cytosol of the field specimens. This method was also used to determine the Cd^{2+} content of the water samples. Zinc and copper concentrations were determined exclusively by Flame Atomic Absorption Spectroscopy as were samples with a higher concentration of Cd^{2+}, such as fractions from columns loaded with rabbit MT-1, induced crayfish cytosol, and concentrated crayfish MT fractions.

Normal quality control procedures were followed for all samples analyzed by AAS in order to validate the accuracy of the results which could be affected by factors such as furnace degradation, optical misalignment, and improper injection. For example, standard curve correlation coefficients had to meet the criteria of being at least 0.99 in

order to proceed with subsequent analysis. Two duplicates were run per sample; if graphite furnace-AAS relative standard deviations were above 25%, or above 10% (flame-AAS), then samples were reanalyzed. After each series of analyses, standards were analyzed in the same fashion as samples to ensure analyte was not being lost as a result of instrument performance or the analytical method. If the experimental value of the standard didn't match the expected concentration, then results obtained during the session were considered suspect and samples were either rerun or values were adjusted to allow for any discrepancies.

The detection limit and the limit of quantitation values of the flame and graphite furnace atomic absorption spectrophotometers were determined for each of the metals by analyzing 2% nitric acid ten times in duplicate. The standard deviation of the mean concentration values for 2% nitric acid was used to calculate the detection limit and the limit of quantitation. To obtain a detection limit, the standard deviation was multiplied by 3.30. The limit of quantitation was determined by multiplying the standard deviation by 10. Table 2 shows the detection limit and the limit of quantitation values for all metals analyzed by flame and/or graphite furnace atomic absorption spectroscopy.

The Cd^{2+} concentrations in fractions 4-14 collected from columns loaded with crayfish gill cytosol were determined by Graphite Furnace Atomic Absorption Spectroscopy on a Perkin Elmer AAnalyst 300 HGA-800 containing a platform graphite furnace. The non-linear standard curve was created from 0.5, 5.0, 10.0, and 20.0 ppb cadmium standards. Solutions consisting of 10 µl sample, 15 µl diluent (2% nitric acid) and 5 µl of matrix modifier (0.4% ammonium phosphate in 2% nitric acid) were injected into the furnace at 30 °C. The temperature was increased to 120 °C over a 10 second period and held there

for 50 seconds to dry the sample. Organic material was burned away at 900 °C for 30 seconds before atomizing the sample at 2000 °C for 6 seconds. The furnace was brought to 2700 °C and held at this temperature for 5 seconds to remove any residual sample. The amount of cadmium present in the water samples was determined using Graphite Furnace Atomic Absorption Spectroscopy (Perkin Elmer AAnalyst 300 HGA 800). At the beginning of the analysis a linear curve was constructed using 0.50, 1.50, and 2.50 ppb Cd standards. Solutions comprising 30 µl of sample, 10 µl diluent (2% Nitric Acid) and 5 µl matrix modifier (0.4% ammonium phosphate in 2% nitric acid), were injected into the furnace at 30 °C. Except for holding the atomizing temperature at 2000 °C for 5 seconds (instead of 6) the furnace conditions remained the same as those used for measuring cadmium in the crayfish cytosol.

Flame-AAS was used to determine the amount Cd^{2+} present in rabbit MT-1 and cytosol of induced crayfish. The linear standard curve included 0.02, 0.04, 0.10, and 0.40 ppm Cd^{2+} standards. Zinc and copper in the water samples and rabbit MT-1 were measured using Flame Atomic Absorption Spectroscopy (Perkin Elmer Model 5100 PC). The linear standard curve used to determine copper consisted of 4 points and included 0.04, 0.10, 0.40 and 1.00 ppm copper standards. The Zn^{2+} standards, 0.05, 0.20, 0.50, 1.00 and 2.00 ppm, were used to create a linear standard curve when measuring zinc.

XII. Statistical Analysis

The Statistical Package for the Social Sciences (SPSS 13.0, SPSS Inc., Chicago, IL) was the software program used to perform all statistical analysis. Kruskal-Wallis One Way Analysis of Variance on Ranks (SPSS 13.0, SPSS Inc., Chicago, IL) was used to compare the mean metallothionein concentrations (µmole MT/g gills) among the four

groups of crayfish. Differences in means were considered statistically significant if $p < 0.05$. Dunn's Method was used to detect the pairwise comparisons showing significant difference in metallothionein concentration. Once again the level of significance was set to 0.05. Stepwise linear regression (SPSS 13.0, SPSS Inc., Chicago, IL) was performed to investigate any correlations that existed between the dependent variable, metallothionein concentration, and individual heavy metals (Zn^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+}), total metal content, sites, mass, and various combinations of these variables. Correlations were considered significant at a 95% confidence level.

Chapter Three: Results

I. Specimen Collection

In order to establish a correlation between metal exposure and metallothionein concentration, variation in specimens was minimized by collecting adult intermoult male crayfish of the same species. With the exception of Deer Lake specimens, deviation in mass was kept to a minimum by selecting crayfish between 3-5.5 g. Because of the collection method and sparse population of crayfish at Deer Lake, variation in mass was greater for specimens collected at this site. Stress was kept to a minimum by limiting the time crayfish were exposed to air. Table 3 contains information about the specimens used in this study.

The cadmium saturation method was employed to quantify the concentration of metallothionein found in the gills of crayfish. The protein was indirectly quantified by measuring the concentration of metallothionein-bound Cd^{2+} present in the samples. Because all aspects of this research were embedded in metal analysis by atomic absorption spectroscopy, parameters were set so the validity of the results could be assessed. Table 2 includes the detection limit and limit of quantitation values for each metal measured on the Flame AAS, Graphite Furnace AAS, and Inductively Coupled Plasma Mass Spectroscopy (ICP-MS).

Table 2: Detection limits and limit of quantitation values for metals measured by atomic emission spectroscopy (Hg), and by atomic absorption spectroscopy (Zn, Cd, Cu).

	Zn (ppm)	Cu (ppm)	Cd (ppm)	Cd (ppb)	Hg (ppt)
Standard Deviation	0.0006742	0.00052	0.0380088	0.01275	0.0606
Detection Limit	0.00222486	0.001717	0.1254292	0.04209	0.2
Limit of Quantitation	0.006741999	0.005203	0.3800884	0.12754	0.61

Table 3: Species, gender, mass, and elapsed time from capture to sacrifice for specimens of *Orconectes propinquus*.

Site	Specimen ID	Gender	Species	Mass (g)	Elapsed Time (seconds)
Dead River	DR 1	Male	*Op*	3.7	53
Dead River	DR 2	Male	*Op*	4.2	62
Dead River	DR 3	Male	*Op*	3.6	57
Dead River	DR 4	Male	*Op*	3.4	64
Dead River	DR 5	Male	*Op*	3.1	41
Dead River	DR 6	Male	*Op*	3.0	62
Dead River	DR 7	Male	*Op*	4.9	58
Dead River	DR 8	Male	*Op*	5.5	66
Dead River	DR 9	Male	*Op*	3.9	65
Dead River	DR 10	Male	*Op*	4.6	53
Dead River	DR 12	Male	*Op*	3.4	48
Deer Lake	DL 1	Male	*Op*	10.1	N/A
Deer Lake	DL 2	Male	*Op*	4.7	N/A
Deer Lake	DL 4	Male	*Op*	9.0	N/A
Deer Lake	DL 5	Male	*Op*	3.9	N/A
Deer Lake	DL 6	Male	*Op*	4.4	N/A
Deer Lake	DL 8	Male	*Op*	3.3	N/A
Deer Lake	DL 10	Male	*Op*	5.3	N/A
Deer Lake	DL 11	Male	*Op*	10.4	N/A
Deer Lake	DL 12	Male	*Op*	9.5	N/A
Deer Lake	DL 13	Male	*Op*	7.7	N/A
East Branch Escanaba River	EBER 1	Male	*op*	3.6	64
East Branch Escanaba River	EBER 5	Male	*op*	3.4	56
East Branch Escanaba River	EBER 6	Male	*op*	4.2	22
East Branch Escanaba River	EBER 7	Male	*Op*	4.8	19
East Branch Escanaba River	EBER 8	Male	*Op*	4.3	60
East Branch Escanaba River	EBER 10	Male	*Op*	3.2	61
East Branch Escanaba River	EBER 11	Male	*Op*	5.4	50
East Branch Escanaba River	EBER 17	Male	*Op*	3.1	52
East Branch Escanaba River	EBER 18	Male	*Op*	3.4	43
East Branch Escanaba River	EBER 19	Male	*Op*	4.6	38
Laughing Whitefish River	LWR 1	Male	*Op*	5.0	75
Laughing Whitefish River	LWR 3	Male	*Op*	4.6	25
Laughing Whitefish River	LWR 7	Male	*Op*	4.5	40
Laughing Whitefish River	LWR 9	Male	*Op*	4.7	46
Laughing Whitefish River	LWR 10	Male	*Op*	5.9	46
Laughing Whitefish River	LWR 14	Male	*Op*	3.6	47
Laughing Whitefish River	LWR 16	Male	*Op*	3.4	50
Laughing Whitefish River	LWR 17	Male	*Op*	4.7	40
Laughing Whitefish River	LWR 18	Male	*Op*	3.1	60
Laughing Whitefish River	LWR 21	Male	*Op*	5.0	78

II. Molecular Weight Comparison of Rabbit and Crayfish Metallothionein

Before quantifying metallothionein in crayfish collected from the field, preliminary experiments were conducted to validate chromatographic methods, to investigate the effects of heat shock and Chelex 100 on samples, and to characterize metallothionein. To obtain high concentrations of metallothionein crayfish were injected with cadmium to induce MT synthesis in the hepatopancreas, and this organ was used in the preliminary experiments. The first experiment was conducted to compare the molecular weight of MT-1 (~4.0 µM) from rabbit kidney to MT found in the hepatopancreas of crayfish. Purified rabbit MT-1 (~4.0 µM) and hepatopancreatic crayfish cytosol were individually applied to a Sephadex G-75 column, which separates globular proteins between 1,000 and 50,000 MW. If the retention time of the crayfish MT matched rabbit MT-1, it was assumed that the molecular weights of the proteins were similar, and that rabbit MT-1 (~4.0 µM) could be used to calibrate the Sephadex G-15 columns used in subsequent size exclusion experiments. Upon applying the purified rabbit MT-1 (~4.0 µM) to the column, fractions of eluate with a volume of 1 ml were collected. Determination of the amount of cadmium present in each fraction allowed an elution profile of cadmium binding molecules and free metal to be constructed by plotting elution volume versus cadmium concentration. The elution profiles of both rabbit MT-1 and crayfish MT are illustrated in Figure 2 and Figure 3, respectively.

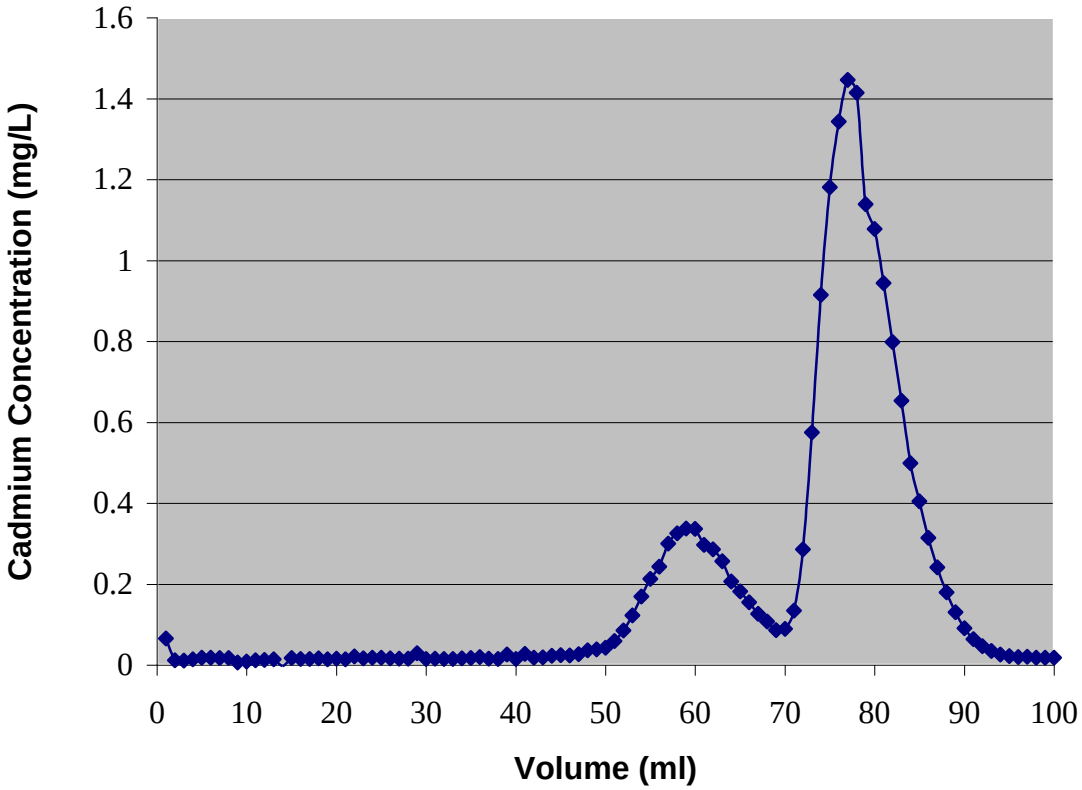


Figure 2: Elution profile for the size chromatographic separation (Sephadex G-75) of metallothionein-bound cadmium and free metal found in 1 ml of rabbit MT-1 (~4.0 µM).

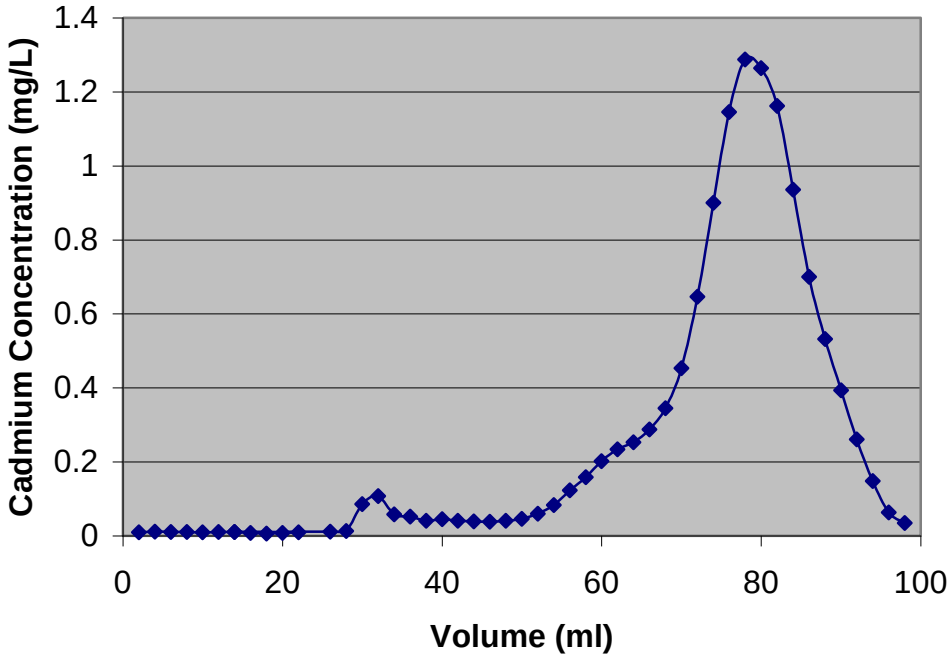


Figure 3: Elution profile for the size chromatographic separation (Sephadex G-75) of cadmium bound molecules and free metal found in hepatopancreatic cytosol (1 ml) of an induced crayfish.

Gel-filtration chromatography (size exclusion chromatography) was used to determine the presence of MT and metals in a sample. This method takes advantage of the principle that components of a mixture elute from a column according to size, and that higher molecular weight molecules come off the column first. In the present work high molecular weight proteins eluted first, followed by metallothionein, and by free metals. Application of purified rabbit metallothionein-1 (~4.0 µM), which had been spiked with cadmium, resulted in an elution profile (Figure 2) consisting of two peaks, one starting at 47 and the other at 70 milliliters which represented metallothionein-bound cadmium and free metals, respectively. The column loaded with hepatopancreatic cytosol from an induced crayfish (Figure 3) produced a slightly different profile. In this graph, an

additional peak, between 28-44 ml, signified cadmium bound to high molecular weight molecules, and unlike the profile for rabbit MT-1, the free metals peak (66-98 ml) in Figure 3 is somewhat unresolved. The shoulder (48-64 ml) on the left side of the free metals peak represents hepatopancreatic metallothionein.

III. Effects of Chelex

The weak cationic chelating resin, Chelex 100 is commonly used to sequester free and loosely bound metals present in a solution in studies which implement metal saturation techniques to quantify metallothionein. Here, removal of loosely bound Cd^{2+} from crayfish cytosol by the negatively-charged Chelex 100 ensured that the cadmium present in the eluted column fractions was bound exclusively to metallothionein. Since the metallothionein present in gill cytosol of crayfish is indirectly quantified via the presence of cadmium, it is pertinent that Chelex 100 does not remove any metal bound to MT. To investigate whether or not addition of the chelating agent affects the metallothionein-bound Cd^{2+}, untreated and Chelex 100 treated rabbit-MT (~4.0 μM) was applied to a Sephadex G-15 column (50 cm X 1.5 cm). Figures 4 and 5 depict the elution profiles of the two samples.

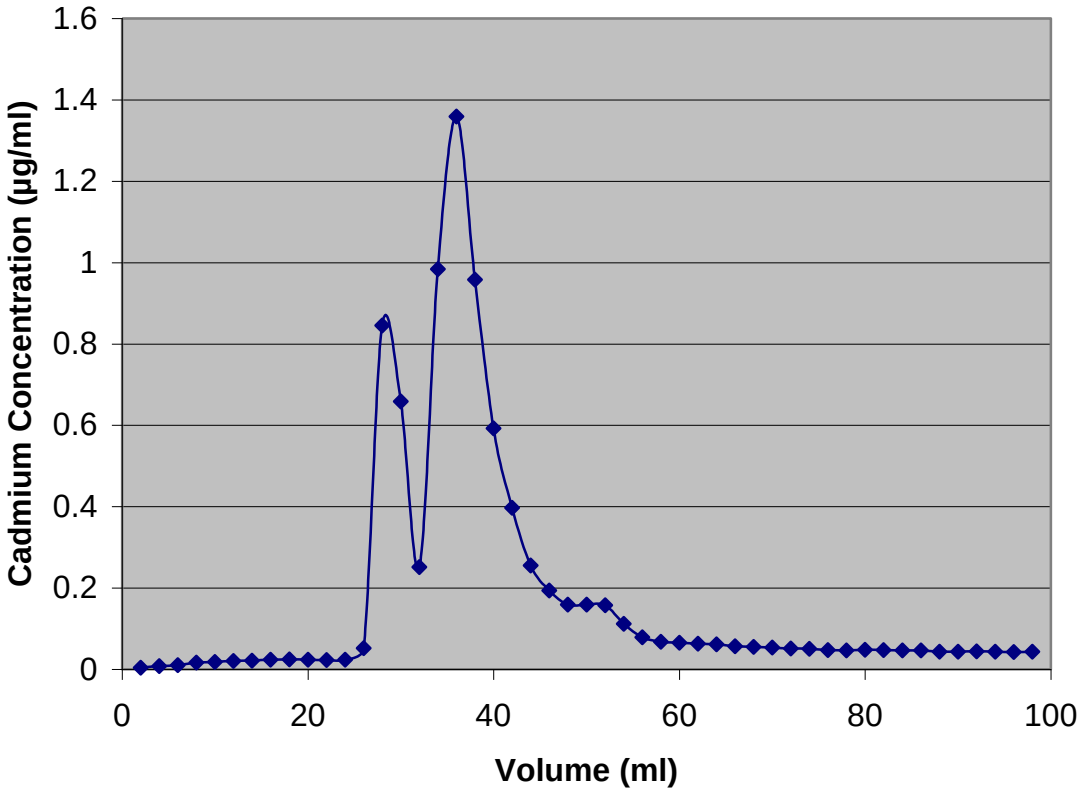


Figure 4: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in 1 ml of rabbit MT-1 (~4.0 µM) spiked with cadmium.

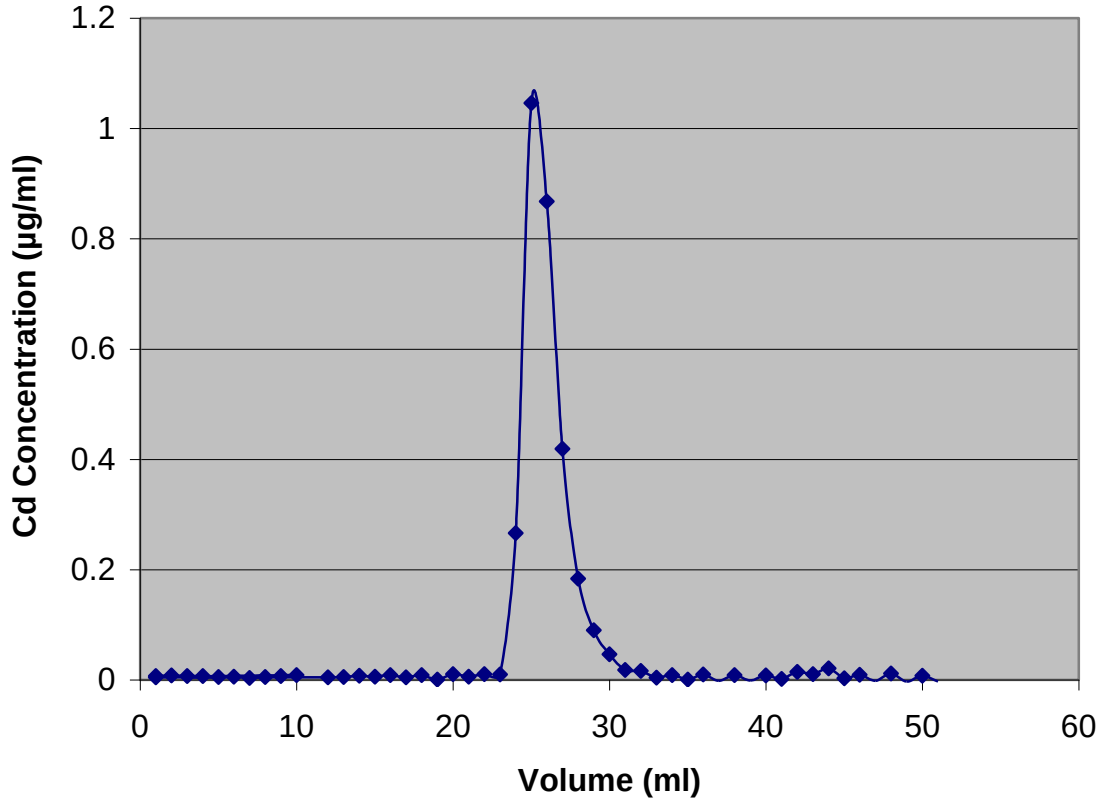


Figure 5: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in 0.8 ml of rabbit MT-1 (~4.0 µM) spiked with cadmium and treated with Chelex-100.

Application of the untreated rabbit MT-1 (1 ml) to the Sephadex G-15 column resulted in the separation of the protein from free metal (Figure 4). The metallothionein-bound cadmium began eluting at 24 milliliters; the peak to the right, beginning at 32 milliliters, represented free cadmium. Figure 5 depicts the effect of treating rabbit MT-1 with Chelex 100. In this elution profile, only the peak (23-32 ml) corresponding to cadmium bound to rabbit MT-1 is present.

Table 4: The concentration of Cd^{2+} measured in metallothionein fractions collected from a Sephadex G-15 column loaded with 1 ml of rabbit MT-1 (~4.0 µM) spiked with 20 µl of Cd^{2+} (1000 ppm).

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (µg/ml)
12	0.0239
13	0.0518
14	0.8451
15	0.6588
16	0.2514
Total Concentration Of Cadmium In All Fractions (µg/ml)	**1.831**
Total Mass Of Cadmium In All Fractions (µg)	**3.662**

Table 4 lists the fractions that contained rabbit MT-1 and their corresponding cadmium concentrations, but before chelexation with Chelex 100. The total concentration of cadmium in fractions 12-16 (Figure 4) was 1.831 µg/ml. To determine the total mass of Cd^{2+} within the fractions, the total concentration, 1.831 µg/ml, was multiplied by the fraction volume (2 ml). As a result of loading 1 ml of untreated rabbit MT-1 onto a Sephadex G-15 column, 3.662 µg of Cd^{2+} was recovered. The calculation below was used to determine the µmoles of rabbit MT-1 contained in fractions 12-16. Based on this calculation, a total of 0.00465 µmoles of rabbit MT-1 eluted from the column loaded with 1 ml of protein.

µmoles MT = µg Cd x (1 g/10^6 µg) x (1 mole Cd/112.4g) x (1 mole MT/7 moles Cd) x (10^6 µmoles/mole)

Table 5: The concentration of Cd^{2+} measured in metallothionein fractions collected from a Sephadex G-15 column loaded with 0.8 ml of rabbit MT-1 (~4.0) spiked with 20 µl of Cd^{2+} (1000 ppm) and treated with Chelex-100.

Fraction # (1.00 ml per fraction)	**Cadmium Concentration** (µg/ml)
23	0.0098
24	0.2663
25	1.046
26	0.8675
27	0.4187
28	0.1836
29	0.09
30	0.0463
31	0.0182
32	0.016
Total Concentration Of Cadmium In All Fractions (µg/ml)	**2.962**
Total Mass Of Cadmium In All Fractions (µg)	**2.962**

Table 5 lists the fractions that contained chelexed rabbit MT-1 and their corresponding cadmium concentrations. The peak in Figure 5 illustrates that metallothionein eluted in fractions 23-32. The total concentration of cadmium in these fractions was 2.962 µg/ml. Based on a fraction volume of 1 ml, the total mass of Cd^{2+}present in the fractions is 2.962 µg. According to the above calculation, 0.00377 µmoles of rabbit MT-1 was recovered when 0.80 ml of chelexed rabbit MT-1 was applied to a Sephadex G-15 column.

Chelex 100 is used to sequester free and loosely bound metals present in solution. Before this reagent could be incorporated into the sample prep it had to be experimentally proven that the chelating agent would not remove any metal bound to metallothionein. If

Chelex 100 does not interfere with Cd^{2+} bound to metallothionein, then the moles of rabbit MT-1 recovered, relative to the volume loaded onto the column, should be the same for the untreated and chelexed samples. Because 0.00465 µmoles of rabbit MT-1 was recovered when the column was loaded with 1 ml of untreated protein, application of 0.80 ml of the Chelex 100 treated protein theoretically should yield 0.00372 µmoles of rabbit MT-1 (0.00465 µmoles x 0.80). As previously mentioned, 0.00377 µmoles of rabbit MT-1 eluted from the column loaded with 0.80 ml of the treated protein sample.

IV. Metal Displacement

Indirect quantification of crayfish metallothionein by the cadmium saturation method relies on the principle that all metals, more specifically copper, bound to metallothionein will be displaced when the protein is exposed to an excess of Cd^{2+} at high temperatures. To test this conjecture, zinc-MT-2 from rabbit liver (provided by Dr. John Ejnik, NMU) was converted to copper-MT by infusing the protein solution with an excess amount of Cu^{1+}. Converting copper metallothionein to cadmium-MT was achieved by adding an excess of cadmium metal to copper-MT, heat shocking, and treating the supernatant with Chelex 100. When all three types of MT had been prepared, zinc, copper, and cadmium measurements were taken on the protein samples. Table 6 summarizes the results of the displacement reactions.

Table 6: Zinc, copper, and cadmium concentration measurements taken on Zn-MT-2, Cu-MT-2, Cd-MT-2 (A) (two Chelex 100 treatments), and Cd-MT-2 (B) (three Chelex 100 treatments).

Protein	Actual Metal Concentrations			Expected Metal Concentrations		
	Zn (ppm)	Cu (ppm)	Cd (ppm)	Zn (ppm)	Cu (ppm)	Cd (ppm)
Zn-MT-2	0.614	0.0605	0.0124	Reference	0	0
Cu-MT-2	0.086	8.052	0.0063	0	0.5967 (min) 1.705 (max)	0
Cd-MT-2 (A)	0	1.06	0.4278	0	0	1.056
Cd-MT-2 (B)	N/A	0.0873	1.27	0	0	1.27

The Zn-MT-2 solution contained 0.614 ppm Zn^{2+}. Taking into consideration that one mole of MT-2 binds 7 moles of zinc (Guo et al., 2005), this value translates into a 1.34 µM of rabbit MT-2. This value was used as a reference to calculate the ppm values expected for both copper and cadmium metallothionein. Stoichiometry experiments conducted by Chen et al. (1996) revealed that MT-2 from rabbit liver can bind 7-20 moles of copper. Depending on how many moles of copper bind to the protein, measurements taken on 1.34 µM copper-MT-2 should range from 0.5967-1.705 ppm. According to Guo et al. (2005), rabbit metallothionein-2a binds 7 moles of Cd^{2+}. If the 1.34 µM of copper-MT-2 is completely converted to Cd_7-MT-2, then 1.056 ppm of Cd^{2+} should be present in solution. Assuming all metals not bound to metallothionein were chelated out of solution readings of 0 ppm would be expected for cadmium and copper when zinc-metallathionein was measured. Similarly, cadmium-MT should be void of copper and zinc and measurements taken on Cu-MT should be zero except for copper. The following calculations were employed to determine the expected metal concentrations for Cd_7-MT-2, Cu_7-MT-2, and Cu_{20}-MT-2.

Zn_7-MT-2 (M) = (0.614 mg/L) x (1 g/1000 mg) x (1 mole Zn^{2+}/65.39 g) x (1 mole MT/7 mole Zn^{2+})

Cu (ppm) = (Zn_7-MT-2 M) x (moles Cu^{1+}/moles MT) x (63.546 g Cu^{1+}/mole) x (1000 mg/1 g)

Cd (ppm) = (Zn_7-MT-2 M) x (moles Cd^{2+}/moles MT) x (112.41 g Cd^{2+}/mole) x (1000 mg/1 g)

The results of this experiment show that cadmium can displace copper from metallothionein under appropriate treatment conditions.

V. Isolation and Quantification of Metallothionein from Field Specimens

Before isolating metallothionein from the gills of the crayfish collected from the field sites, the Sephadex G-15 (desalting) columns were calibrated with rabbit MT-1 (~4.0 µM) to determine the volume at which metallothionein-bound cadmium would begin eluting.

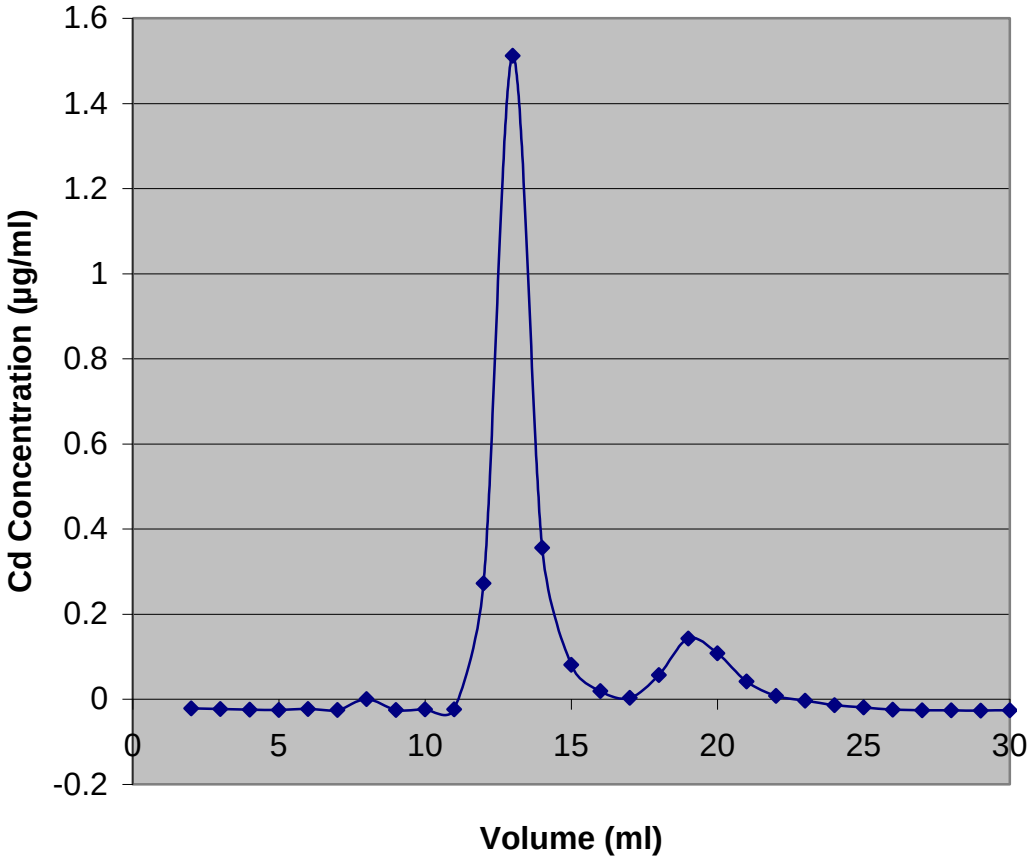


Figure 6: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in 0.8 ml of rabbit MT-1 (~4.0 µM) spiked with cadmium and treated with Chelex 100.

The first peak (12-16 ml) represents rabbit MT-1, whereas the fractions immediately following (17-22 ml) contain free Cd^{2+} (Figure 6). Based on this elution profile MT from crayfish gills should begin eluting in fraction #6 when collecting 2 milliliters of eluate per fraction. For the purposes of conserving time and reagents, only fractions 4-14 were analyzed since cadmium bound to MT didn't begin eluting until a volume of 12 ml was eluted. Figure 7 is an example Cd^{2+} elution profile for the chromatographic separation of metallothionein-bound cadmium and free metal found in one of the gill cytosol

preparations excised from a crayfish collected from Laughing Whitefish River. Appendix A (Figures A1-A45) contains the graphs for the remaining specimens.

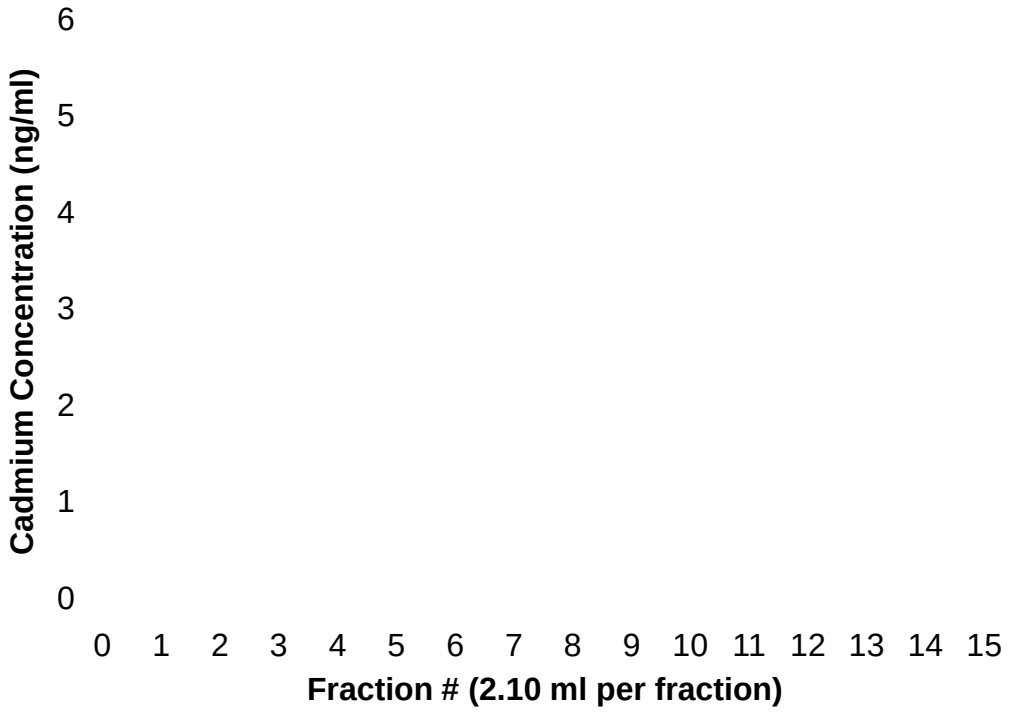


Figure 7: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) of Laughing Whitefish River #21 crayfish.

Fractions 6-8 and 10-14 contained metallothionein-bound cadmium and free metals, respectively (Figure 7). The cadmium concentration of the eleven fractions (4-14) analyzed are listed in Table 7. Tables for the remaining specimens analyzed can be found in Appendix B (Tables B1-B45).

Table 7: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from a crayfish from the Laughing Whitefish River #21.

Fraction # (2.10 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.394
5	0.337
6	0.432
7	5.624
8	0.387
9	0.404
10	0.379
11	4.935
12	1.885
13	0.879
14	0.708
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**6.443**
Total Mass Of Cadmium In MT Fractions (ng)	**13.5303**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**1.20366E-10**
Total Moles of MT In Fractions (moles)	**2.00609E-11**
Mass Of Gills (g)	**0.1958**
Moles MT/g Gills	**1.02456E-10**
µMoles MT/g Gills	**0.000102456**

Cadmium bound to MT eluted in fractions 6-8 (Figure 7), so only these fractions were used to determine the moles of cadmium bound to MT.

Indirect quantification of metallothionein by the presence of metals bound to the protein depends not only on the metal species but also on the source of the tissue. Rabbit metallothionein binds seven cadmium atoms, whereas metallothionein isolated from invertebrates such as lobsters contain six moles of Cd^{2+} per mole of MT. The following

calculations explain how the cadmium concentration of the fractions was used to determine the concentration of metallothionein in the gills.

Cd^{2+} bound to MT (ng) = Total Concentration of Cd^{2+} in MT Fractions (ng/ml) x Fraction volume (ml)

Moles of Cd^{2+} bound to MT = Cd^{2+} bound to MT (ng) x (1g/10^9 ng) x (1 mole/112.41 g)

Moles of MT = Moles of Cd^{2+} bound to MT x (1 mole MT/ 6 moles Cd^{2+})

µMoles of MT/g gills = Moles of MT /mass of gills (g) x (10^6 µmoles/1 mole)

The concentration of MT isolated from the gills of crayfish collected from Dead River (DR), Deer Lake (DL), East Branch of the Escanaba River (EBER), and Laughing Whitefish River (LWR) is shown in Table 8, along with the µmoles MT/g gill averages and standard deviations for each site.

Table 8: Metallothionien concentration in gills of crayfish, average MT concentration, and standard deviation values for Dead River (DR), Deer Lake (DL), East Branch of the Escanaba River (EBER), and Laughing Whitefish River (LWR).

Sample ID	Conc MT (µmole MT/g gills)	Average MT (µmole MT/g gills)	Standard Deviation
DR 1	0.00118296		
DR 2	0.002355292		
DR 3	0.002501769		
DR 4	0.001295806		
DR 5	0.00645537		
DR 6	0.007226629		
DR 7	0.000857		
DR 8	0.000444233		
DR 9	0.00032029		
DR 10	0.000439862		
DR 12	0.001396378	**0.002225054**	**0.002397894**
DL 1	0.000266539		
DL 2	0.000163356		
DL 4	0.000180853		
DL 5	0.007076761		
DL 6	0.007342617		
DL 8	0.003773527		
DL 10	0.003366887		
DL 11	8.66705E-05		
DL 12	9.00009E-05		
DL 13	9.65898E-05	**0.00224438**	**0.002969343**
EBER 1	0.000277305		
EBER 5	0.000366191		
EBER 6	0.000849875		
EBER 7	0.001395453		
EBER 8	0.000735634		
EBER 10	0.000521661		
EBER 11	0.000410741		
EBER 17	0.000127264		
EBER 18	9.35986E-05		
EBER 19	9.23123E-05	**0.000487003**	**0.000411545**
LWR 1	0.000359604		
LWR 3	0.000322572		
LWR 7	0.00037243		
LWR 9	0.000177027		
LWR 10	0.000230034		
LWR 14	5.35266E-05		
LWR 16	0.000649403		
LWR 17	0.000250456		
LWR 18	0.000193863		
LWR 21	0.000102465	**0.000271138**	**0.000168841**

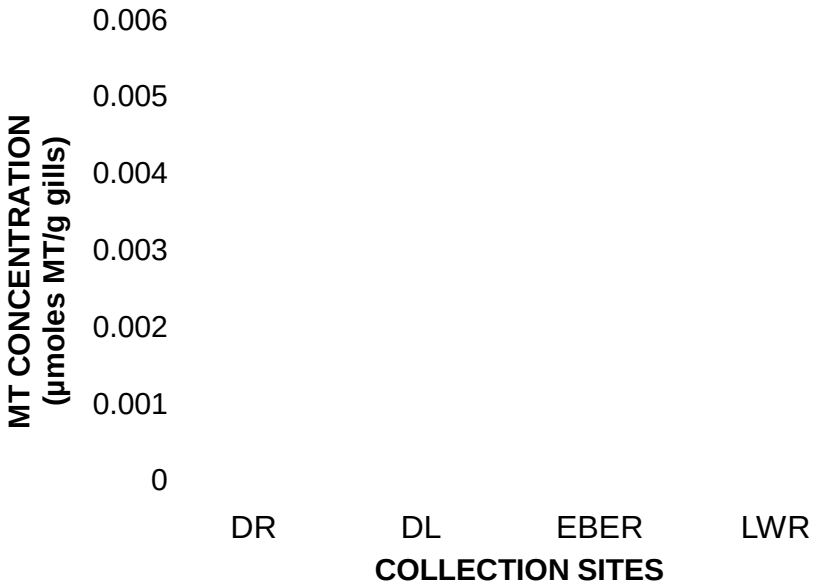


Figure 8: Average metallothionein concentrations and standard deviation (error bars) values for crayfish from Dead River (DR, n=11), Deer Lake (DL, n=10), East Branch of the Escanaba River (EBER, n=10), and Laughing Whitefish River (LWR, n=10).

Crayfish from Deer Lake and Dead River exhibited the highest concentration of metallothionein in the gills with averages of 0.00224438 and 0.002225054 µmoles MT/g gills and there was very little difference, $1.9326E^{-}5$, between the two sites (Table 8). The average metallothionein concentration of these sites was approximately 4.6 times higher than that determined for crayfish collected from the East Branch of the Escanaba River, 0.000487003 µmoles MT/g gills (Table 8). The lowest average metallothionein concentration was found in crayfish collected from Laughing Whitefish River (0.000271138 µmoles MT/g gills) which is approximately 8 times lower than the value determined for Deer Lake and Dead River (Table 8).

The standard deviations calculated for Dead River and Deer Lake were greater than the averages 0.002397894 and 0.002969343, respectively (Table 8). The standard deviations for specimens collected from the East Branch of the Escanaba River and

Laughing Whitefish River were less than the average 0.000411545 and 0.000168841 respectively. The implication of these abnormal distributions and its effect on significance was further examined.

VI. Cadmium-Mercaptide Bond UV Absorption

A control experiment was conducted to investigate whether fractions containing metallothionein exhibit increases in cadmium concentration and UV absorption at 250 nm. Before the hepatopancreatic cytosol from the induced crayfish was applied to the Sephadex G-25/G-75 column, the column was calibrated with rabbit MT-1 (~4.0 μM) to determine the volume at which MT-bound cadmium would begin eluting. Figure 9 illustrates the chromatographic separation of metallothionein-bound cadmium and free metal.

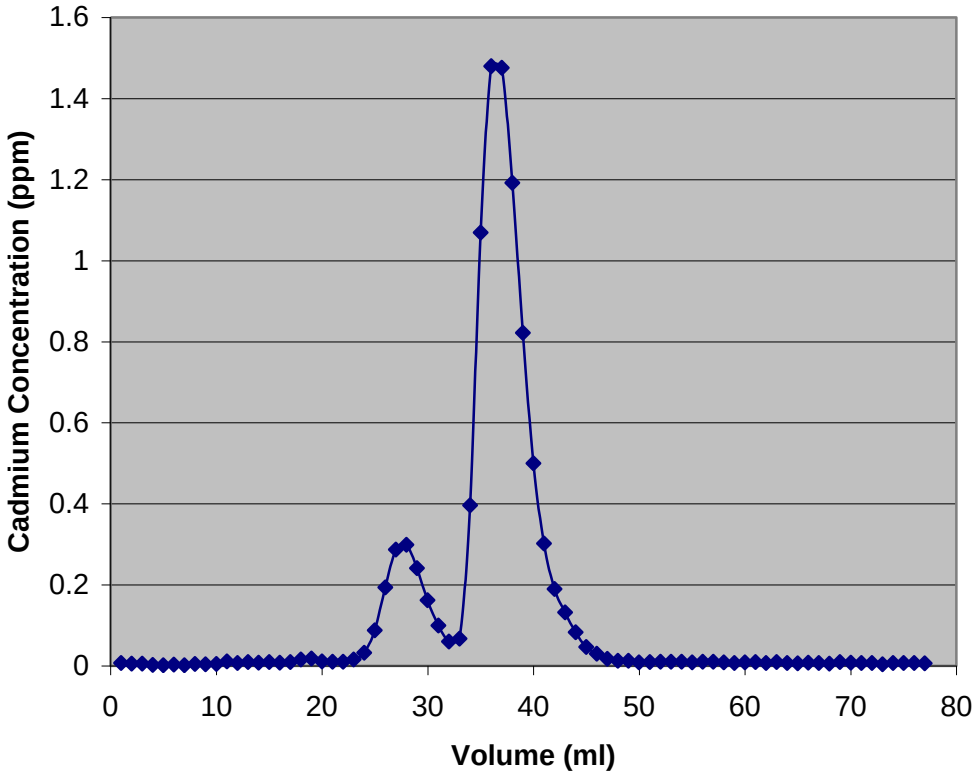


Figure 9: Elution profile for the size chromatographic separation (Sephadex G-25/G-75) of metallothionein-bound cadmium and free metal found in 0.75 ml of ~2.0 µM rabbit metallothionein-1 solution (750 µl rabbit MT-1, 750 µl tissue prep buffer).

Application of rabbit metallothionein-1 solution (~2.0 µM) which had been spiked with cadmium resulted in an elution profile (Figure 9) consisting of two peaks. The peaks starting at 24 and 33 milliliters represent metallothionein-bound cadmium and free metals, respectively. The metallothionein-bound cadmium in the hepatopancreatic cytosol of the induced crayfish was therefore suspected to elute at approximately 24 milliliters.

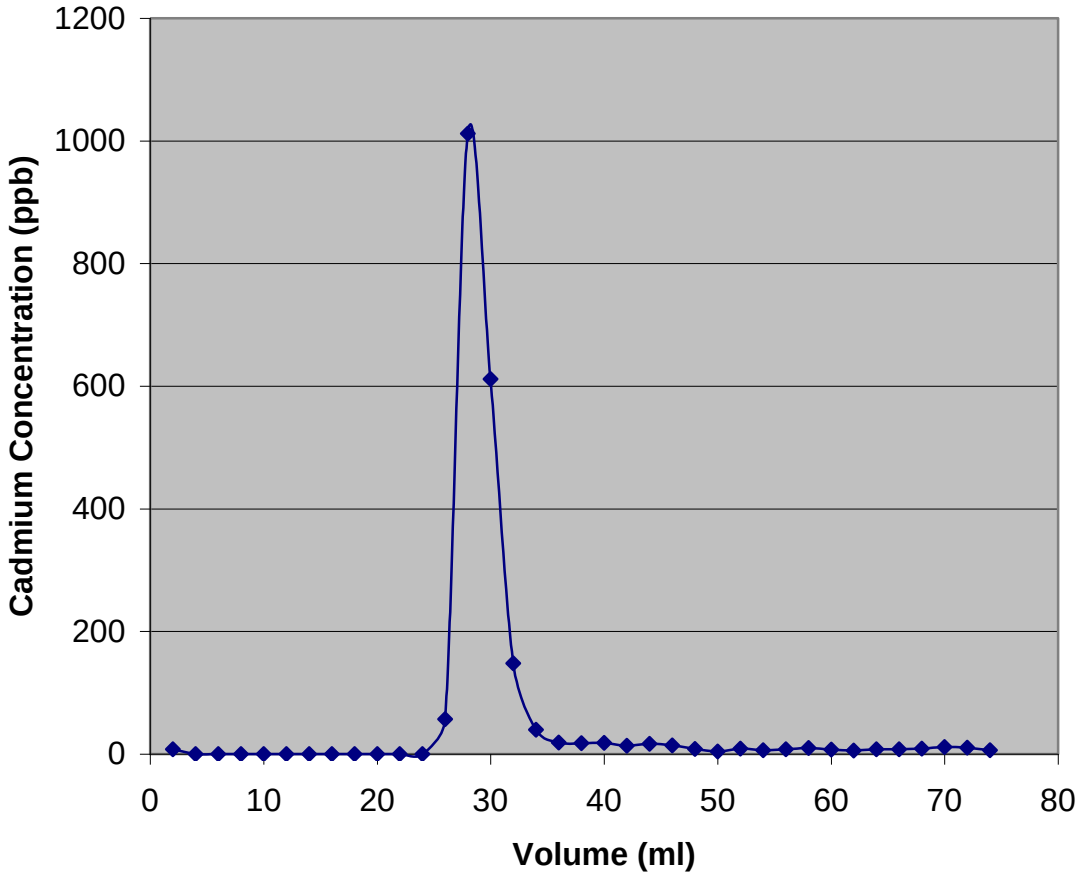


Figure 10: Elution profile for the size chromatographic separation (Sephadex G-25/G-75) of metallothionein-bound cadmium and free metal found in the chelexed hepatopancreatic cytosol (0.8 ml) of an induced crayfish.

The hepatopancreatic metallothionein from the induced crayfish eluted between 26 and 34 milliliters (Figure 10). Table 9 includes the absorbance values at 250 nm and cadmium concentration of the collected fractions.

Table 9: Absorbance (250 nm) and Cd^{2+} concentration of fractions (2.00 ml) collected from a Sephadex G-25/G-75 column loaded with ~0.8 ml of hepatopancreatic cytosol from an induced crayfish. The highlighted fractions contain cadmium bound to MT (Figure 10).

Fraction # (2.00 ml per fraction)	**Elution Volume** (ml)	**Cadmium Concentration** (ng/ml)	**Absorbance** (250 nm)
10	20	0	-0.02
11	22	0	-0.006
12	24	0	0.001
13	26	57	0.533
14	28	1012	2.504
15	30	611.6	1.357
16	32	147.9	0.493
17	34	39.5	0.323
18	36	18.6	0.596
19	38	18	0.54
20	40	18.4	0.354
21	42	13.6	0.176
22	44	16.7	0.144
23	46	14.3	0.113
24	48	8.3	0.143
25	50	4.1	0.15
26	52	8.9	0.154
27	54	6.1	0.167

UV absorption at 250 nm is characteristic of cadmium-mercaptide bonds. Table 9, in conjunction with Figure 10, shows the correlation between absorbance at 250 nm and the presence of Cd-MT. Fractions comprising the metallothionein peak, #13-17, exhibit not only an elevated absorption at 250 nm, but also an increase in cadmium concentration.

VII. Determination of Sulfhydryl:Metal Ratio

To verify the presence of cadmium-MT in the fractions which showed an increase in UV absorption at 250 nm and in cadmium concentration, the ratio of Cd^{2+} to cysteine residues was determined. Because DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) reacts

with thiol groups, this reagent can be used to quantify the concentration of cysteine residues present in a protein solution. The absorbance at 220 nm and 250 nm of the gill cytosol from three induced crayfish applied to a column is shown in Table 10.

Table 10: UV absorbance readings (220 nm and 250 nm) of fractions (highlighted fractions contain metallothionein) collected from a Sephadex G-15 column loaded with gill cytosol from an induced crayfish.

Fraction #	Absorbance (220 nm)	Absorbance (250 nm)
1	2.557	2.502
2	2.525	2.469
3	2.516	2.467
4	2.51	2.464
5	2.517	2.463
6	2.499	2.463
7	2.505	2.465
8	2.522	2.47
9	2.512	2.464
10	2.51	2.468
11	2.551	2.583
12	2.779	3.218
13	2.813	3.252
14	2.681	3.112
15	2.644	2.978
16	2.646	2.878
17	2.579	2.825
18	2.558	2.726
19	2.549	2.666
20	2.549	2.66
21	2.549	2.633
22	2.52	2.566
23	2.515	2.592
24	2.583	2.773
25	2.572	2.695
26	2.542	2.636
27	2.54	2.585
28	2.541	2.588
29	2.544	2.611
30	2.519	2.604

Relative to the other fractions measured #12-18 showed an increased absorbance at both 220 nm and 250 nm (Table 10) and so these fractions were pooled, concentrated, and then reacted with DTNB. Figure 11 shows the kinetic profile for a reaction between 0.1 ml of 10 mM DTNB and a solution consisting of 0.1 ml crayfish MT and 0.8 ml 0.1 mM Tris, pH 7.49.

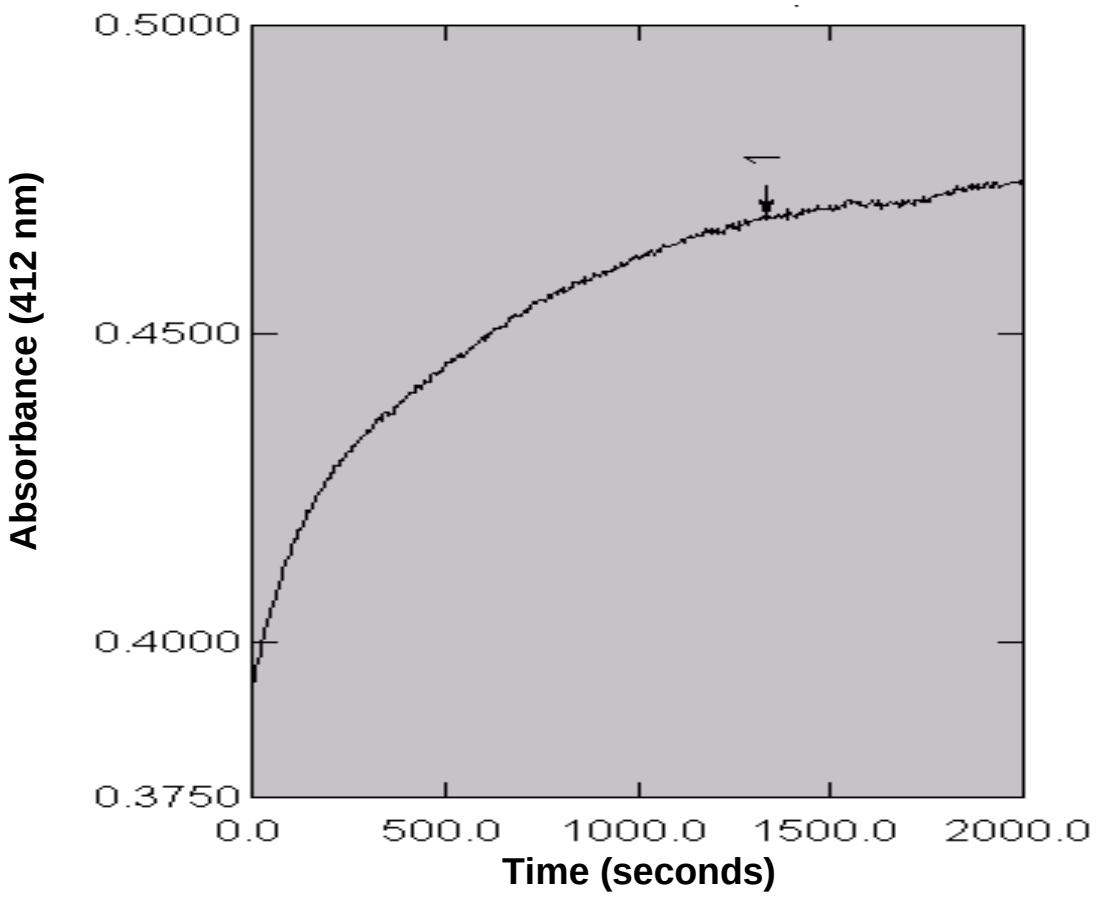


Figure 11: Time course graph for a DTNB reaction with metallothionein from gill tissue of induced crayfish.

The absorbance readings for the blank and diluted crayfish MT sample at the end of the run were 0.234 and 0.482, respectively. Subtracting the blank from the protein sample gave an absorbance change of 0.248. This increase in absorbance was attributed

to the reaction of DTNB with the sulfhydryl groups of cysteine residues present in metallothionein. More specifically, an increase in the reaction's product was responsible for the absorbance change. The product, 5-thio-2-nitrobenzoate, absorbs at 412 nm with a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ (Chen et al., 1996). The concentration of sulfhydryl groups was determined using Beer's Law, $A= \varepsilon b c$. Using a pathlength of 1 cm and 13,600 $M^{-1}cm^{-1}$, the sulfhydryl concentration was determined to be 1.82_E^{-4} M for the undiluted solution. The cadmium content of the gill cytosol was measured by Flame Atomic Absorption Spectroscopy. The cadmium concentration was calculated to be 6.3_E^{-5} M and therefore, the ratio, cysteines:Cd^{2+}, was 2.9:1.

VIII. Metal Analysis of Water Samples

The concentration of zinc, copper, cadmium, and mercury measured in water samples collected from the Dead River (DR), Deer Lake (DL), East Branch of the Escanaba River (EBER), and Laughing Whitefish River (LWR) are shown in Table 11. Atomic absorption spectroscopy was used to determine the metal concentrations in the water samples.

Table 11: Concentration of metals (ppm) measured in water samples taken from the four specimen collection sites (Dead River, Deer Lake, East Brach of the Escanaba River, Laughing Whitefish River). Total Metal is the sum of zinc, copper, cadmium, and mercury measured in the water samples.

Sites	Zn (ppm)	Cu (ppm)	Cd (ppm)	Hg (ppm)	Total Metal (ppm)
DR	6.56E-03	5.81E-03	8.60E-05	0	1.25E-02
DL	4.65E-03	3.30E-03	1.66E-05	5.15E-06	7.97E-03
EBER	3.44E-03	7.36E-03	1.22E-04	6.80E-06	1.09E-02
LWR	2.99E-03	4.30E-03	6.67E-05	1.98E-06	7.36E-03

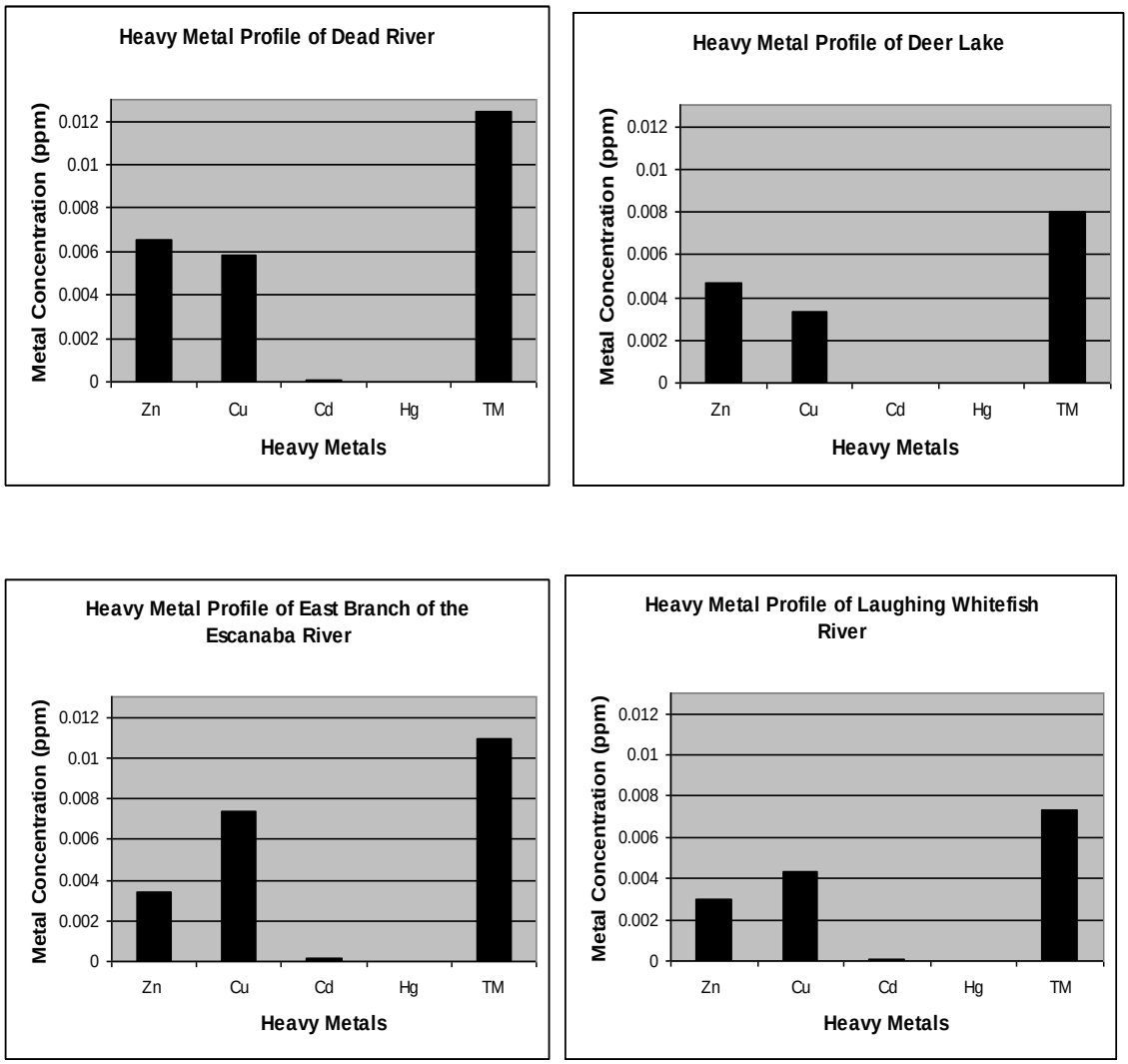


Figure 12: Heavy metal profiles for the Dead River, Deer Lake, East Branch of the Escanaba River, and Laughing Whitefish River. TM (total metals) refers to the sum of zinc, copper, cadmium, and mercury measured in the water.

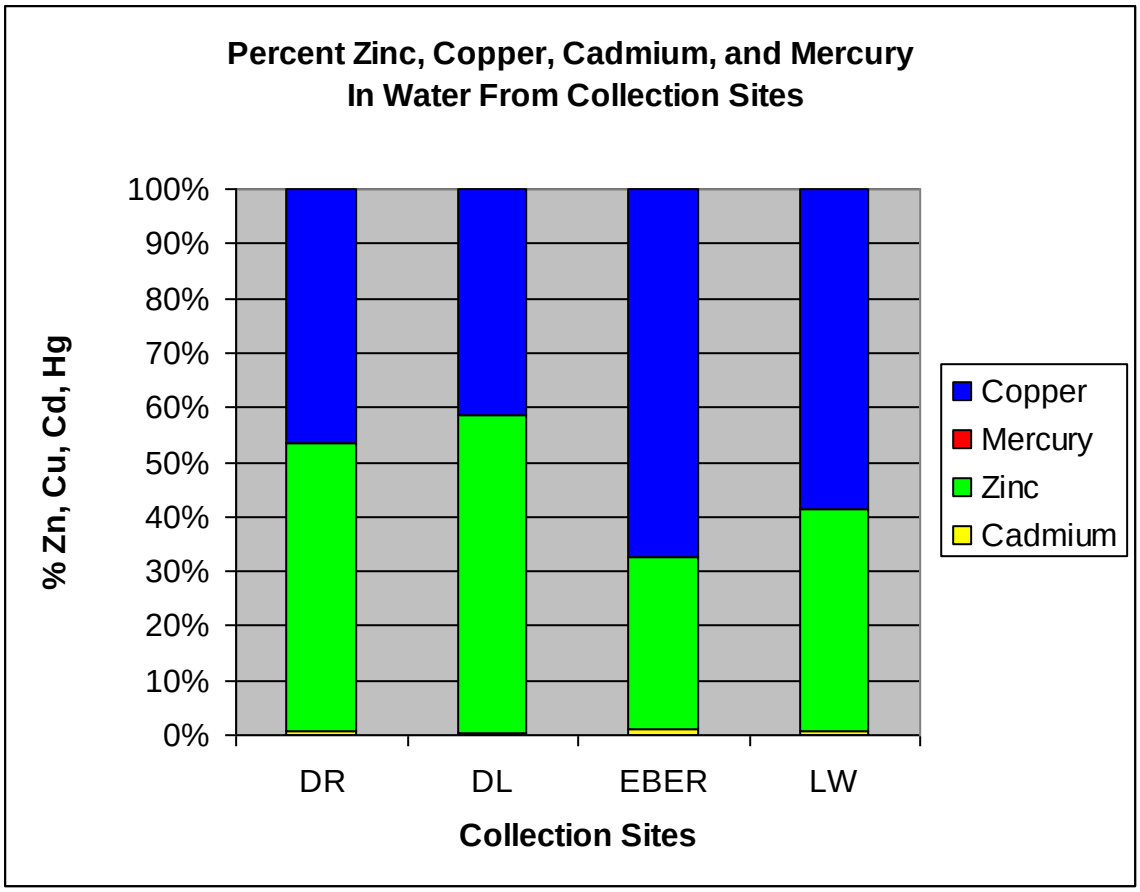


Figure 13: Histogram showing the percent of copper, zinc, mercury, and cadmium in the water from Dead River (DR), Deer Lake (DL), East Branch of the Escanaba River (EBER), and Laughing Whitefish River (LWR).

Table 12: Percentages of zinc, copper, cadmium, and mercury in water samples from Dead River (DR), Deer Lake (DL), East Branch of the Escanaba River (EBER), and Laughing Whitefish River (LWR).

PERCENT HEAVY METALS IN COLLECTION SITES				
	DR	DL	EBER	LWR
% Zn	52.66	58.31	31.47	40.64
% Cu	46.65	41.41	67.36	58.43
% Cd	0.69	0.21	1.11	0.91
% Hg	0	0.065	0.062	0.027

IX. Statistical Analysis

Metallothionein concentrations within treatment groups did not display a normal distribution pattern so Kruskal-Wallis non- parametric one way ANOVA on ranks (SPSS 13.0, SPSS Inc., Chicago, IL) was used to detect group differences. In order for differences in metallothionein concentration to be considered significant the observed significance level had to be less than 0.05. The one-way analysis of variance conducted on all four test sites resulted in an overall p value of 0.015. Dunn's method (SPSS 13.0, SPSS Inc., Chicago, IL) was used to determine which combinations of sites displayed a significant difference. Of the six combinations tested, only Dead River and Laughing Whitefish River showed a significant difference in metallothionein concentration.

Linear regression analysis (SPSS 13.0, SPSS Inc., Chicago, IL) was used to examine the association between metallothionein concentration and potential predictors. The dependent metallothionein concentration was analyzed against individual heavy metals (Zn^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+}), total metal content, sites, mass, and various combinations of these variables. Based on a level of significance set at 95%, regression analysis revealed a significant relationship between the dependent variable, metallothionein concentration, and zinc content of the water samples ($F_{1,39}$ =7.089, P = 0.011). The relationship between the two variables was given by the equation y = 0.576x-0.001 (R^2 = 0.154).

Chapter Four: Discussion

The goals of the present study were to develop a cost effective and time efficient method for metallothionein quantification and to investigate the utility of crayfish gill metalollothionein as a biomarker for aquatic metal pollution in the Upper Peninsula of Michigan. The cadmium saturation method employed to quantify the concentration of metallothionein in crayfish gills indirectly quantified proteins by measuring the concentration of metallothionein-bound Cd^{2+} present in the samples. Because the validity of the results relied on accurate metal measurements, it was necessary to determine parameters such as the detection limit and limit of quantitation for Flame AAS, Graphite Furnace AAS, and Inductively Coupled Plasma Mass Spectroscopy. It was found that detection limit values reflected the sensitivity of the instrument, and that the limit of quantitation values set the lowest amount of analyte that could be quantitatively measured with accuracy and precision. Table 2 contains these values for the aforementioned instruments. Since the cadmium measurements of the column fractions were above the limit of quantitation determined for Cd^{2+} (ppb) these values were considered accurate. The same was true for the zinc, copper, cadmium, and mercury measurements used to determine the concentration of these metals in the water samples and the cadmium measurement of the crayfish metallothionein used in the DTNB reaction.

Before the developed method was employed to quantify metallothionein in specimens collected from the field, a series of control experiments were conducted to investigate the effects of heat shock and Chelex 100 treatments, to validate chromatographic and metal displacement methods, and to characterize metallothionein. Because purified crayfish

metallothionein was not commercially available, purified MT-1 from rabbit kidney was used to calibrate the Sephadex columns. To validate rabbit metallothionein's utility as a reference, the molecular weights of crayfish MT and rabbit MT were compared by running both on a Sephadex G-75 column. Although metallothionein has a molecular weight of 6-6.5 kDa, its dumbbell shape causes it to elute at approximately 10,000 Da (Vasak, 1991). Because rabbit and crayfish MT share the same tertiary structure, the calibration of the columns with rabbit MT was more accurate than using molecular weight standards. Figure 2 shows the elution profile for MT-1 from rabbit kidney where two peaks represent rabbit MT-1 (left) and free metals (right). Figure 3 shows the elution profile for crayfish metallothionein extracted from the hepatopancreas, and three peaks are present, the left one, representing cadmium bound to high molecular weight proteins, and the shoulder on the left hand side of the free metal peak corresponding to crayfish MT. The overlap of the metallothionein and free metal peaks is due to an excessive amount of free metals present in the sample applied to the column. Better resolution of the two peaks would have been achieved by treatment of the sample with Chelex 100. Because rabbit MT and crayfish MT began eluting at similar volumes, 47 ml and 48 ml, respectively, it was concluded that the molecular weights of the two metallothioneins were the same and that rabbit metallothionein could be used to calibrate the Sephadex columns. The one milliliter difference in elution volume was attributed to variation in loading the column.

Chelex 100 is commonly used to sequester free and loosely bound metals present in a solution when metal saturation techniques are used to quantify metallothionein. Because cadmium concentrations were used to quantify metallothionein, Chelex 100 must not

remove metals bound to metallothionein or the results would be distorted. Figures 4 and 5 represent the elution profiles for untreated and Chelex 100 treated rabbit MT, respectively. The elution profile for untreated rabbit MT contains both metallothionein and free metal peaks, and the shoulder on the right hand side of the free metal peak was most likely additional free metals and an artifact of loading the column. The absence of a free metal peak in Figure 5 illustrates the effectiveness of Chelex 100 treatments in sequestering free and loosely bound metals. The information in Tables 4 and 5 was used to assess whether or not Chelex 100 removed metal bound to metallothionein. Based on the calculations previously discussed in the results section, 0.00465 µmoles of protein was recovered from the column loaded with 1 ml of rabbit MT. Therefore, a theoretical recovery of 0.00372 µmoles of rabbit MT was expected when 0.8 ml of Chelex 100 treated sample was loaded onto the column. An actual recovery of 0.00377 µmoles of rabbit MT confirms the chelating agent's effectiveness in removing free and loosely bound metal without interfering with the metals bound to metallothionein.

Successful quantification of metallothionein using the cadmium saturation method depends upon the complete displacement of metals bound to metallothionein by cadmium. Because the affinities of metal ions for metallothionein follow the order of Hg(II)>Cu(I)>Cd(II)>Zn(II) (Mouneyrac et al., 2001), adding excess cadmium to the sample prep resulted in complete displacement of zinc from metallothionein. Although Cu(I) has a greater binding affinity than Cd(II), the research results of Lacorn et al. (2001) confirmed an exchange of Cu(I) for cadmium. These findings were further substantiated by the copper displacement control experiment conducted in this study. Table 6 contains the results of this experiment. Complete conversion of 1.34 µM of Zn-

MT-2 into Cu-MT-2 would result in a copper concentration ranging from 0.5967 ppm to 1.705 ppm. However, the actual concentration of copper measured in the converted Cu-MT-2 sample was 8.052 ppm. Although the actual copper concentration was significantly higher than the expected value, the success of the conversion was reflected in the subsequent decrease in zinc concentration. The original Zn-MT-2 sample contained 0.614 ppm of zinc. However, only 0.086 ppm of zinc was measured in the Cu-MT-2 sample. The increase in copper concentration and subsequent decrease in zinc concentration confirms the displacement of metallothioenin-bound zinc by copper. Although the synthesized Cu-MT-2 sample wasn't expected to contain zinc or cadmium, the residual amounts measured were most likely present in the buffer and additional treatments with Chelex 100 would have removed the unbound metal. If Cu-MT-2 was completely converted into Cd-MT (A), then 1.056 ppm of cadmium was expected to be present in the sample. Even though only 0.4278 ppm of cadmium was measured after the first Chelex 100 and heat shock treatments, the increase in cadmium and subsequent decrease in copper concentration (1.06 ppm) indicates that copper was displaced by cadmium. Additional treatments of Cu-MT-2 with Chelex 100 before the addition of excess cadmium resulted in a significantly greater conversion of Cu-MT-2 into Cd-MT-2 (B). The displacement of metallothionein-bound copper by cadmium is apparent from the decrease in copper (0.0873 ppm) and concomitant increase in cadmium concentration (1.27 ppm). As expected, the Cd-MT-2 sample was void of zinc. Even though copper also should have been absent from the sample, the residual amount measured (0.0873 ppm) was most likely present as free metal in the buffer. The displacement of metallothionein-bound copper by cadmium is attributed to the effects of heat shock.

When metallothionein is subjected to high temperatures, the protein unfolds and liberates the bound metal. At this point, the Cu(I) originally bound to metallothionein is oxidized to Cu(II) by oxidizing agents present in the solution. Because Cu(II) doesn't bind to metallothionein, the excess cadmium becomes incorporated into metallothionein as the sample cools and the protein folds back into its original three dimensional structure.

For the reaction of DTNB with crayfish metallothionein, fractions containing MT were identified by measuring absorption at 250 nm which is characteristic of mercaptide-cadmium bonds (Willner et al., 1987). A control experiment was conducted to investigate whether fractions containing metallothionein exhibit not only an increase in cadmium concentration, but also elevated absorbance at 250 nm. A Sephadex G-25/G-75 column was first calibrated with rabbit metallothionein to determine the volume that crayfish MT was expected to elute from the column. According to the elution profile for rabbit metallothionein (Figure 9), the hepatopancreatic MT from crayfish was expected to elute at approximately 24 ml. Figure 10 displays the elution profile for the crayfish MT cytosol applied to the column. In this graph, only the peak representing metallothionein-bound cadmium is present. Effective removal of free cadmium from solution by Chelex 100 is responsible for the absence of the free metal peak. The high molecular weight protein peak, which preceded metallothionein in Figure 3, is also absent. Unlike the crayfish cytosol loaded onto the Sephadex G-75 column (Figure 3), this hepatopancreatic cytosol was subjected to heat shock treatment. Exposure of the hepatopancreatic cytosol to an elevated temperature resulted in the denaturation of high molecular weight proteins. Because metallothionein is heat stable, this protein remained intact and soluble while the high molecular weight proteins precipitated out of solution. Although the crayfish MT

began eluting at 26 ml, instead of the expected 24 ml, this discrepancy can be attributed to mechanical differences in loading the column. Table 9 displays both the cadmium concentrations and UV absorbances at 250 nm for fractions #10-27. The fractions comprising the metallothionein peak are highlighted. Fractions 13-17 exhibit not only an elevated absorbance at 250 nm, but also an increase in cadmium concentration. Therefore, UV spectroscopic analysis at 250 nm was used to identify fractions containing metallothionein chromatographically isolated from gill cytosol.

The presence of metallothionein in the gill cytosol of crayfish was confirmed by determining the sulfhydryl:cadmium ratio of fractions chromatographically isolated on a Sephadex G-15 column. Because DTNB reacts with sulfhydryl groups, this reagent is commonly used to quantify the concentration of cysteine residues present in a protein solution. Only fractions showing concomitant increases in UV absorbance at both 220 nm and 250 nm were used in the DTNB reaction (Table 10). An increase in absorbance at 220 nm is characteristic of proteins. The partial double bond character of peptide bonds is responsible for UV absorbance at this wavelength. The kinetic profile shown in Figure 11 is characteristic of a reaction between DTNB and metallothionein. DTNB displaces metals bound to metallothionein. As a result, metallothionein gets oxidized while the DTNB gets reduced to 5-thio-2-nitrobenzoate which absorbs at 412 nn. If DTNB reacted with other reducing agents such as apo-MT or β-mercaptoethanol, the reaction would be instantaneous instead of taking place over a 30 minute period. According to Zhu et al. (1994), each domain of lobster metallothionein is comprised of a Cd_3S_9 cluster. Therefore, a sulfhydryl:Cd^{2+} ratio of 18:6 (3:1) was expected to be present in the crayfish fractions. The sulfhydryl and cadmium concentrations of the fractions

were determined to be $1.83_E{}^{-4}$ M and $6.3_E{}^{-5}$ M, respectively. The experimental ratio of $1.83_E{}^{-4}$ M:$6.3_E{}^{-5}$ M (2.9:1), validated the presence of metallothionein in the collected fractions.

Different sizes of Sephadex (G-75/G-25, G-75, G-15) were used during method development to increase peak separation and minimize run time. Because Sephadex G-15 columns maintained optimal resolution of the metallothionein and free metal peaks and reduced run time, these columns were used to isolate metallothionein present in the gill cytosol of field specimens. Calibration of the Sephadex G-15 columns with rabbit metallothionein demonstrated that crayfish metallothionein would begin eluting at approximately 12 ml (Figure 6). Figure 7 shows the elution profile for the chromatographic separation of metallothionein-bound cadmium and free metals found in the gill cytosol of Laughing Whitefish River #21 crayfish specimen. For this specimen, metallothionein began eluting in fraction #6. Because the volume of each fraction was 2.1 ml, MT eluted from the column at 12.6 ml which agrees with the expected volume (12 ml) determined by calibration of the column with rabbit MT. Metallothoinein in the gill cytosol of Laughing Whitefish River #21 crayfish specimen was indirectly quantified by determining the concentration of cadmium present in fractions comprising the MT peak. The fractions comprising the metallothionein peak are highlighted (Table 7). Table 7 also shows the information used to calculate the concentration of metallothionein present in the gill tissue (µmoles MT/g gills). The graphs and tables for the remaining specimens are located in Appendix A and Appendix B, respectively.

The Kruskal-Wallis non parametric one way ANOVA test (SPSS 13.0, SPSS Inc., Chicago, IL) used to detect group differences in metallothionein concentration (Table 8)

resulted in an overall p value of 0.015. The Dunn's method (SPSS 13.0, SPSS Inc., Chicago, IL) determined that only Dead River and Laughing Whitefish River showed a significant difference in average metallothionein concentration. Figure 8 illustrates the average metallothionein concentrations and the standard deviations calculated for each site. The lack of significant differences between different combinations of sites, such as Deer Lake/Dead River and Laughing Whitefish River/East Branch of the Escanaba River can be attributed to minimal differences in average metallothionein concentrations. For Deer Lake and Dead River, the difference in average metallothionein concentrations was only $1.9326E^{-}5$ µmoles MT/g gills. The difference between Laughing Whitefish River and East Branch of the Escanaba River was 0.000216 µmoles MT/g gills. A high degree of variance also contributed to the lack of significant differences between the sites (Table 8). Intersite differences are often concealed when high variability exists within a group. This may have been the case when the East Branch of the Escanaba River was compared to both Dead River and Deer Lake and Laughing Whitefish River was compared to Deer Lake. Individual physiological variation of crayfish within the groups was responsible for the large degree of variance found in Dead River and Deer Lake specimens.

Linear Regression analysis revealed that zinc was the best predictor for metallothionein concentration. Although zinc was responsible for only 15.4% of the variance in metallothionein concentration (R^2=0.154), the relationship was significant (P=0.011) and the variance between groups was greater than variance within groups ($F_{1,39}$ = 7.089). Copper and zinc accounted for over 98% of the metal content in the water samples (Table 12). Cadmium and mercury were responsible for the remaining 2% (Table 12). The lack of correlation between metallothionein concentration and the

nonessential metals, Cd^{2+} and Hg^{2+}, was most likely a consequence of these two metals being present at too low of a concentration (less than 1 ppb) to warrant additional synthesis of MT. Under such low exposure conditions, basal levels of MT synthesis would accommodate any influxes of cadmium or mercury via the gills. Zinc and copper concentrations in the water samples were nearly equal. However, linear regression analysis exposed a correlation between metallothionein concentration and environmental exposure to zinc. This relationship is supported by the fact that zinc is a potent inducer of metallothionein synthesis, whereas copper is a weak inducer (Pourang et al., 2004). In addition to the differential inducibility of MT by these metals, alternative metal detoxification pathways also influence metallothionein synthesis. Glutathione (GSH) is a tripeptide known to function as an antioxidant and a pathway for metal detoxification (Downs et al., 2001). GSH prevents hydrogen peroxide and Cu(I) from forming free radicals by sequestering these compounds. In studies referenced by Legras et al. (2000), glutathione is the preferential detoxification pathway for copper in blue crabs and metallothionein synthesis is a secondary chelator. Copper's weak inducibility of metallothionein and preference for GSH explain why metallothionein concentration didn't correlate copper exposure in this study.

Previous field studies have confirmed invertebrate metallothionein's reliability as a biomarker for metal exposure. Pedersen et al. (1997) collected shore crabs, *Carcinus maenas,* from five sites with varying concentrations of zinc, copper, and cadmium. Increases in both Cu-MT and Zn-MT concentrations measured in the gills mimicked the copper and zinc gradients determined for the collection sites (Pedersen et al., 1997). In laboratory experiments conducted by Canli et al. (1997), lobsters were simultaneously

exposed to various concentrations of zinc, copper, and cadmium in the water. Metallothionein concentrations in the gill and hepatopancreas correlated to increases in ambient metal concentrations (Canli et al., 1997). A relationship between cadmium and metallothionein was revealed when metal and metallothionein concentrations were measured in the tissues (Canli et al., 1997). A relationship between cadmium exposure and metallothionein was further verified in a study conducted by Martin-Diaz et al. (2006). Crayfish, *Procambarus clarkii*, were exposed simultaneously to cadmium and zinc levels comparable to concentrations found at a river impacted by a mining spill. An increase in cadmium exposure correlated to an increase in the bioaccumulation of Cd and metallothionein concentration in the hepatopancreas.

In the study conducted by Pedersen et al. (1997) in England, metallothionein concentrations measured in the gills of marine crabs collected from metal contaminated sites and control sites were determined to be ~4-8 nmoles MT/g gills and ~1 nmole MT/g gills, respectively. The metallothionein concentrations measured in the present study were between 0.3 and 2.2 nmoles MT/g gills. The midpoint of this range (1.3 nmoles MT/g gills) agrees with the concentration of MT measured in the gill tissue of marine crabs from the UK. Based on the results from the present study and Pedersen et al (1997), a proposed value of approximately 1 nmole MT/g gills or less would be expected from biomonitors inhabiting a clean aquatic ecosystem, whereas specimens collected from metal contaminated sites are likely to contain a metallothionein concentration of 4 nmoles MT/g gills or above.

The present study revealed only a weak correlation between metallothionein concentration and zinc. However, previous studies have established dose dependant

relationships between metallothionein and exposure to both essential and nonessential metals. Therefore, it is possible to use metallothionein as a biomarker of metal exposure. However, the locations must contain sufficient metal concentrations to induce metallothionein synthesis or the influence of metal exposure on MT production will be overshadowed by the effects of physiological variations within the biomonitors. To establish the effects of metal exposure on metallothionein concentration, the polluted sites must significantly vary in metal content and a reference site containing low levels of heavy metals must be incorporated into the study.

Future research is needed to apply the time efficient and cost effective method developed and validated in this study to an area impacted by severe anthropogenic activity such as mining. The detoxification pathways of crustaceans include metal binding ligands and the incorporation of metals into vacuoles, and a practical expansion of the current method would be to measure the metal concentrations in the tissue. This would allow for a comparison of metals accumulated in the tissue with metallothionein and environmental exposure, and a more complete assessment of metal toxicity would be accomplished.

LITERATURE CITED

Ahearn, G. A., P. K. Mandal, A. Mandal. 2004. Mechanisms of heavy-metal sequestration and detoxification in crustaceans: a review. Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology 174: 439-452.

Bremmer, I. 1991. Nutritional and physiologic significance of metallothionein. Methods in Enzymology 205: 25-35.

Brouwer, M., J. Enghild, T. Hoexum-Brouwer, I. Thogersen, and A. Truncali. 1995. Primary structure and tissue-specific expression of blue crab (*Callinectes sapidus*) metallothionein isoforms. Biochemical Journal 311: 617-622.

Brown, D. A., and T. A. Parsons. 1978. Relationship between cytoplasmic distribution of mercury and toxic effects to zooplankton and chum salmon (*Oncorhynchus keta*) exposed to mercury in a controlled ecosystem. Journal of the Fisheries Research Board of Canada 35: 880-884.

Canli, M., R. M. Stagg, and G. Rodger. 1997. The induction of metallothionein in tissues of the norway lobster *Nephrops norvegicus* following exposure to cadmium, copper and zinc: the relationships between metallothionein and the metals. Environmental Pollution 96: 343-350.

Chen, P., A. Munoz, D. Nettesheim, C. F. Shaw, III, and D. H. Petering. 1996. Stoichiometry and cluster specificity of copper binding to metallothionein: homogenous metal clusters. Biochemical Journal 317: 395-402.

Chen, P., P. Onana, C. F. Shaw, III, and D. H. Petering. 1996. Characterization of calf liver Cu, Zn-metallothionein: naturally variable Cu and Zn stoichiometries. Biochemical Journal 317: 389-394.

Couillard, Y., and L. St-Cyr. 1997. Technical evaluation of metallothionein as a biomarker for the mining industry. Canadian Centre for Mineral and Energy Technology, Ottawa, Ontario. 28-29 pp.

Dalton, T. P., Q. Li, D. Bittel, L. Liang, and G. K. Andrews. 1996. Oxidative stress activates metal-responsive transcription factor-1 binding activity. The Journal of Biological Chemistry 271: 26233-26241.

Downs, C. A., J. E. Fauth, and C. M. Woodley. 2001. Assessing the health of grass shrimp (*Palaeomonetes pugio*) exposed to natural and anthropogenic stressors: a molecular biomarker system. Marine Biotechnology 3: 380-397.

Eisler, R. 1985. Cadmium Hazards to Fish, Wildlife, and Invertebrates: a synoptic Review. U.S. Fish and Wildlife Service, Patuxent Wildlife Research Center, Laurel, Maryland. 2-15 pp.

Eisler, R. 1987. Mercury Hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Fish and Wildlife Service, Patuxent Wildlife Research Center, Laurel, Maryland. 2-42 pp.

Eisler, R. 1993. Zinc hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Department of the Interior Fish and Wildlife Service, Patuxent Wildlife Research Center, Laurel, Maryland. 57 pp.

Eisler, R. 1998. Copper hazards to fish, wildlife, and invertebrates: a synoptic review. U.S. Geological Survey, Patuxent Wildlife Research Center, Laurel, Maryland. 82 pp.

Engel, D. W., and B. A. Fowler. 1979. Factors influencing cadmium accumulation and its toxicity to marine organisms. Environmental Health Perspectives 28: 81-88.

Engel, D. W., and M. Brouwer. 1993. Crustaceans as models for metal metabolism: I. Effects of the molt cycle on blue crab metal metabolism and metallothionein. Marine Environmental Research 35: 1-5

Girard, C. 1852. A revision of the North American, *Astaci*, with observations on their habitats and geographical distribution. Proceedings. Academy of Natural Sciences of Philadelphia 6: 87-91.

Gunderson, J. 1995. Rusty Crayfish: A Nasty Invader. Minnesota Sea Grant, Duluth, Minnesota. 5 pp.

Guo, Y., Y. Ling, B. A. Thomson, and K. W. Michael Siu. 2005. Combined ion-mobility and mass-spectrometry investigations of metallothionein complexes using a tandem mass spectrometer with a segmented second quadrupole. Journal of the American Society for Mass Spectrometry 16: 1787-1794.

Hobbs, III, H. H., and J. P. Jass. 1988. The crayfishes & shrimp of Wisconsin. Milwaukee Public Museum, Milwaukee, Wisconsin. 52-65 pp.

Hogstrand, C., P. M. Verbost, S. E. Wendelaar Bonga, and C. M. Wood. 1996. Mechanisms of zinc uptake in gills of freshwater rainbow trout: interplay with calcium transport. American Journal of Physiology 270: R1141-R1147.

Horton, M. 2004. The role of crayfish in terrestrial aquatic metal biogeochemical cycles. The Geological Society of America, gsa.confex.com/gsa/2004AM/finalprogram/, accessed 24 April 2007.

Howard, C. L., and C. S. Hacker. 1990. Effects of salinity, temperature, and cadmium on cadmium-binding protein in the grass shrimp, *Palaemonetes pugio.* Archives of Environmental Contamination and Toxicology 19: 341-347.

Hyne R. V., and W. A. Maher. 2001. Macroinvertebrate biomarkers: links to toxicosis and changes in population or communities. Cooperative Research Centre for Freshwater Ecology, enterprise.canberra.edu.au, accessed 30 April 2007.

Jin, T., J. Lu, and M. Nordberg. 1998. Toxicokinetics and biochemistry of cadmium with special emphasis on the role of metallothionein. Neurotoxicology 19: 529-535.

Kagi, J. H., and A. Schaffer. 1988. Biochemistry of metallothionein. Biochemistry 27: 8509-8515.

Kling, P.G., and P. Olsson. 2000. Involvement of differential metallothionein expression in free radical sensitivity of RTG-2 and CHSE-214 cells. Free Radical Biology and Medicine 28: 1628-1637.

Kojima, Y. 1991. Definitions and nomenclature of metallothionein. Methods in Enzymology 205: 8-10.

Lacorn, M., A. Lahrssen, N. Rotzoll, T. J. Simat, and H. Steinhart. 2001. Quantification of metallothionein isoforms in fish liver and its implications for biomonitoring. Environmental Toxicology and Chemistry 20: 140-145.

Legras, S., C. Mouneyrac, J. C. Amiard, C. Amiard-Triquet, and P. S. Rainbow. 2000. Changes in metallothionein concentrations in response to variation in natural factors (salinity, sex, weight) and metal contamination in crabs from a metal rich estuary. Journal of Experimental Marine Biology and Ecology 246: 259-279.

Livingstone, D. R. 2003. Oxidative stress in aquatic organisms in relation to pollution and aquaculture. Revue de Médecine. Vétérinaire 154: 427-430.

Lucu C., and V. Obersnel. 1996. Cadmium influx across isolated *Carcinus* gill epithelium interaction of lanthanum and calcium with cadmium influxes. Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology 166: 184-189.

Margoshes, M., and B. L. Vallee. 1957. A cadmium protein from equine kidney cortex. Journal of the American Chemical Society 79: 4813-4814.

Martin-Diaz, M. L., S. R. Tuberty, C. L. McKenney, Jr., J. Blasco, C. Sarasquete, and T. A. Delvalls. 2006. The use of bioaccumulation, biomarkers, and histopathology diseases in *Procambarus clarkii* to establish bioavailability of Cd and Zn after a mining spill. Environmental Monitoring and Assessment 116: 169-184.

Mouneyrac, C., C. Amiard-Triquet, J. C. Amiard, and P. S. Rainbow. 2001. Comparison of metallothionein concentrations and tissue distribution of trace metals in crabs (*Pachygrapsus marmoratus*) from a metal-rich estuary, in and out of reproductive season. Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology, and Endocrinology 129: 193-209.

Munoz, A., F. Laib, D. H. Petering, and C. F. Shaw, III. 1999. Characterization of the cadmium complex of peptide 49-61: a putative nucleation center for cadmium induced folding in rabbit liver metallothionein IIA. Journal of Biological Inorganic Chemistry 4: 495-507.

Munoz, A., D. H. Petering, and C. F. Shaw, III. 2000. Structure-reactivity relationships among metallothionein three-metal domains: role of non-cysteine amino acid residues in lobster metallothionein and human metallothionein-3. Inorganic Chemistry 39: 6114-6123.

Munoz, A., F. H. Forsterling, C. F. Shaw, III, and D. H. Petering. 2000. Determination of the solution three dimensional structure of chemically-synthesized native $^{113}Cd_3S_9$ domain of loster metallothionein by NMR spectroscopy. University of Wisconsin-Madison, www.nmrfam.wisc.edu/~amalia/posters/enc-amp/intro.html, accessed 25 April 2007.

Munoz, A., F. H. Forsterling, C. F. Shaw, III, and D. H. Petering. 2002. Structure of the $^{113}Cd_3\beta$ domains from *Homarus americanus* metallothionein-1: hydrogen bonding and solvent accessibility of sulfur atoms. Journal of Biological Inorganic Chemistry 7: 713-724.

Nielson, K. B., C. L. Atkin, and D. R. Winge. 1985. Distinct metal-binding configurations in metallothionein. Journal of Biological Chemistry 260: 5342-5350.

Pedersen, S. N., K. L. Pedersen, P. Hojrup, M. H. Depledge, and J. Knudsen. 1996. Primary structures of decapod crustacean metallothioneins with special emphasis on freshwater and semi-terrestrial species. Biochemical Journal 319: 999-1003.

Pedersen, S. N., A. K. Lundebye, and M. H. Depledge. 1997. Field application of metallothionein and stress protein biomarkers in the shore crab (*Carcinus maenas*) exposed to trace metals. Aquatic Toxicology 37: 183-200

Pizarro, F., M. Olivares, R. Uauy, P. Contreras, A. Rebelo, and V. Gidi. 1999. Acute gastrointestinal effects of graded levels of copper in drinking water. Environmental Health Perspective 107: 117-121.

Pourang, N., J. H. Dennis, and H. Ghourchian. 2004. Tissue distribution and redistribution of trace elements in shrimp species with the emphasis on the roles of metallothionein. Ecotoxicology 13: 519-533.

Ramo, J. D., A. Pastor, A. Torreblanca, J. Medina, and J. Diaz-Mayans. 1989. Cadmium-binding proteins in midgut gland of freshwater crayfish *Procambarus clarkia* 42: 241-46

Richards, M. P. 1989. Recent developments in trace element metabolism and function: role of metallothionein in copper and zinc metabolism. Journal of Nutrition 119: 1062-1070.

Robbins, A.H., D. E. McRee, M. Williamson, S. A. Collett, N. H. Xuong, W. F. Furey, B. C. Wang, and C. D. Stout. 1991. Refined crystal structure of Cd, Zn metallothionein at 2.0 A resolution. Journal of Molecular Biology 221: 1269-1293.

Serafim, M. A., R. M. Company, M. J. Bebianno, and W. J. Langston. 2002. Effect of temperature and size on metallothionein synthesis in the gill of *Mytilus galloprovicialis* exposed to cadmium. Marine Environmental Research 54: 361-365.

Staessen, J. A., J. P. Buchet, G. Ginucchio, R. R. Lauwerys, P. Lijnen, H. Roels, and R. Fagard. 1996. Public health implications of environmental exposure to cadmium and lead: an overview of epidemiological studies in Belgium. Working Groups. Journal of Cardiovascular Risk 3: 26-41.

Thawley, S., S. Morris, and A. Vosloo. 2004. Zn and Cd accumulation in *Potamonautes warreni* from sites in the North-West Province of South Africa. International Congress Series 1275: 180-188.

Vallee, Bert. 1991. Introduction to metallothionein. Methods in Enzymology 205: 3-11.

M. Vasak. 1991. Criteria of purity for metallothioneins. Methods in Enzymology. 205: 44-47

Waalkes, M. P., and P. L. Goering. 1990. Metallothionein and otherr cadmium-binding proteins: recent developments. Chemical Research in Toxicology 3: 281-288.

Wallace, W. G., T. M. Hoexum Brouwer, M. Brouwer, and G. R. Lopez. 2000. Alterations in prey capture and induction of metallothioneins in grass shrimp fed cadmium-contaminated prey. Environmental Toxicology and Chemistry 19: 962-971.

Walsh, C.T., H. H. Sandstead, A. S. Prasad, P. M. Newberne, and P. J. Fraker. 1994. Zinc: health effects and research priorities for the 1990s. Environmental Health Perspectives 102: 5-46.

Willner, H., M. VašăK, and J. H. R. Kagi. 1987. Cadmium-thiolate clusters in metallothionein: spectroscopic and spectropolarimetric features. Biochemistry 26: 6287-6292.

Zhu, Z., E. F. DeRose, G. P. Mullen, D. H. Petering, and C. F. Shaw, III. 1994. Sequential proton resonance assignments and metal cluster topology of lobster metallothionein-1. Biochemistry 33: 8858-8865.

APPENDIX A

ELUTION PROFILES FOR THE SIZE CHROMATOGRAPHIC SEPARATION OF METALLOTHIONEIN-BOUND CADMIUM AND FREE METAL FOUND IN GILL CYTOSOL FROM SPECIMENS OF *Orconectes propinquus* FROM COLLECTION SITES

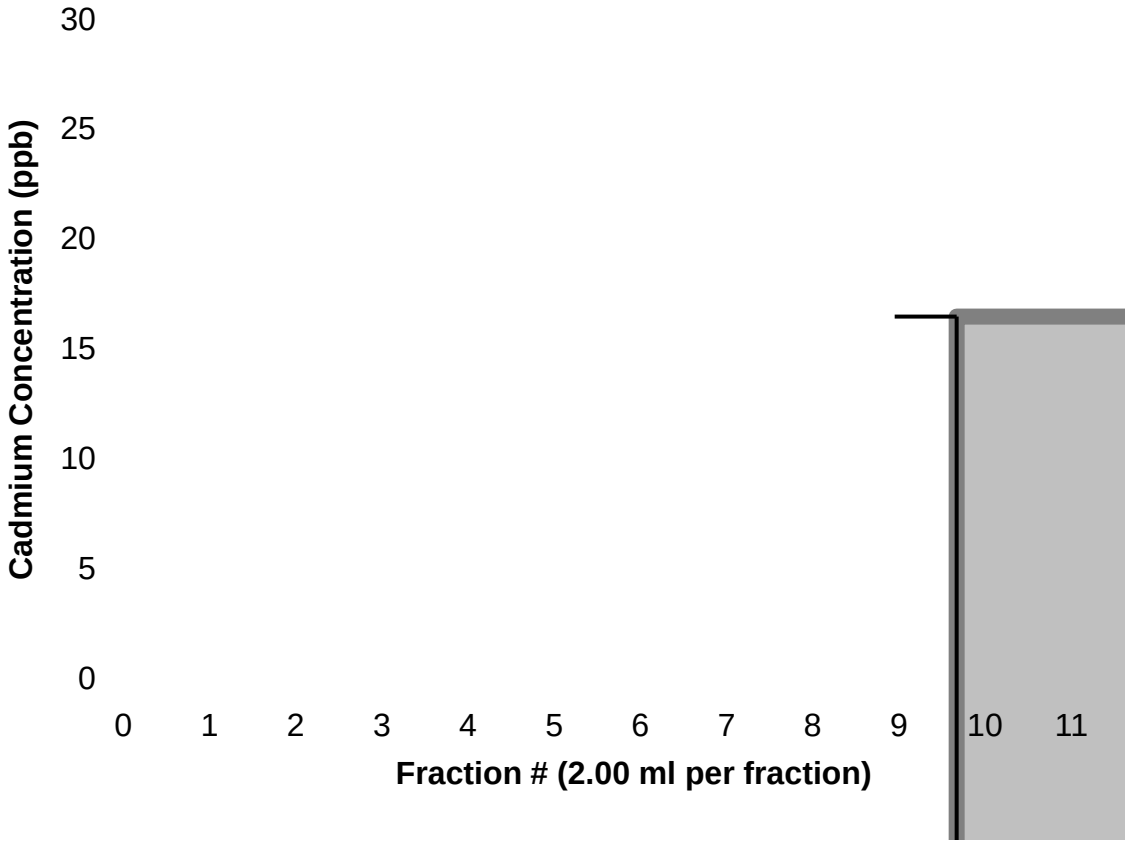


Figure A1: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #1 of *Orconectes propinquus* collected from the Dead River.

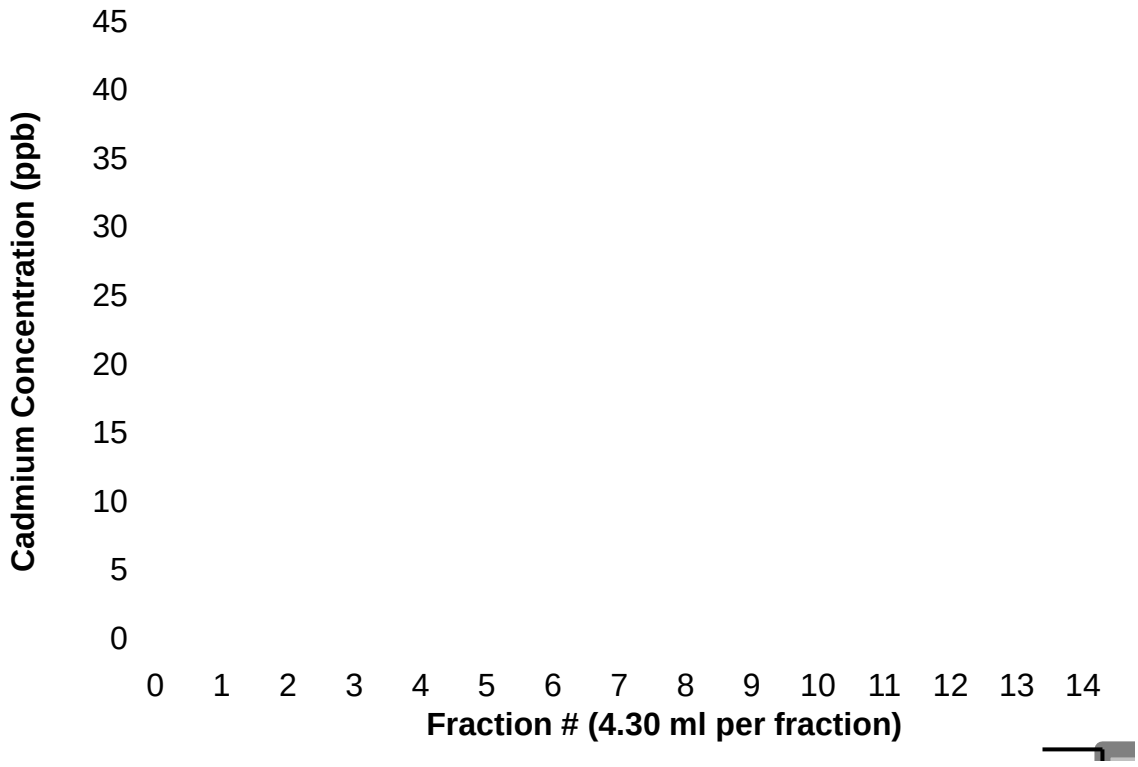


Figure A2: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #2 of *Orconectes propinquus* collected from the Dead River.

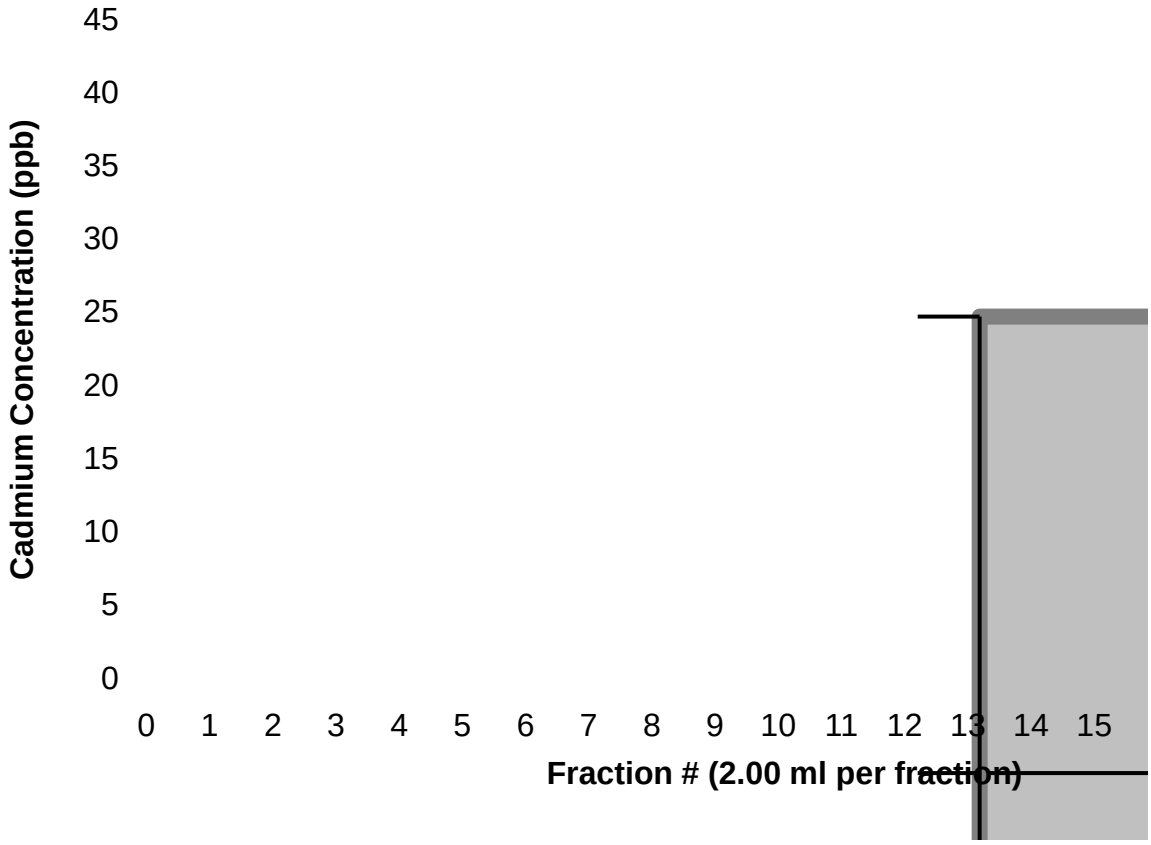


Figure A3: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #3 of *Orconectes propinquus* collected from the Dead River.

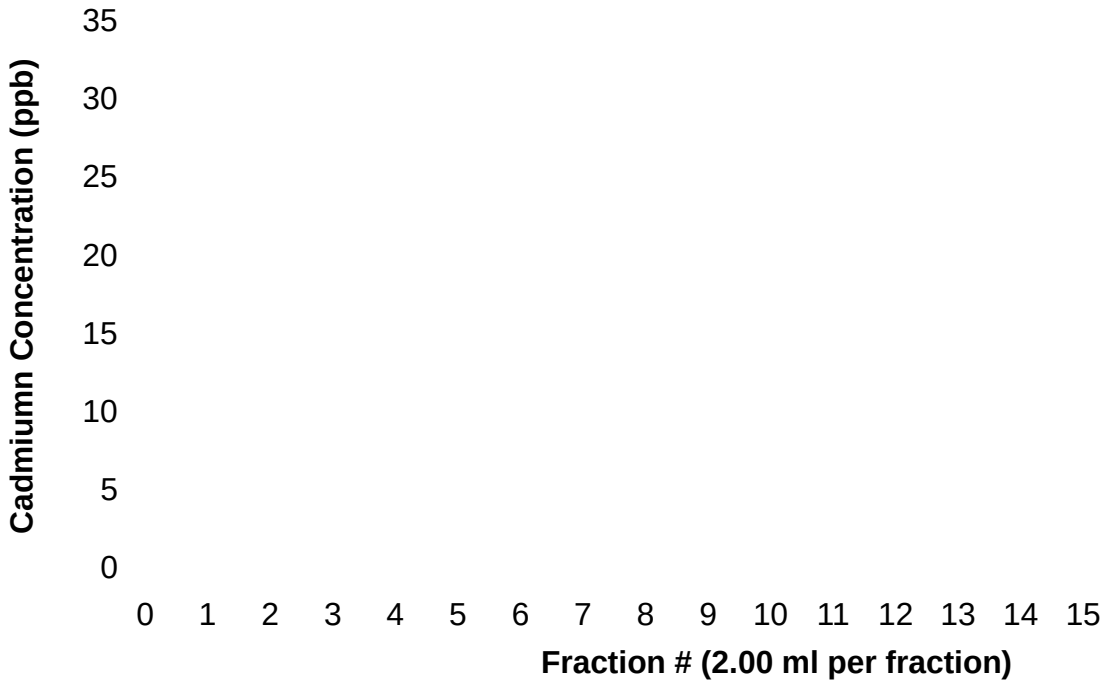


Figure A4: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #4 of *Orconectes propinquus* collected from the Dead River.

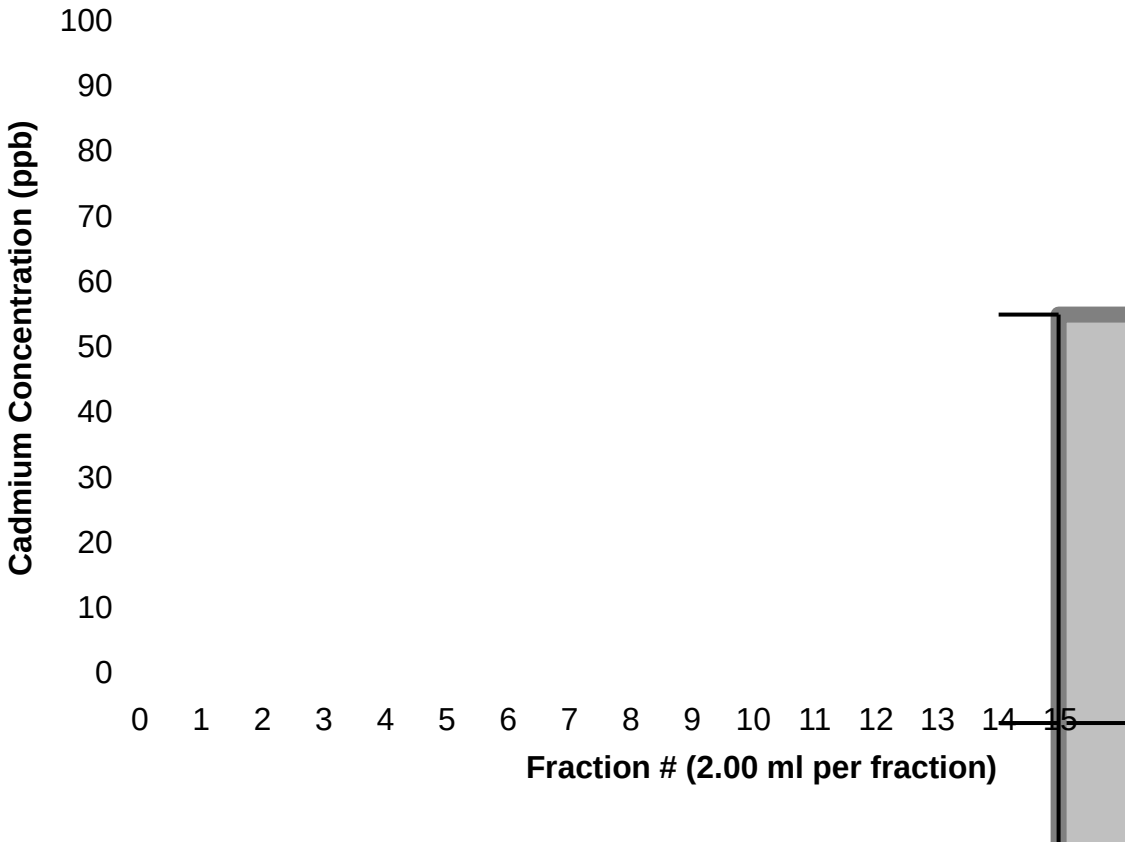


Figure A5: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #5 of *Orconectes propinquus* collected from the Dead River.

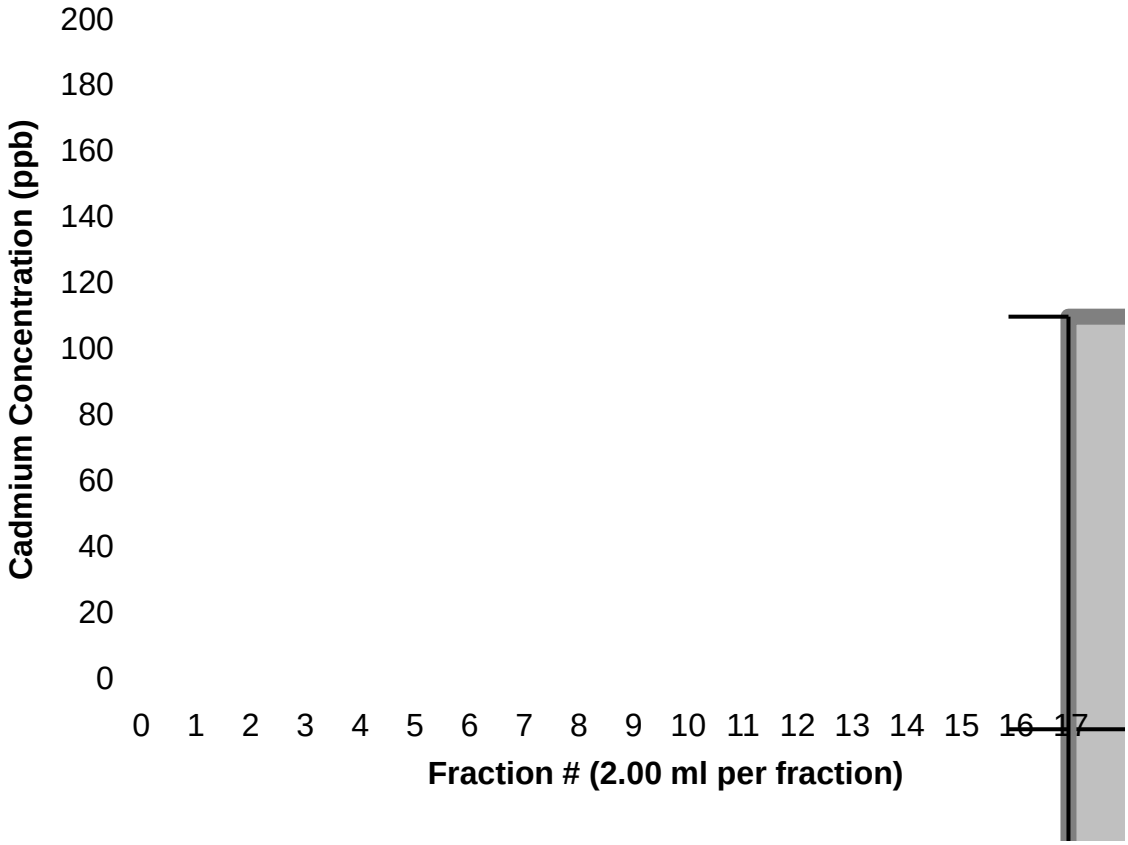


Figure A6: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #6 of *Orconectes propinquus* collected from the Dead River.

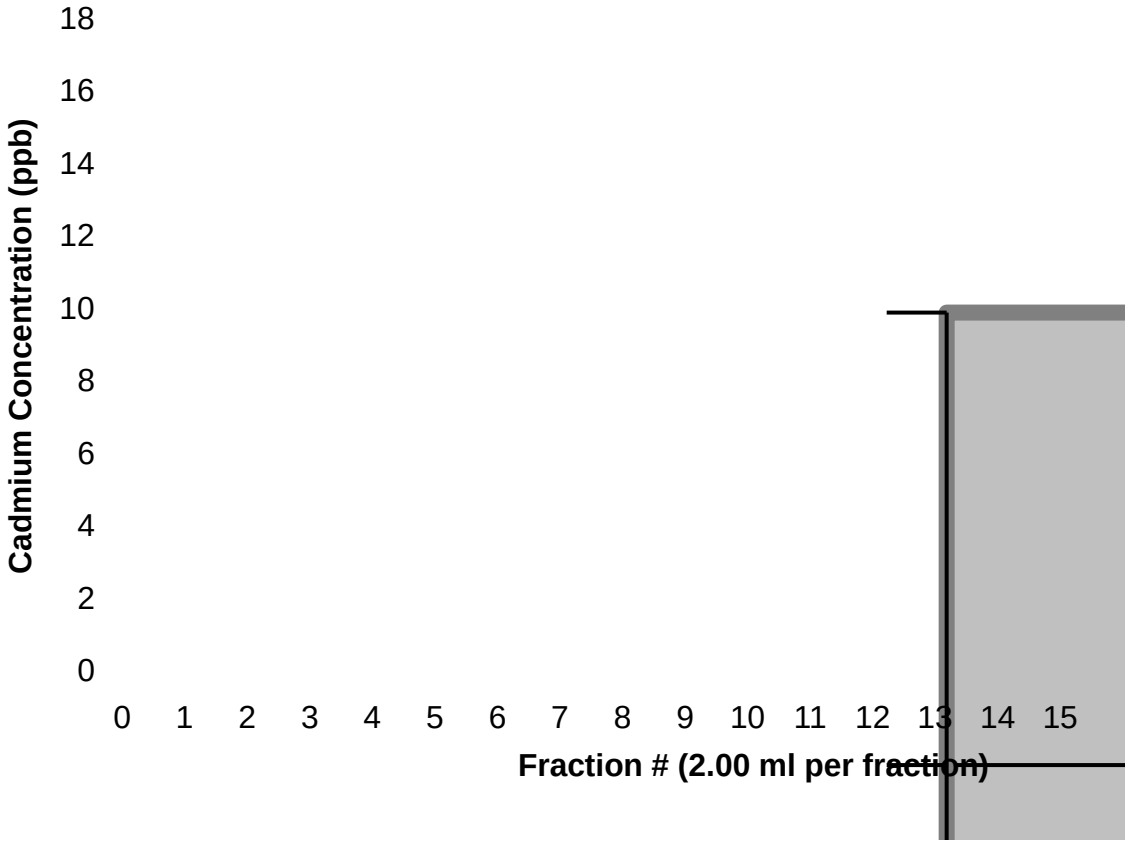


Figure A7: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #7 of *Orconectes propinquus* collected from the Dead River.

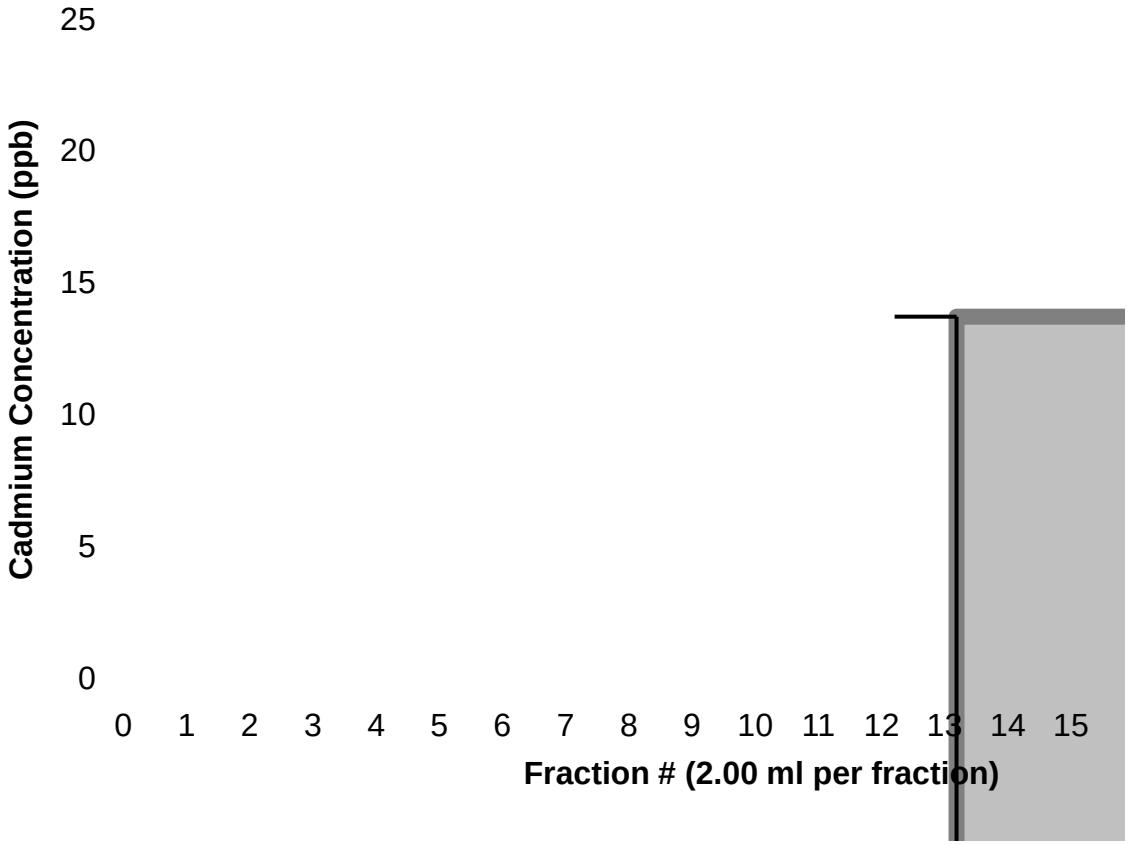


Figure A8: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #8 of *Orconectes propinquus* collected from the Dead River.

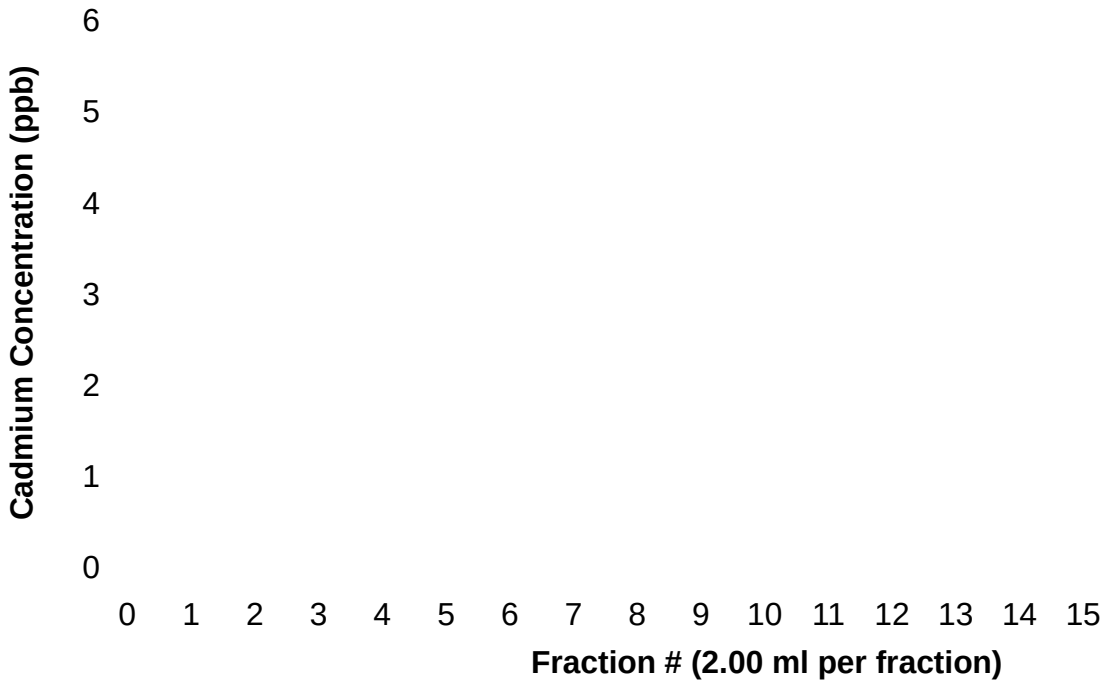


Figure A9: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #9 of *Orconectes propinquus* collected from the Dead River.

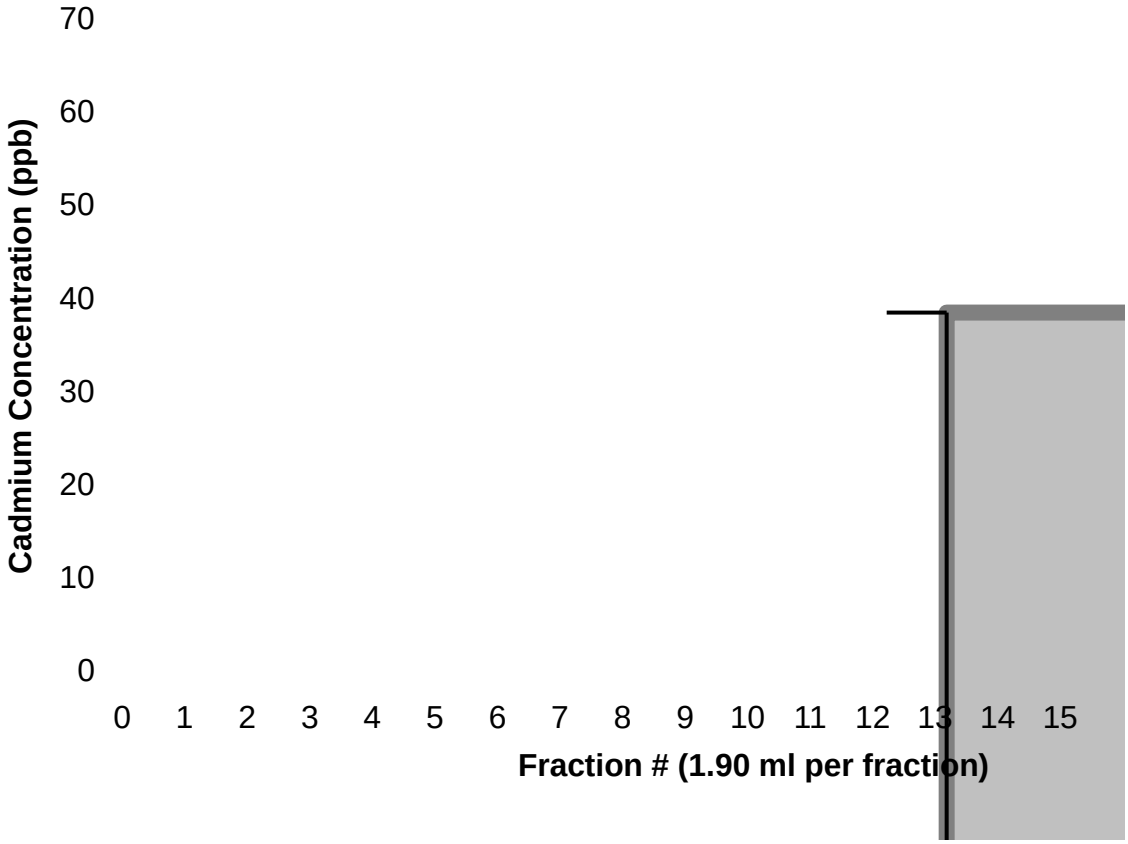


Figure A10: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #10 of *Orconectes propinquus* collected from the Dead River,

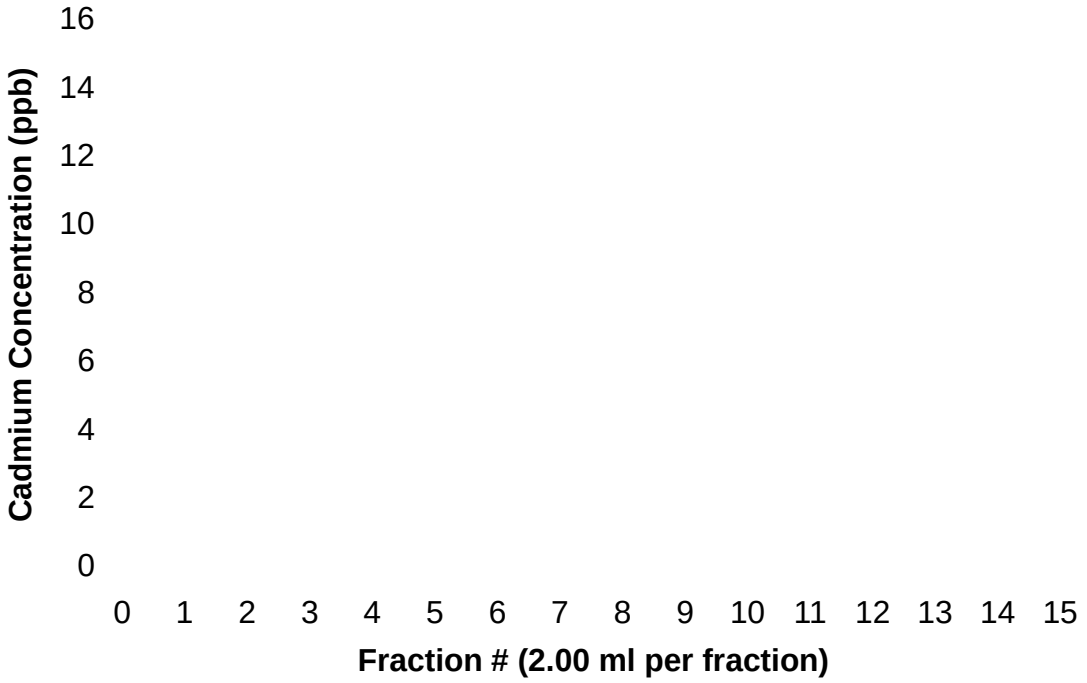


Figure A11: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #12 of *Orconectes propinquus* collected from the Dead River.

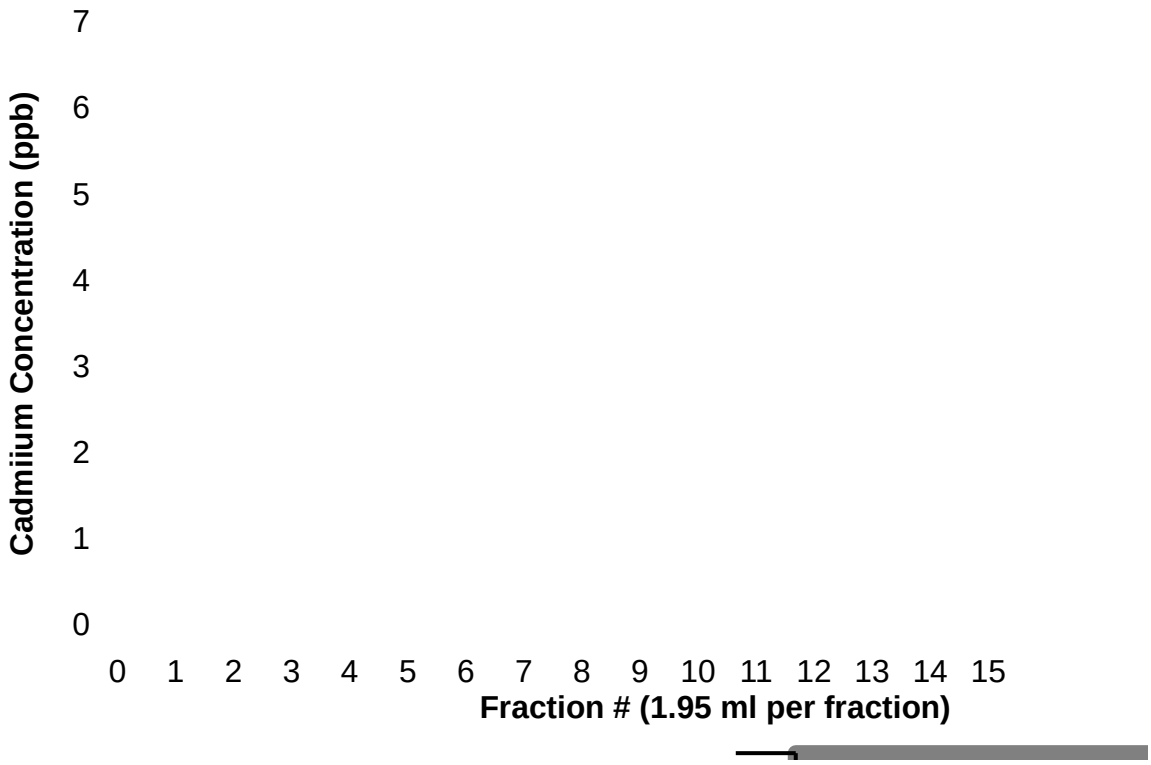


Figure A12: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #1a of *Orconectes propinquus* collected from Deer Lake.

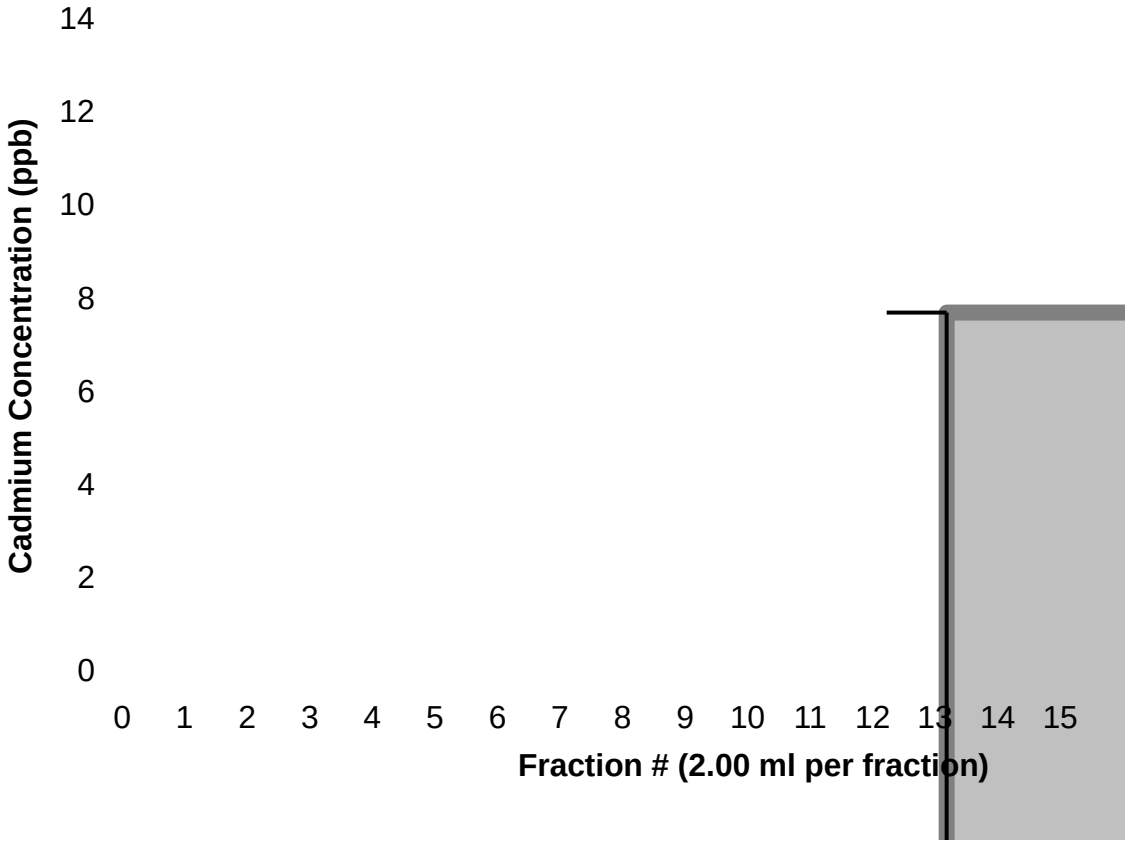


Figure A13: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #1b of *Orconectes propinquus* collected from Deer Lake.

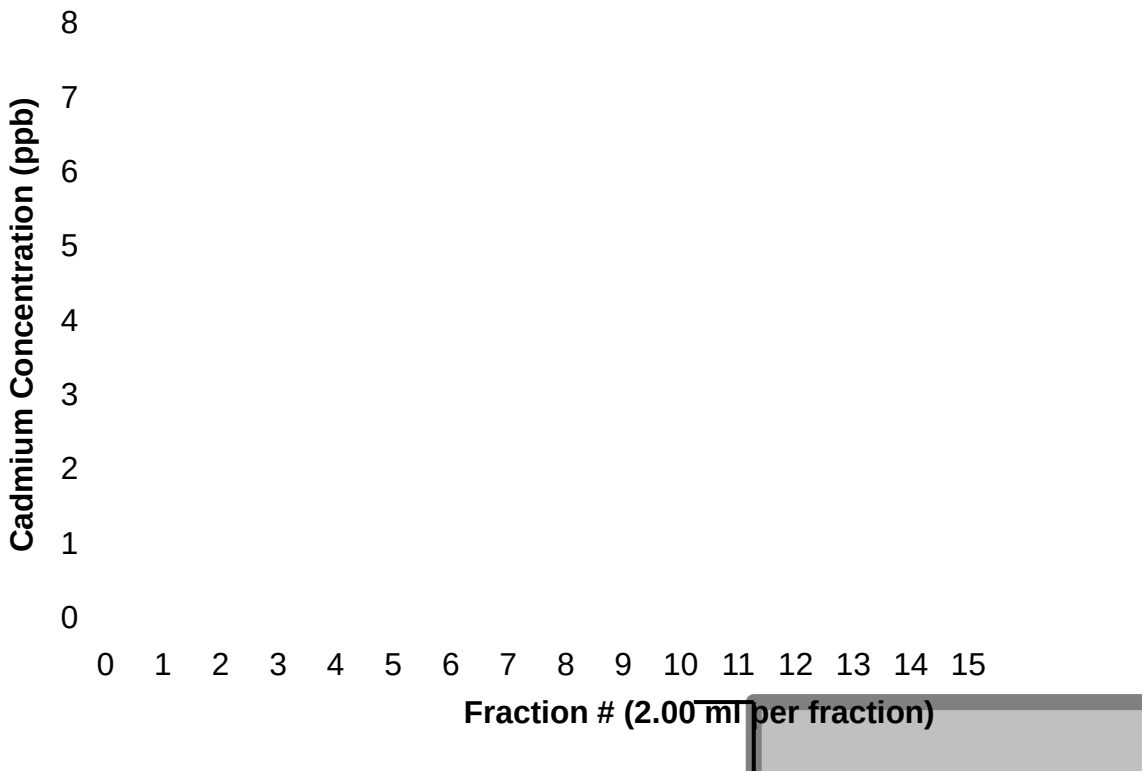


Figure A14: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #2 of *Orconectes propinquus* collected from Deer Lake.

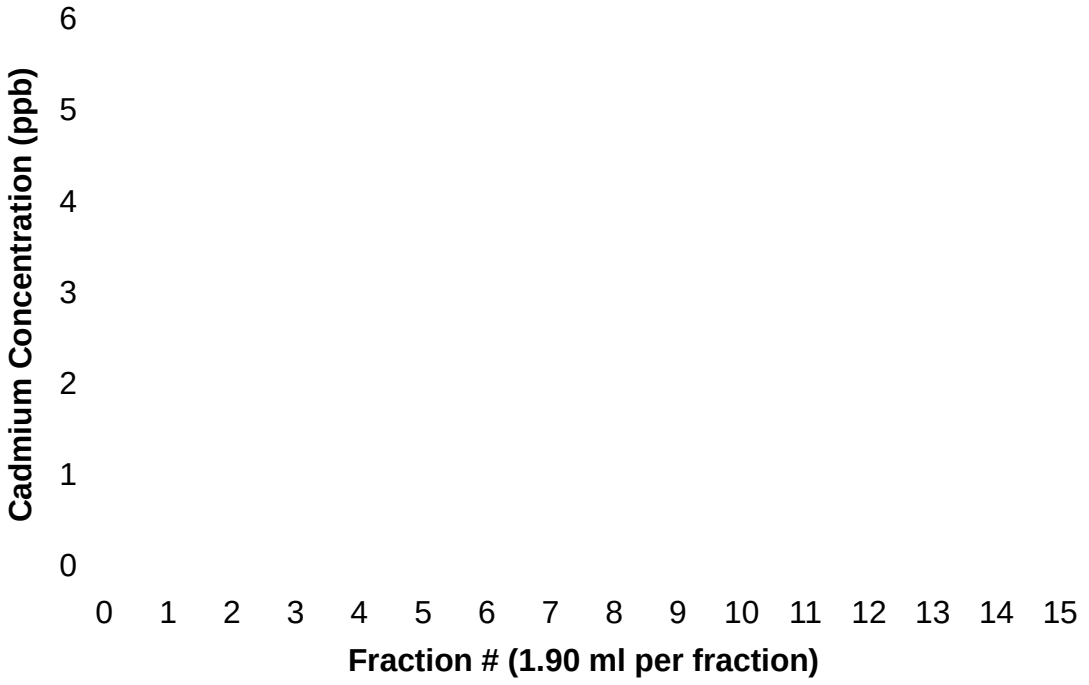


Figure A15: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #4a of *Orconectes propinquus* collected from Deer Lake.

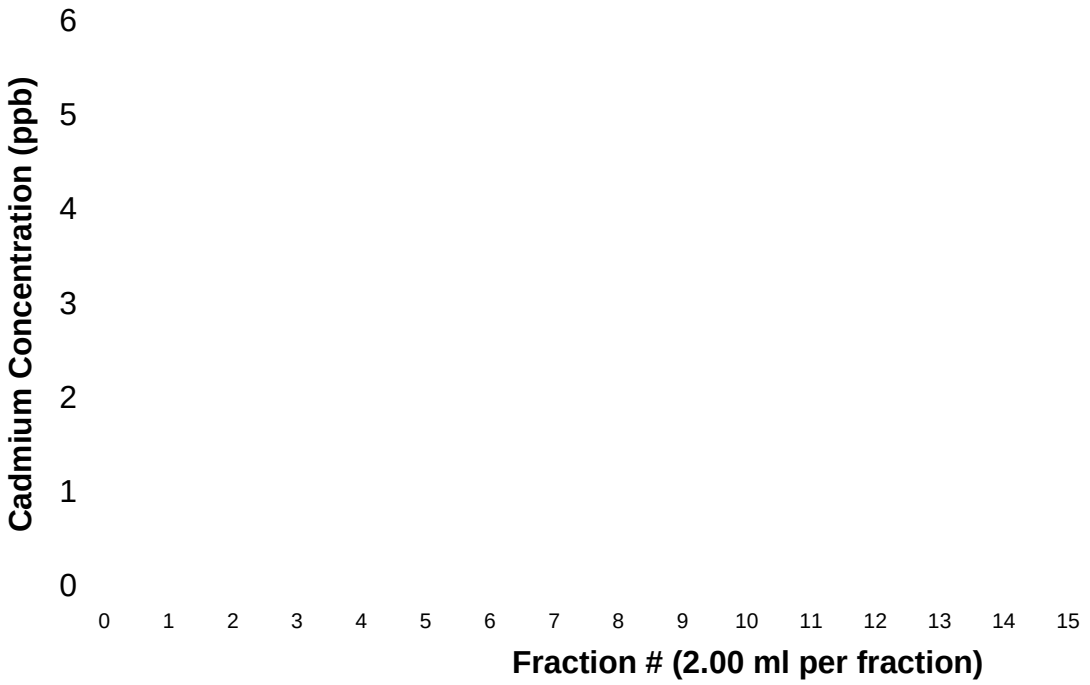


Figure A16: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #4b of *Orconectes propinquus* collected from Deer Lake.

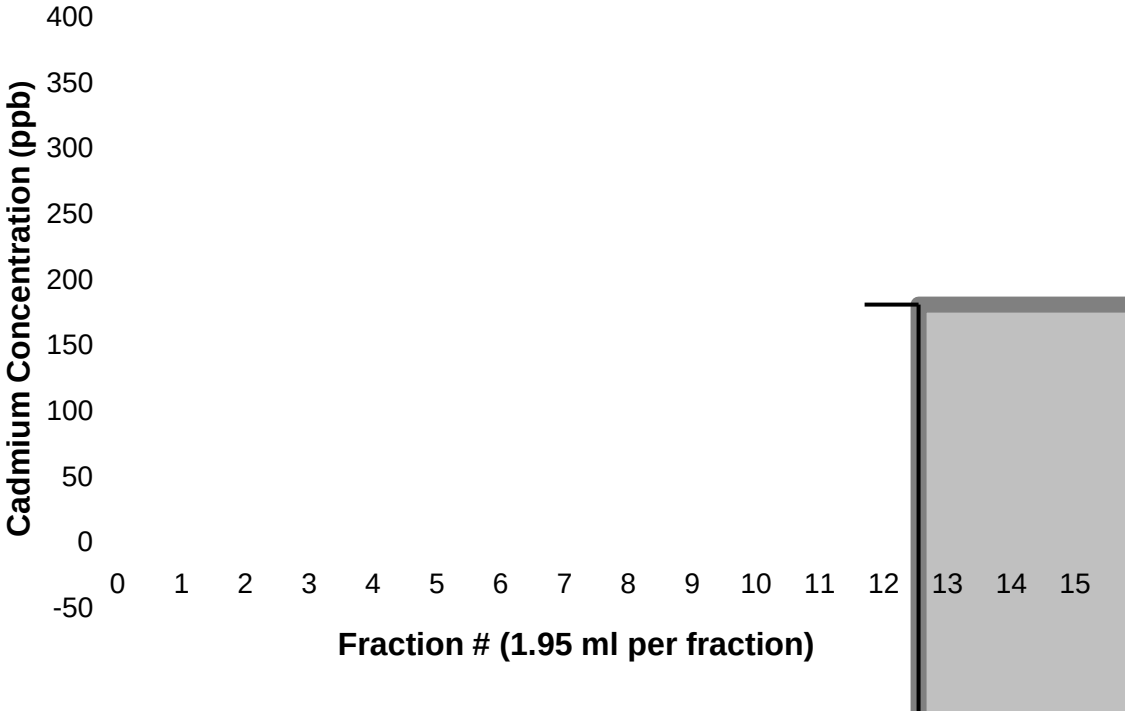


Figure A17: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #5 of *Orconectes propinquus* collected from Deer Lake.

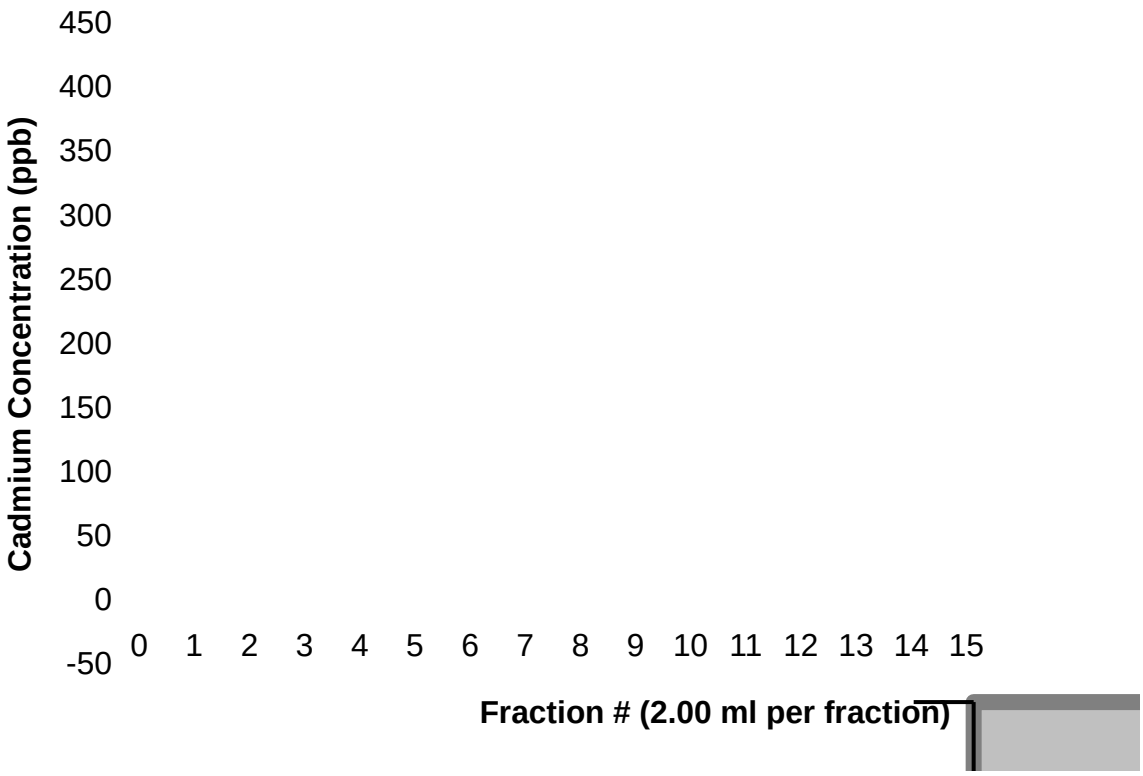


Figure A18: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #6 of *Orconectes propinquus* collected from Deer Lake.

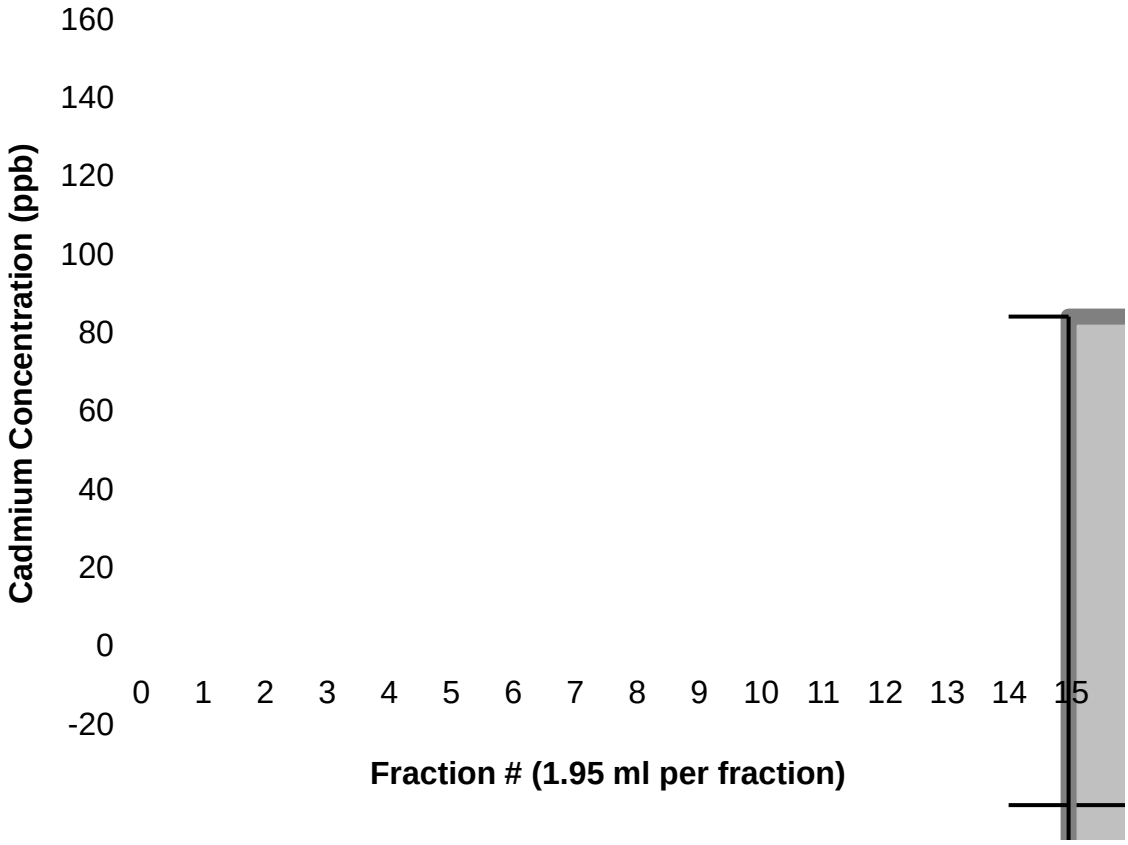


Figure A19: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #8 of *Orconectes propinquus* collected from Deer Lake.

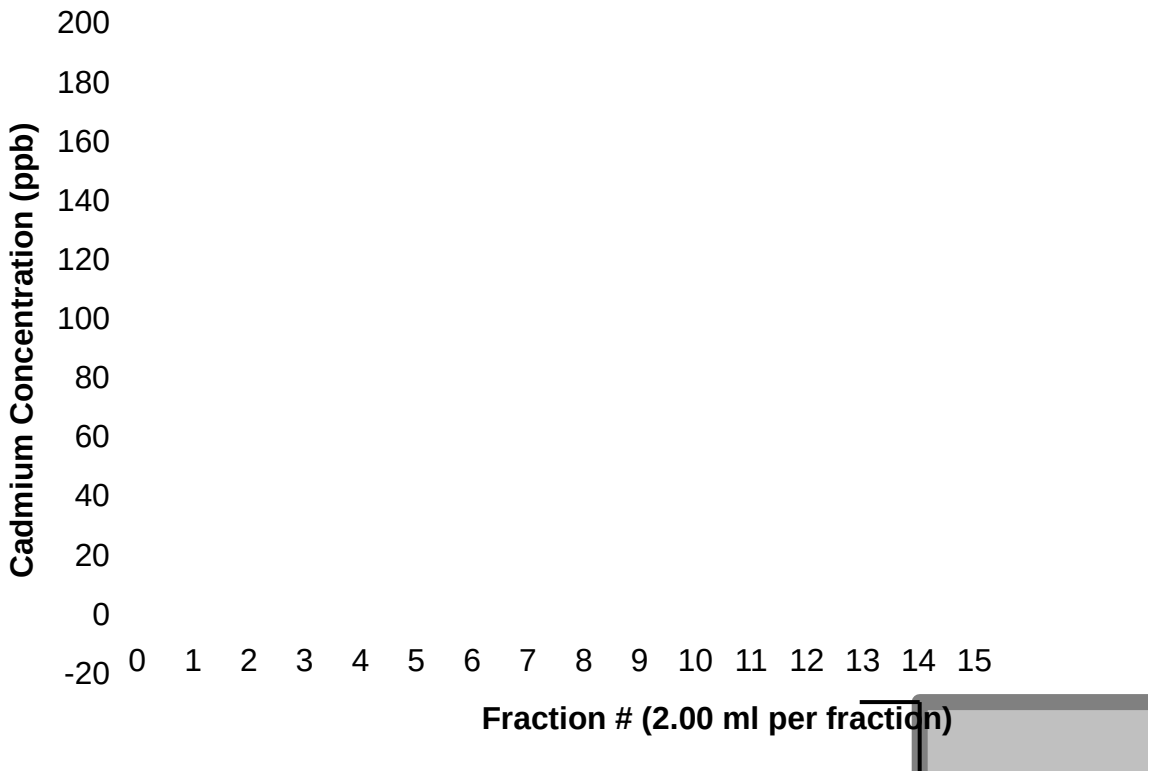


Figure A20: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #10 of *Orconectes propinquus* collected from Deer Lake.

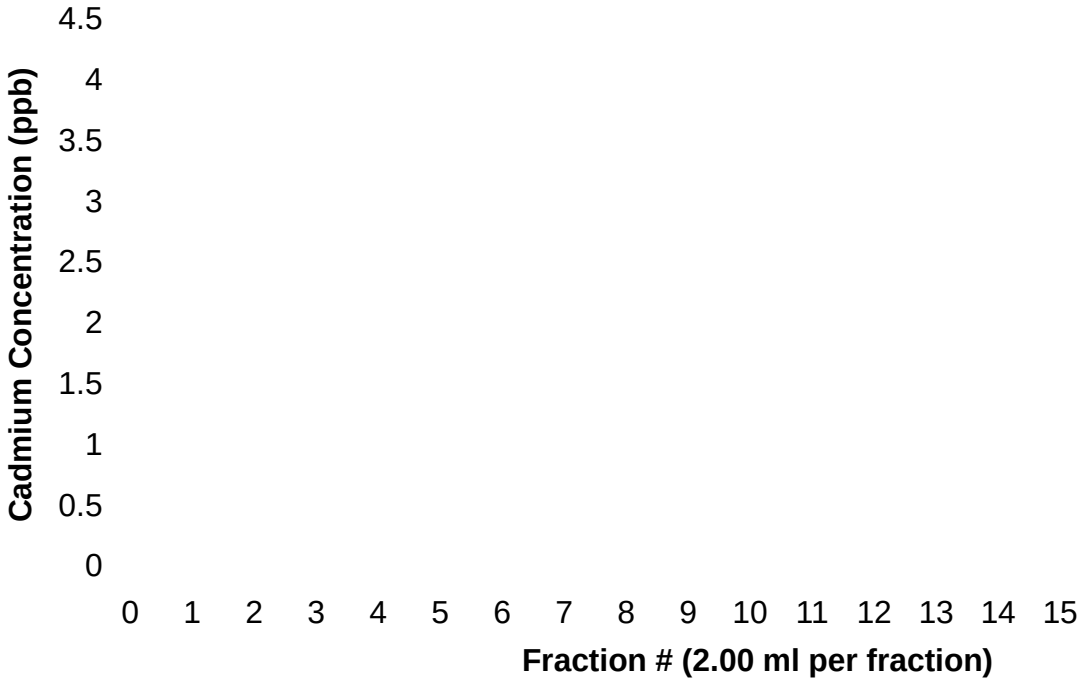


Figure A21: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #11a of *Orconectes propinquus* collected from Deer Lake.

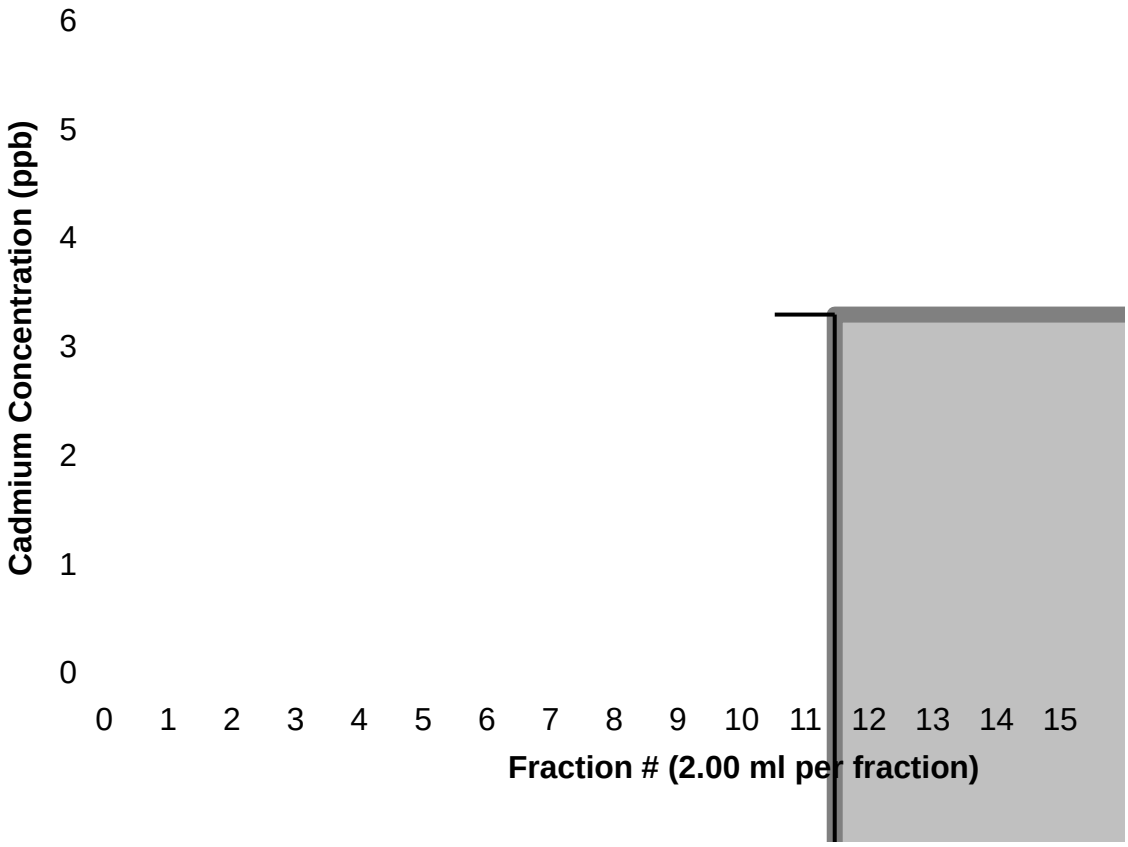


Figure A22: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #11b of *Orconectes propinquus* collected from Deer Lake.

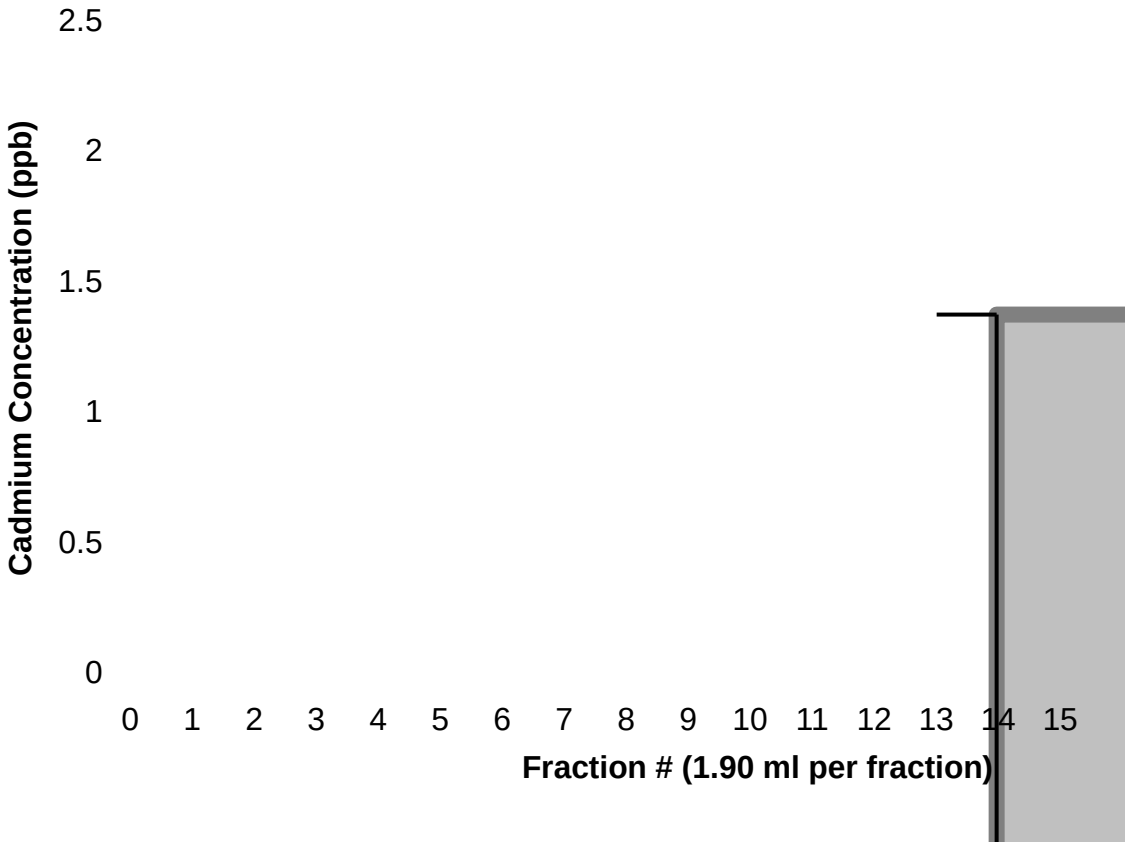


Figure A23: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #12a of *Orconectes propinquus* collected from Deer Lake.

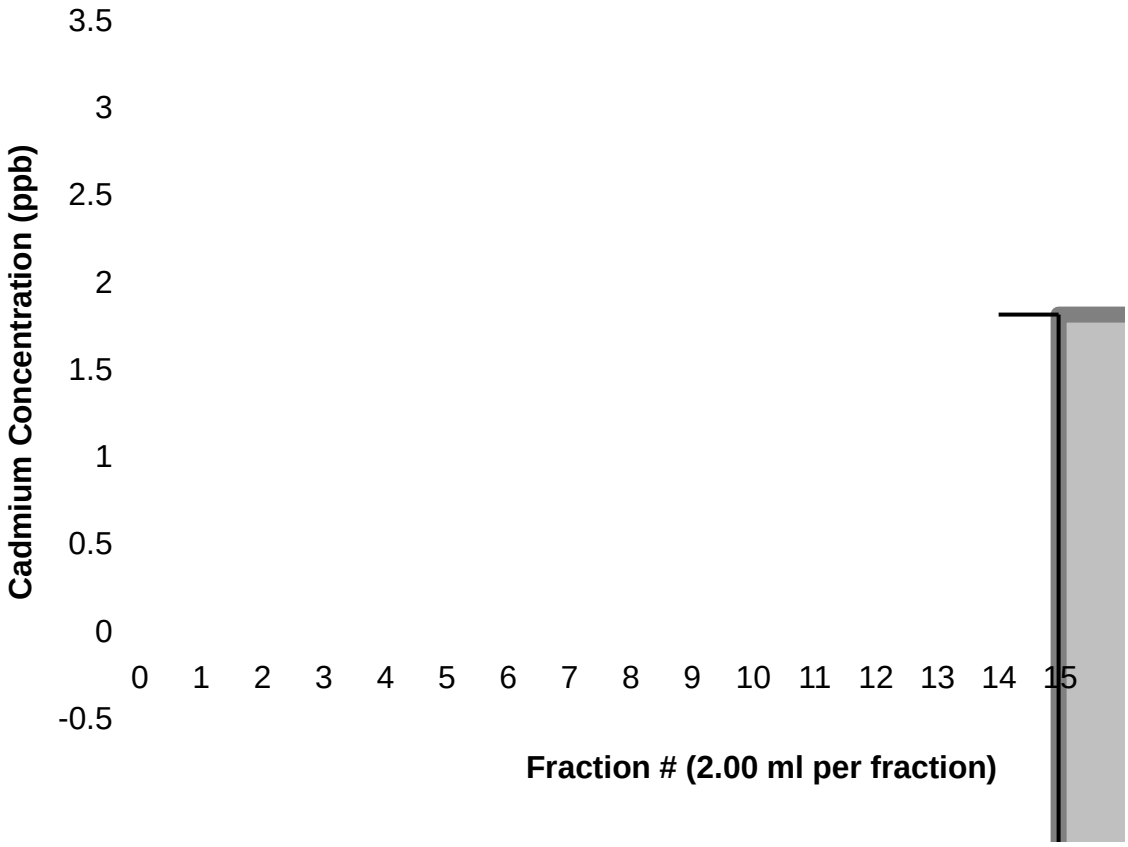


Figure A24: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #12b of *Orconectes propinquus* collected from Deer Lake.

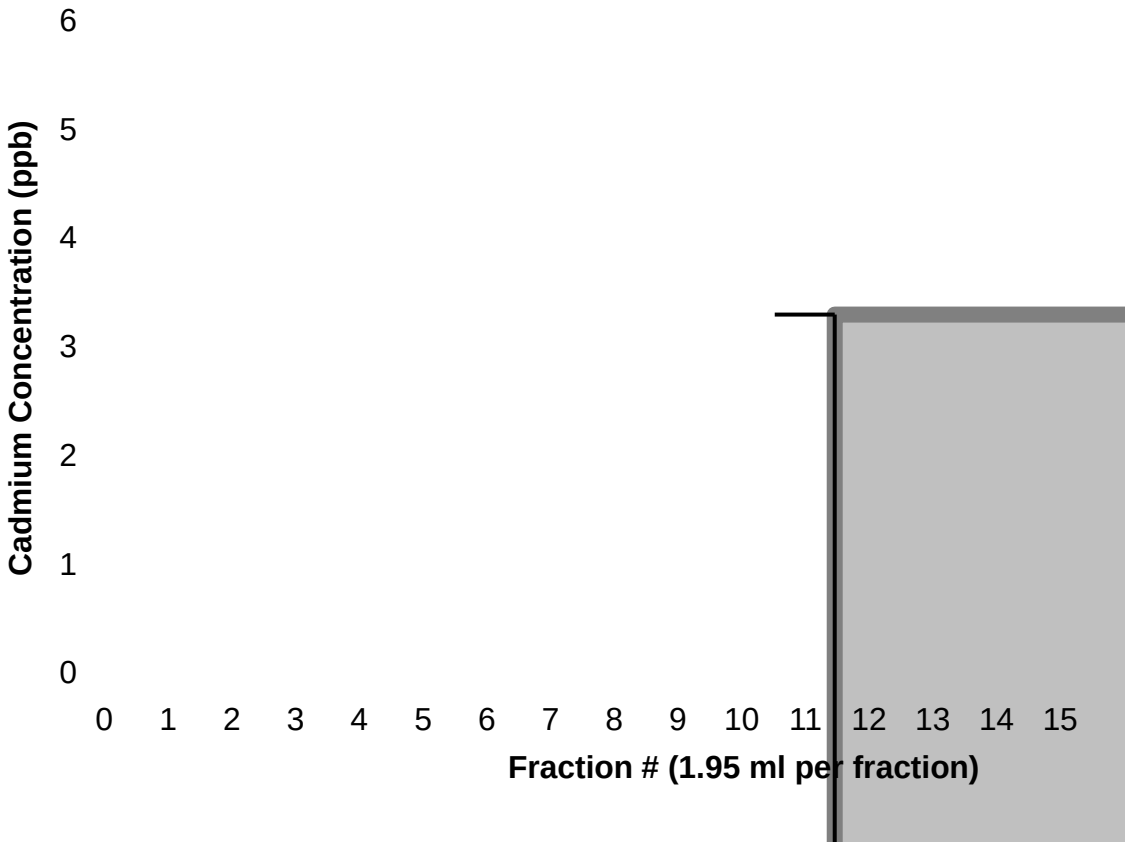


Figure A25: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #13a of *Orconectes propinquus* collected from Deer Lake.

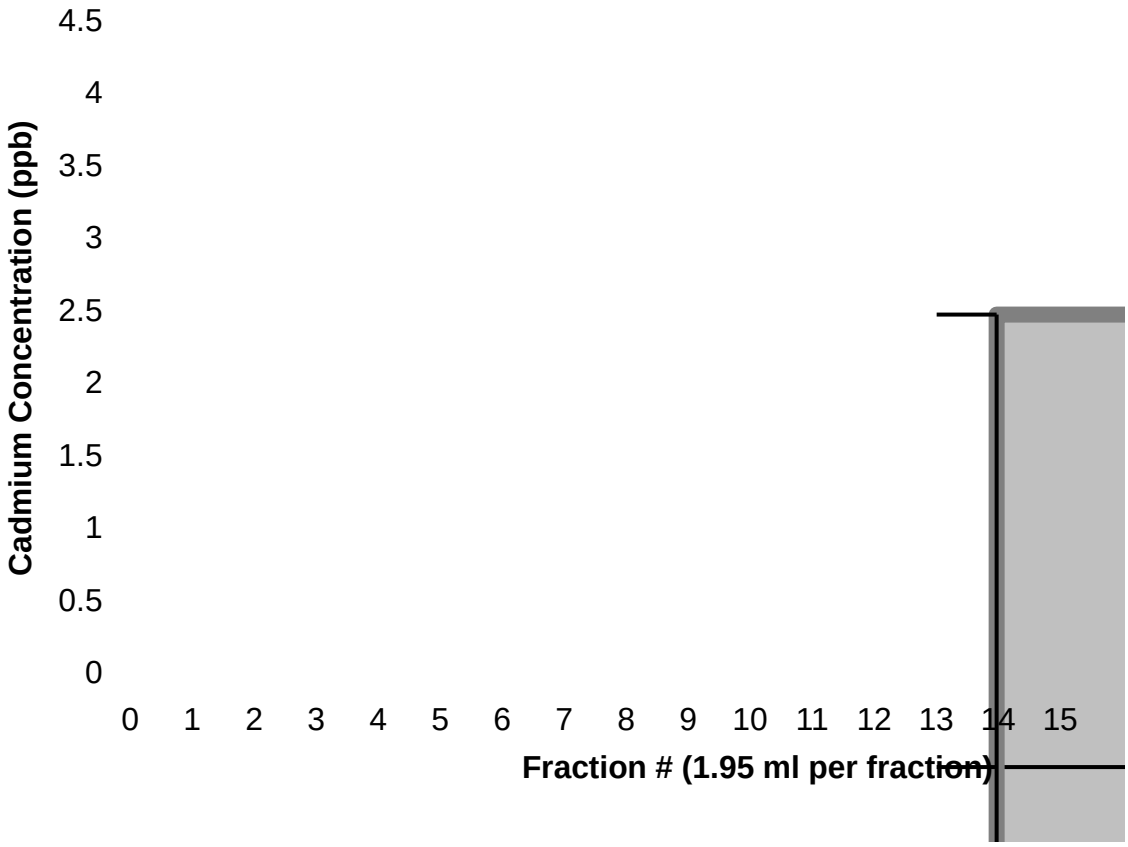


Figure A26: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #13b of *Orconectes propinquus* collected from Deer Lake.

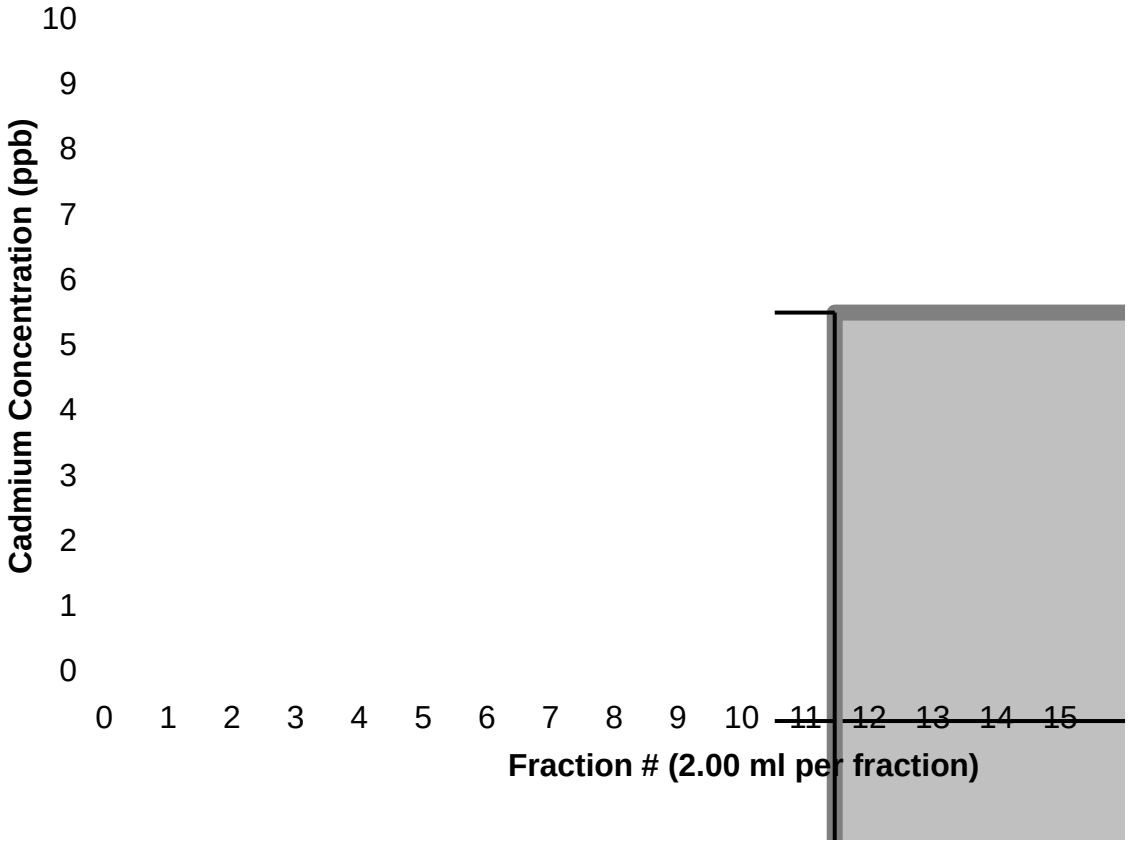


Figure A27: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #1 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

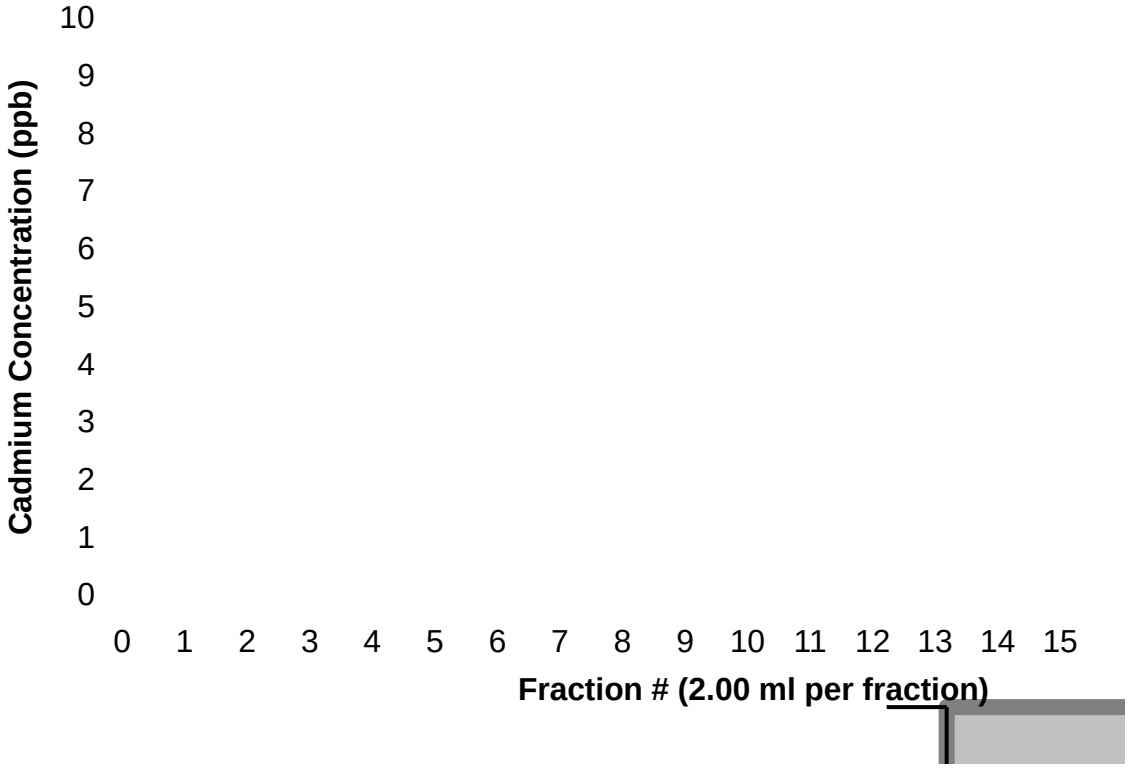


Figure A28: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #5 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

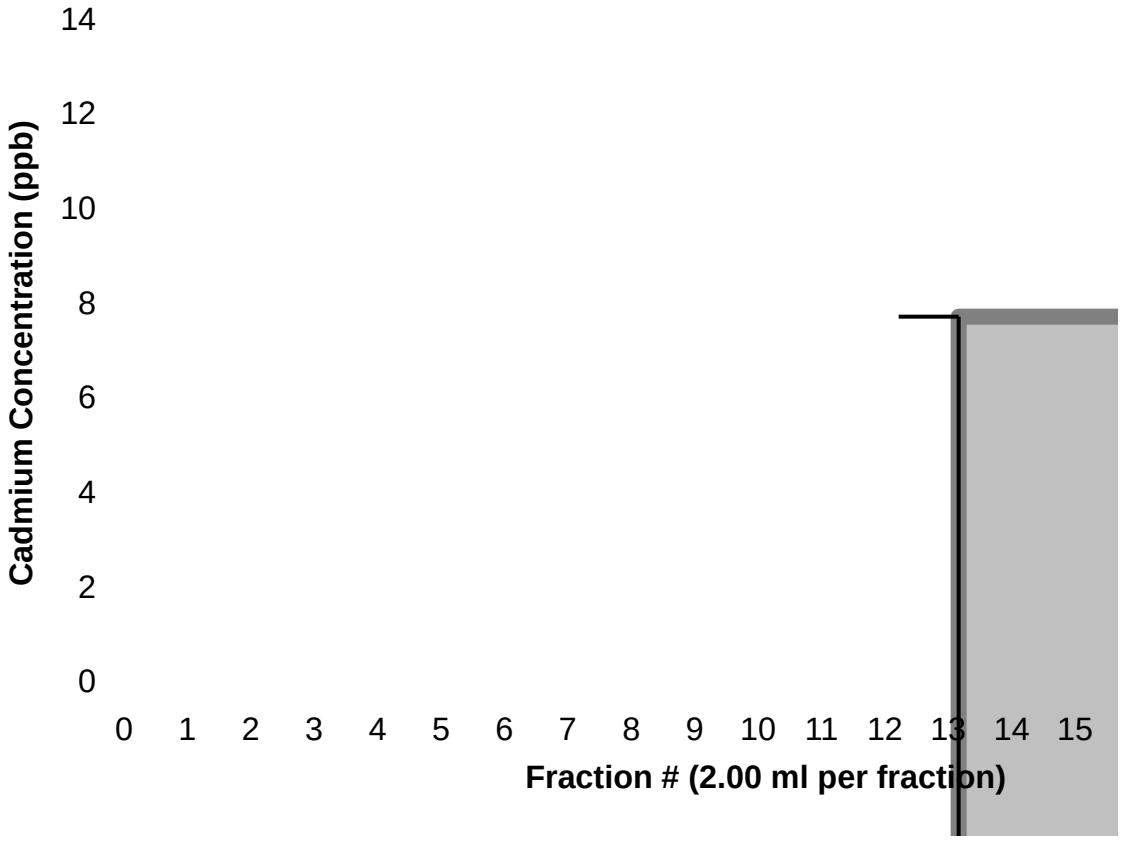


Figure A29: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #6 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

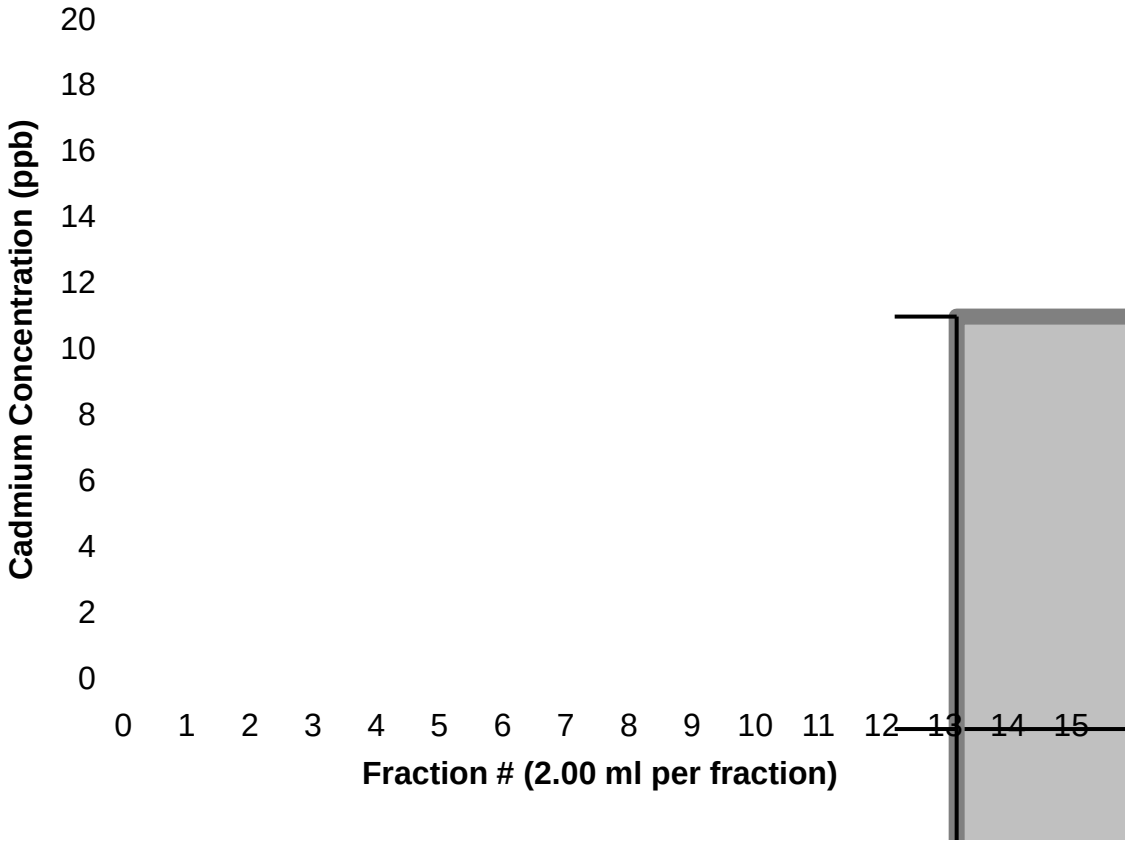


Figure A30: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #7 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

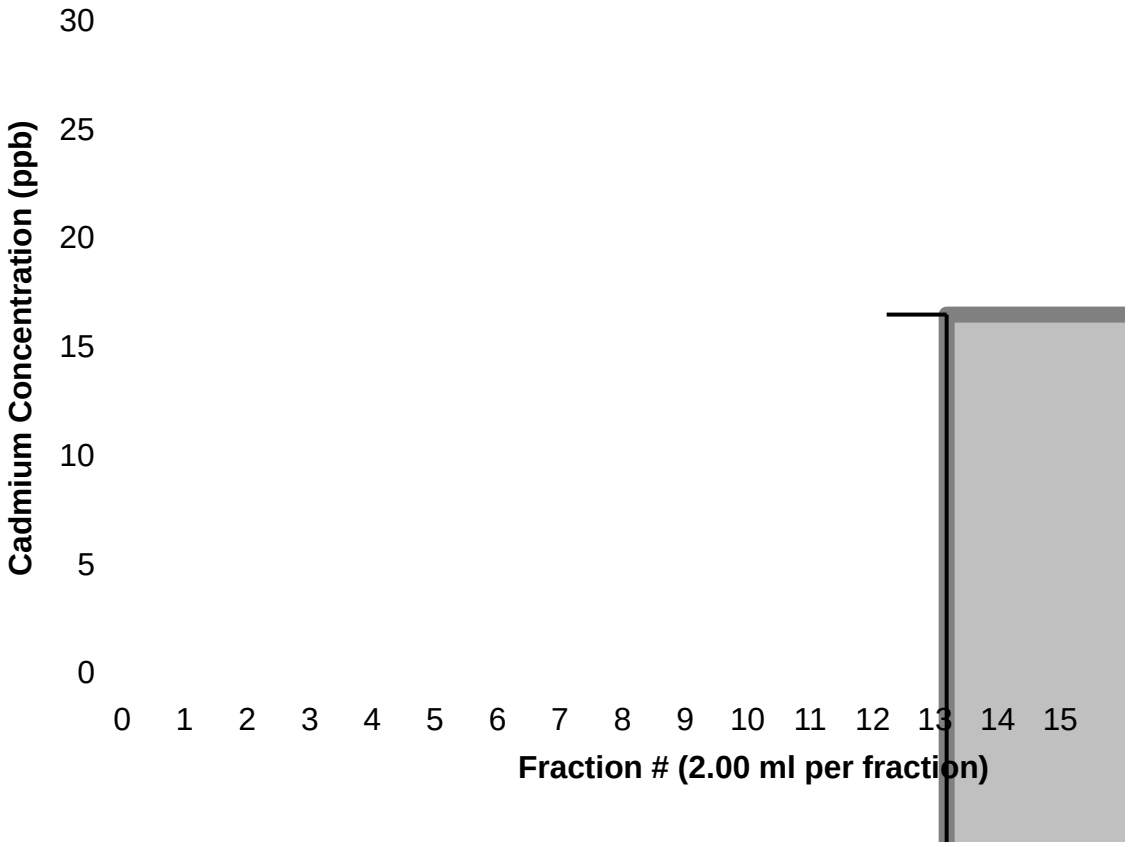


Figure A31: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #8 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

Figure A32: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #10 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

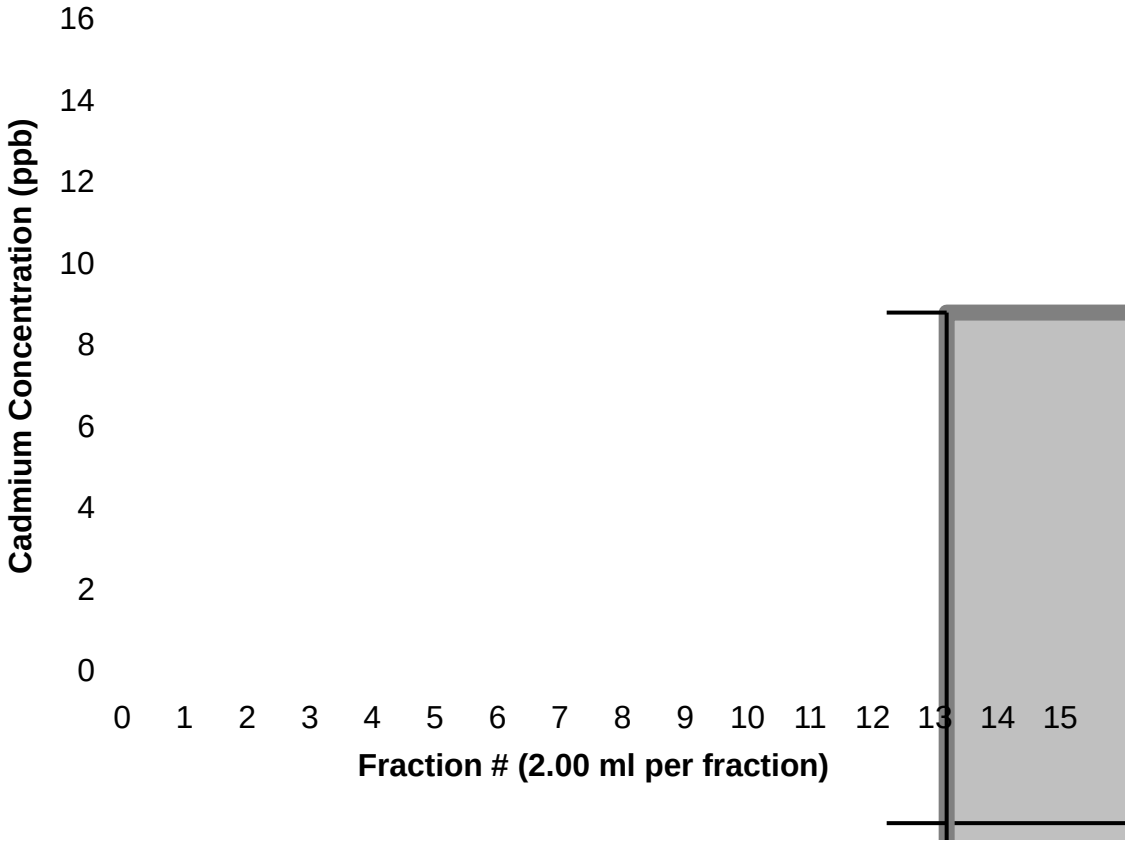


Figure A33: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #11 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

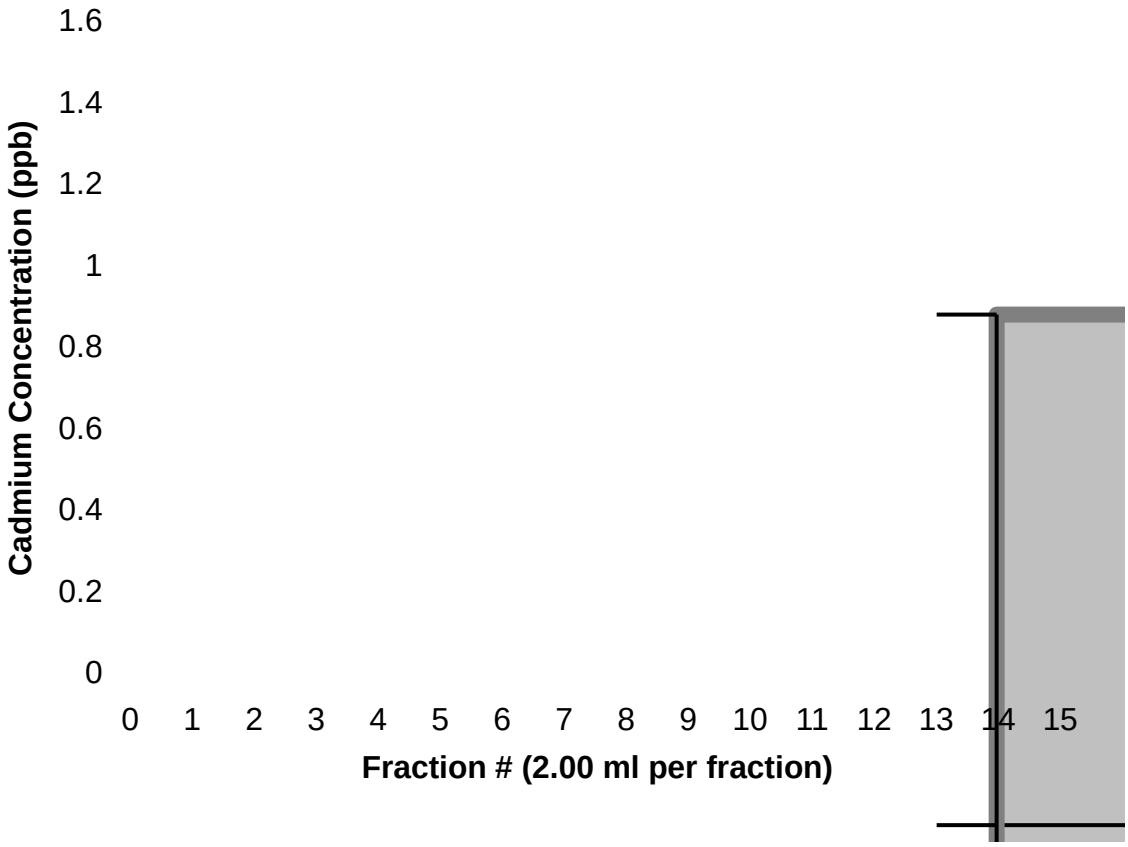


Figure A34: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #12 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

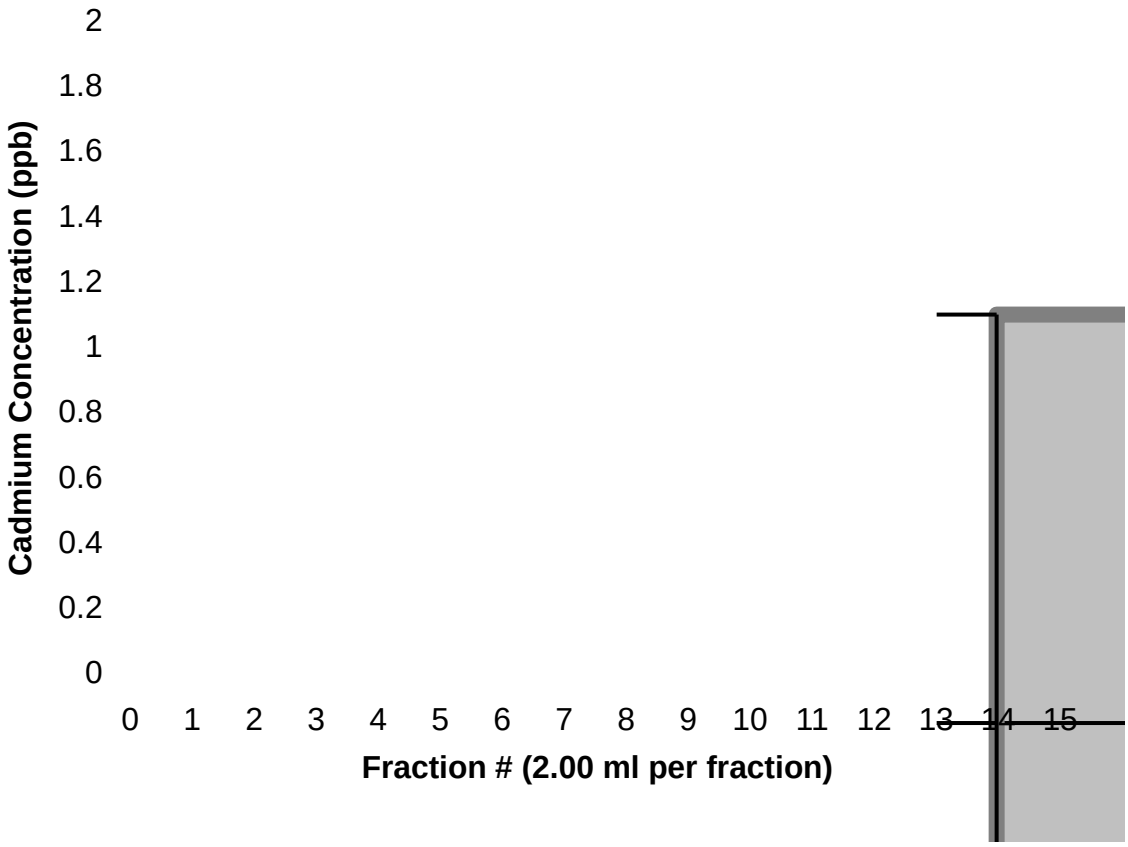


Figure A35: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #18 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

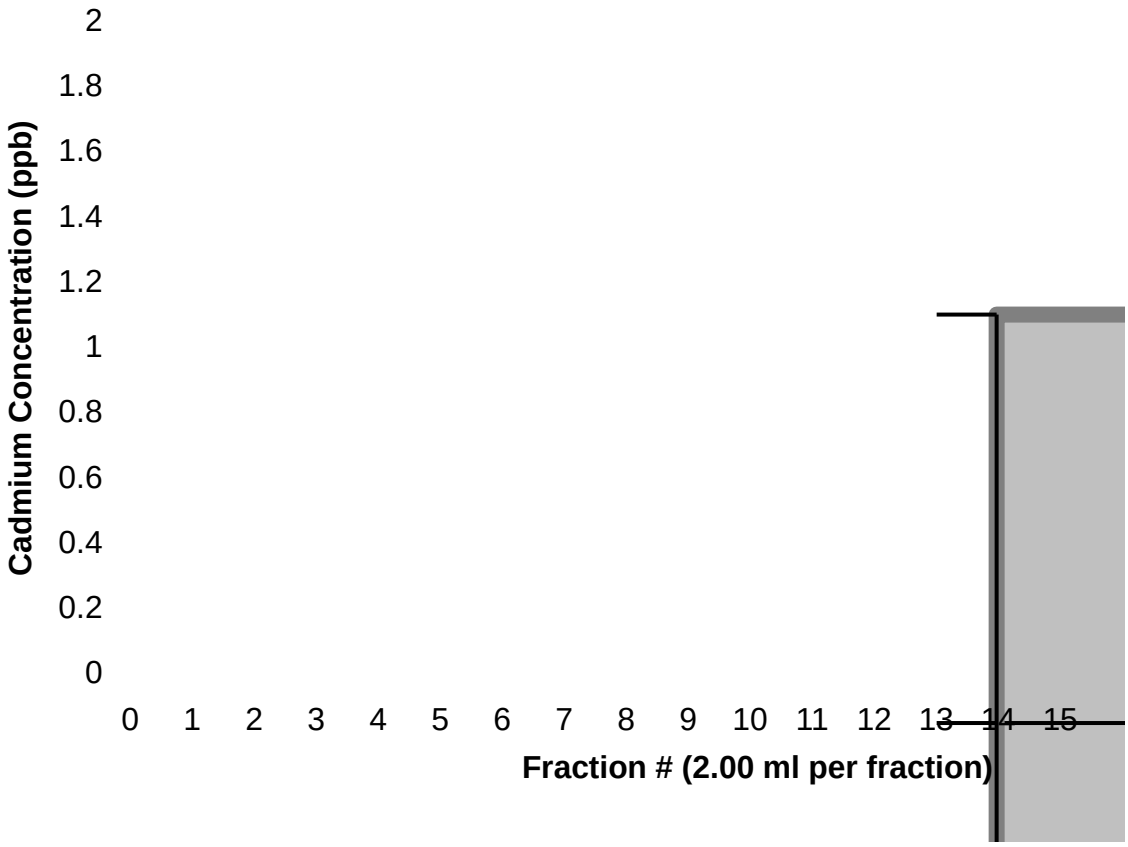


Figure A36: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #19 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

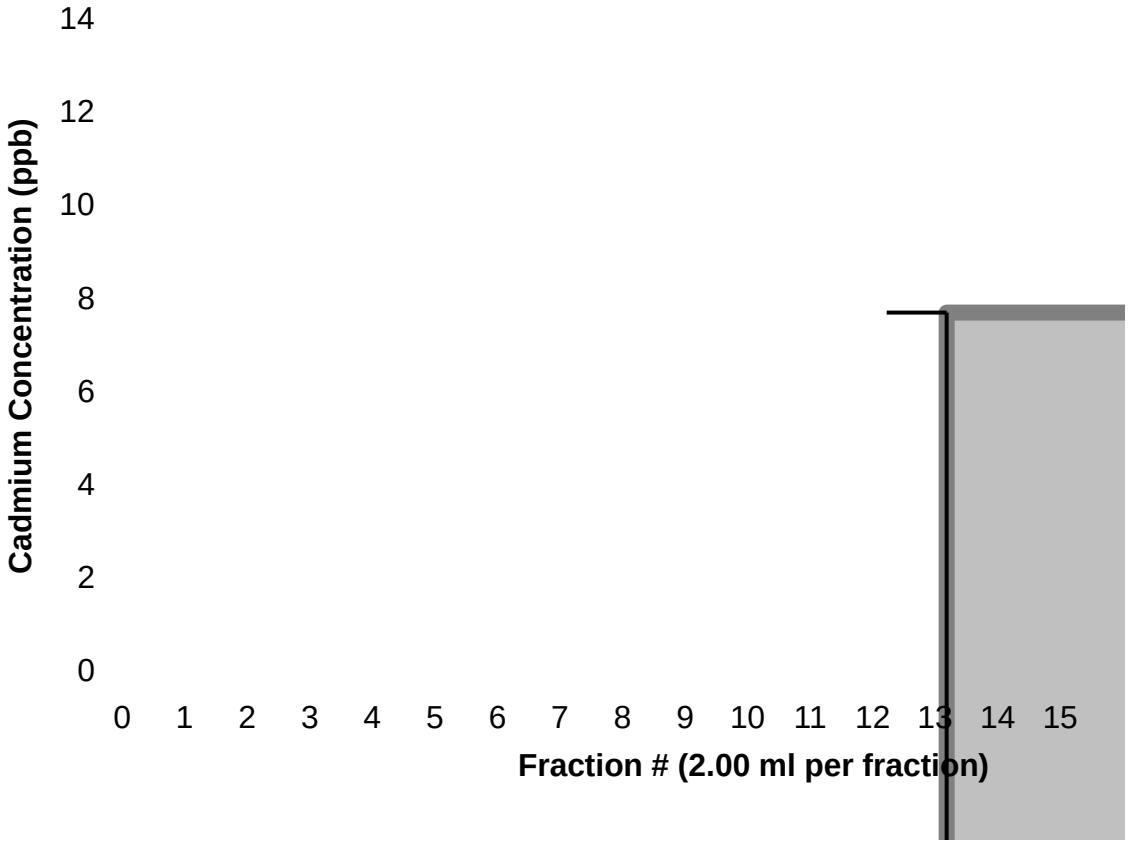


Figure A37: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #1 of *Orconectes propinquus* collected from the Laughing Whitefish River.

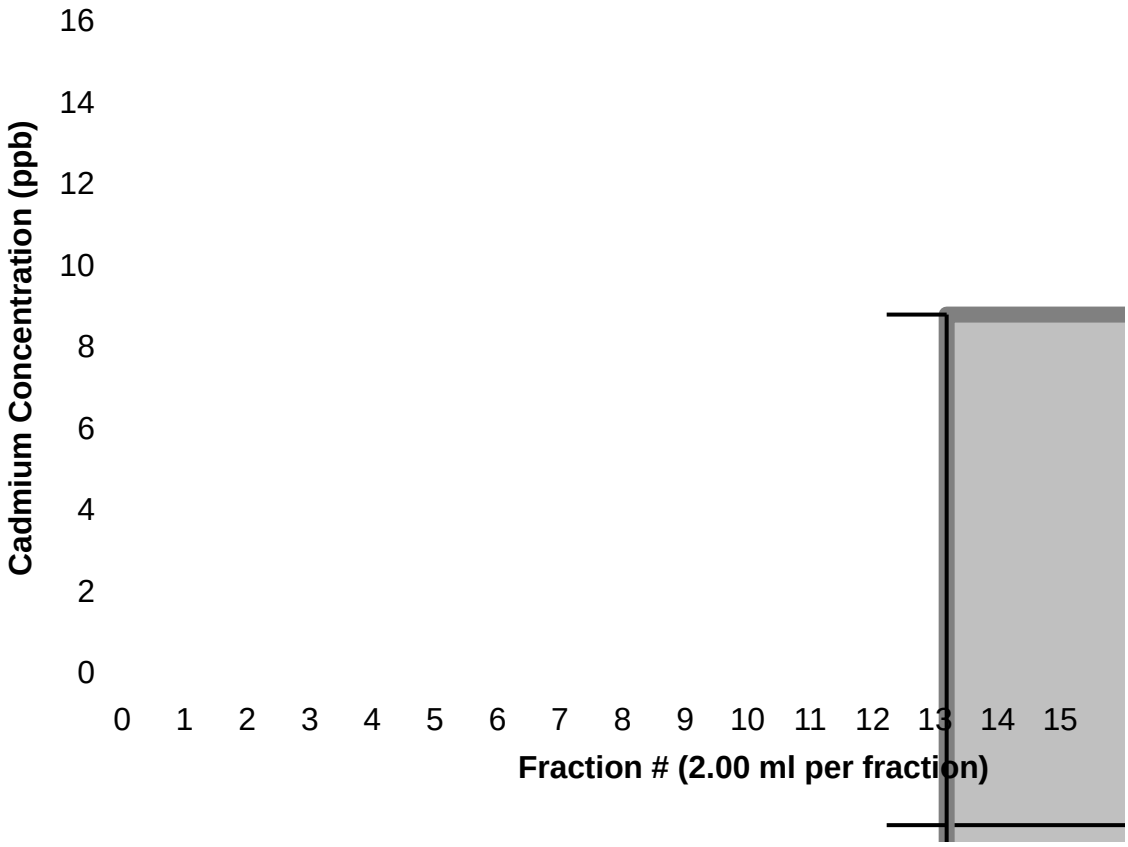


Figure A38: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #3 of *Orconectes propinquus* collected from the Laughing Whitefish River.

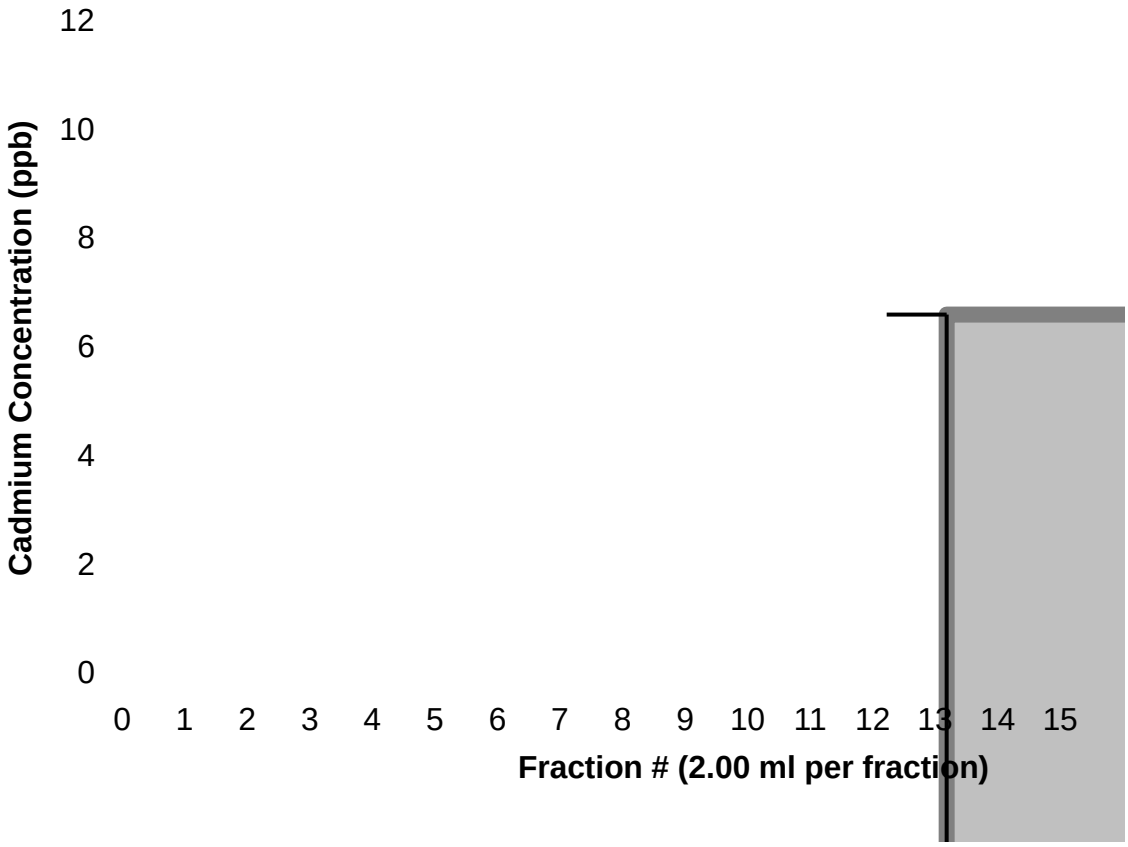


Figure A39: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #7 of *Orconectes propinquus* collected from the Laughing Whitefish River.

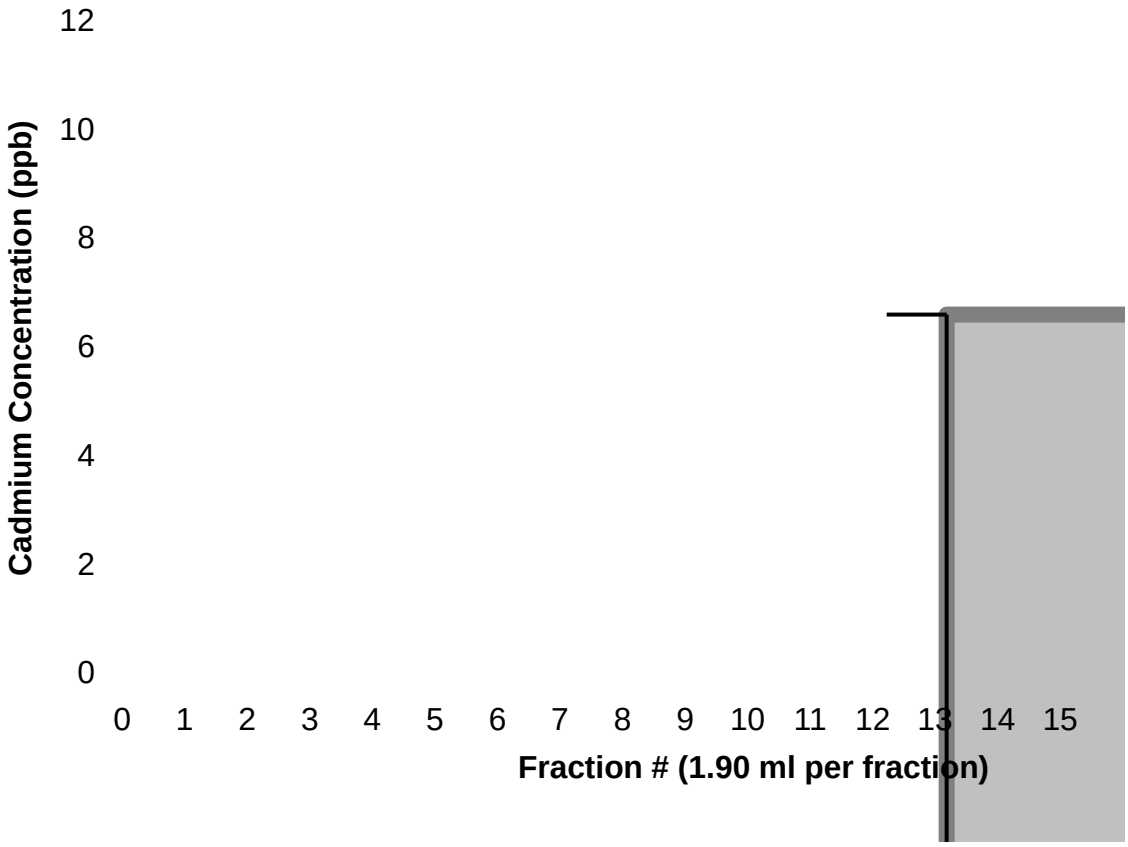


Figure A40: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #9 of *Orconectes propinquus* collected from the Laughing Whitefish River.

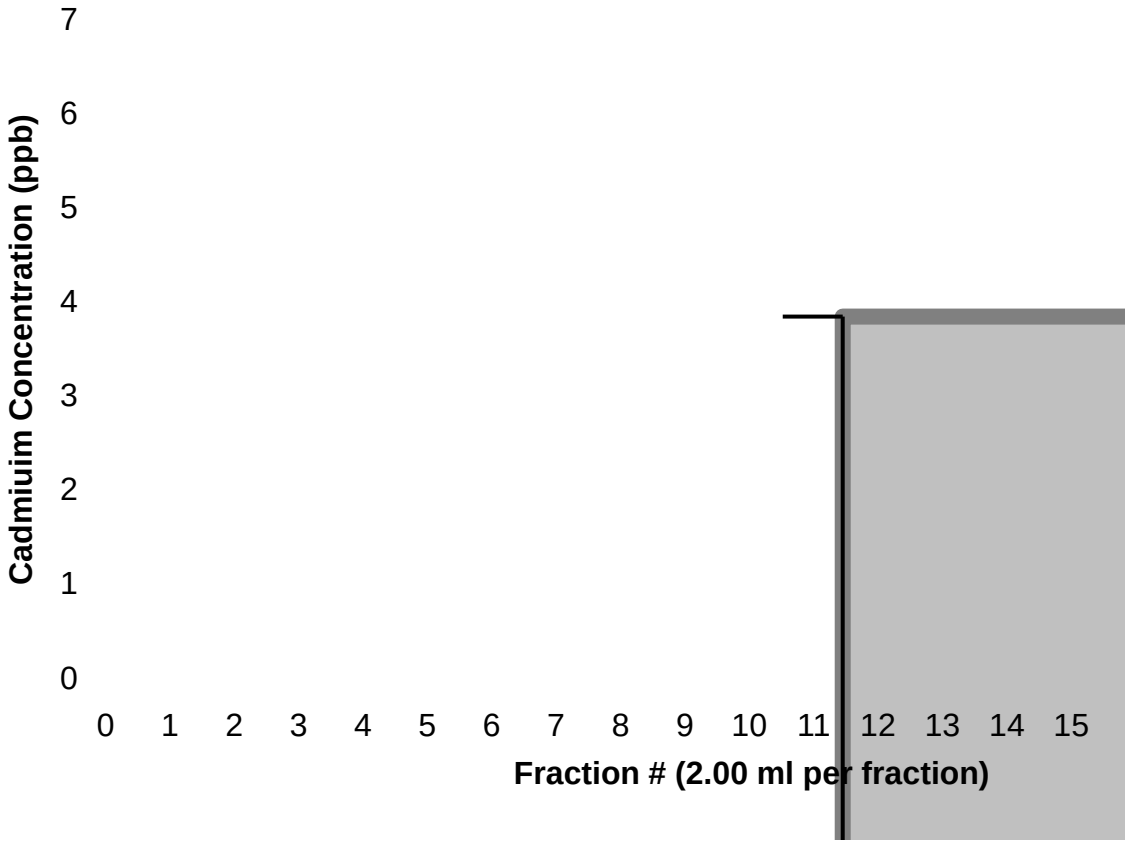


Figure A41: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #10 of *Orconectes propinquus* collected from the Laughing Whitefish River.

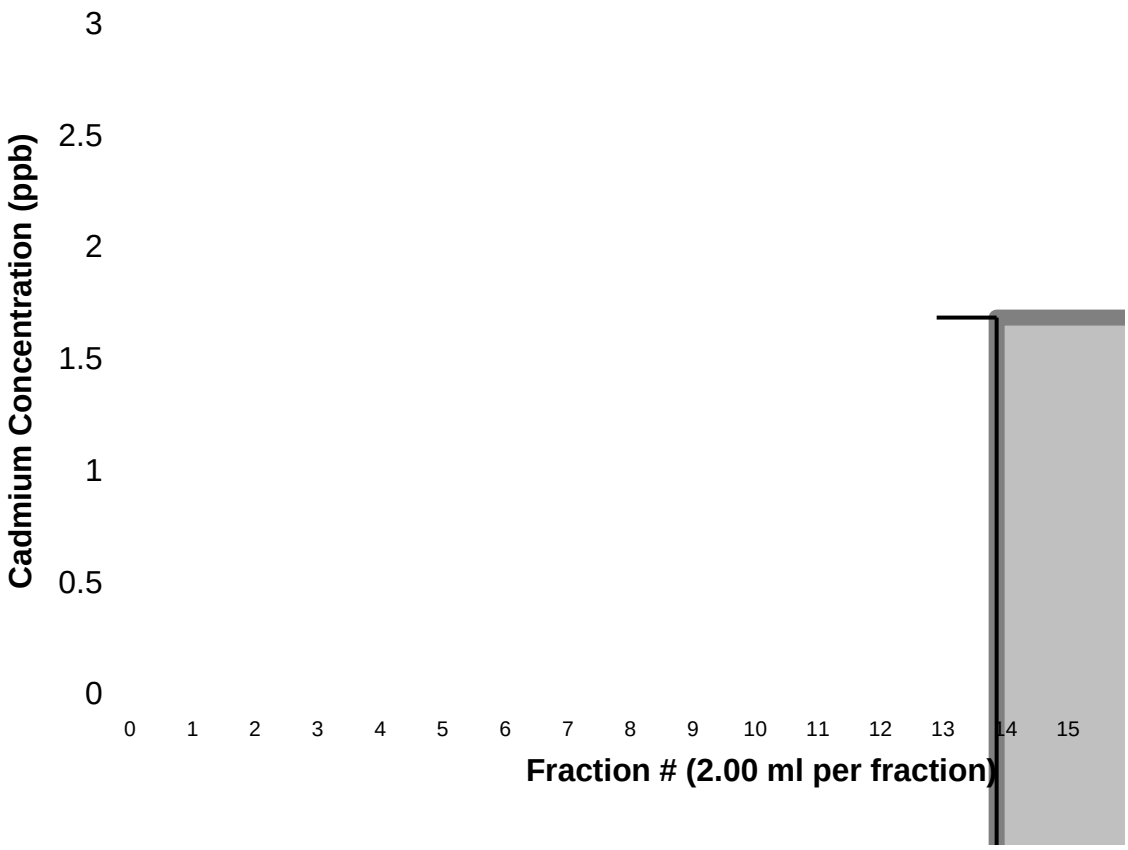


Figure A42: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #14 of *Orconectes propinquus* collected from the Laughing Whitefish River.

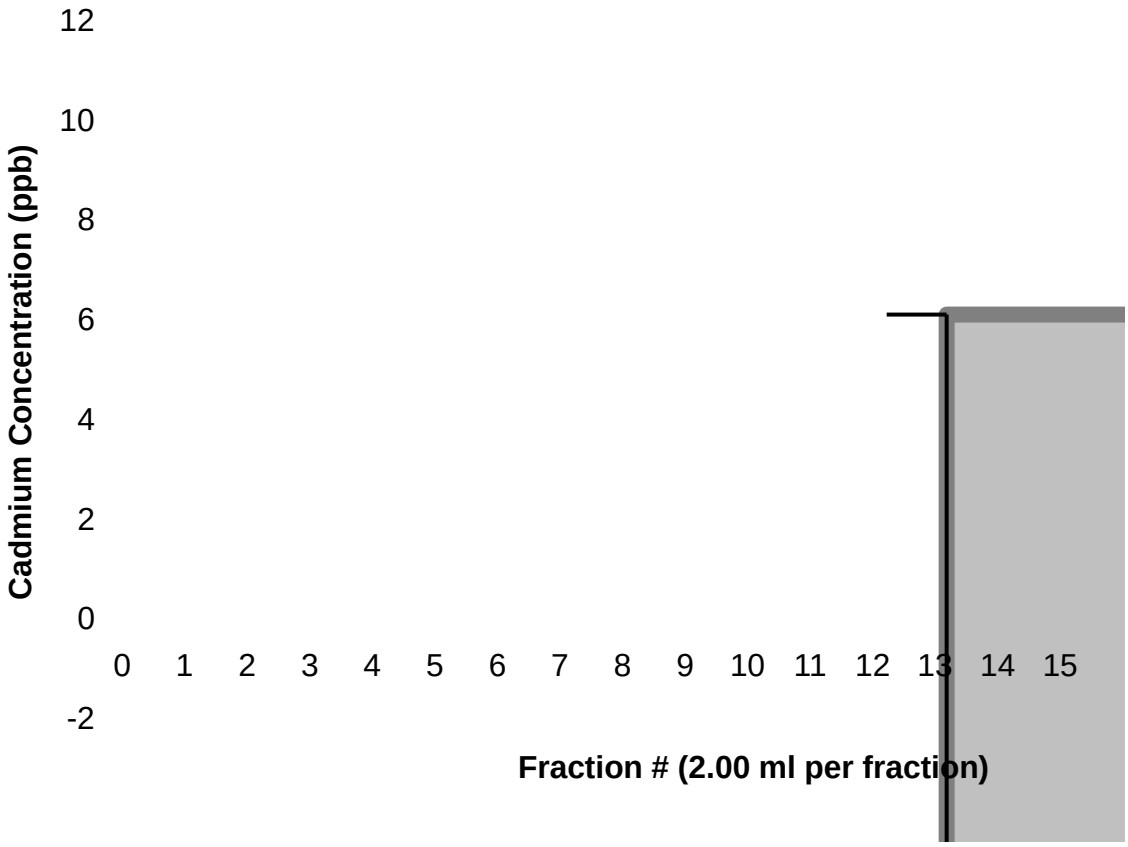


Figure A43: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #16 of *Orconectes propinquus* collected from the Laughing Whitefish River.

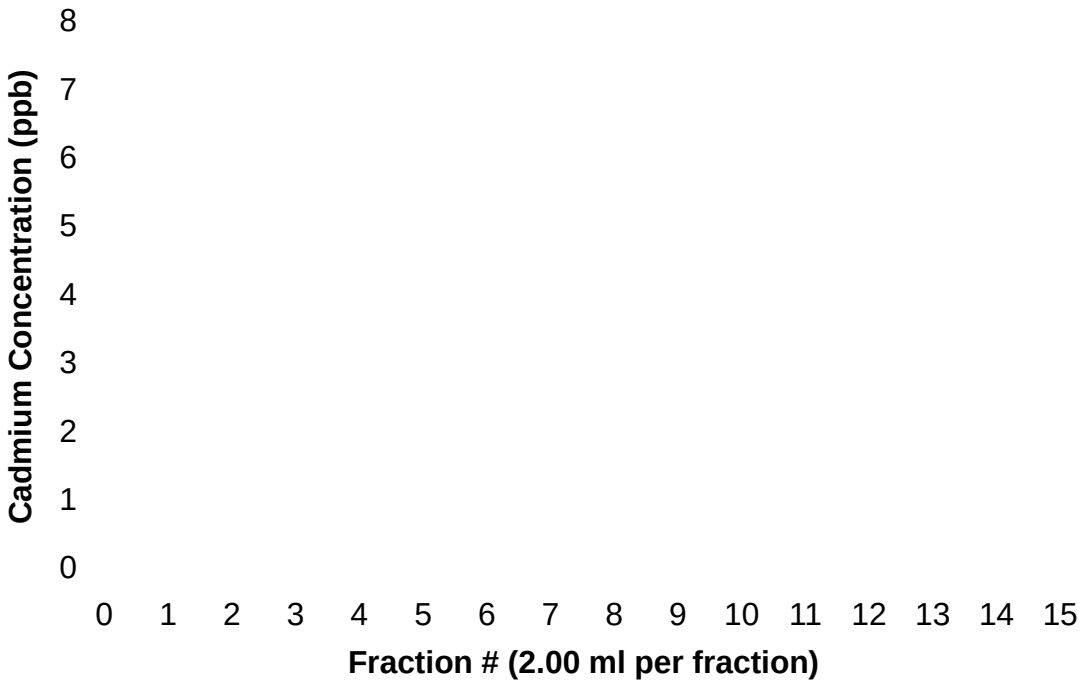


Figure A44: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #17 of *Orconectes propinquus* collected from the Laughing Whitefish River.

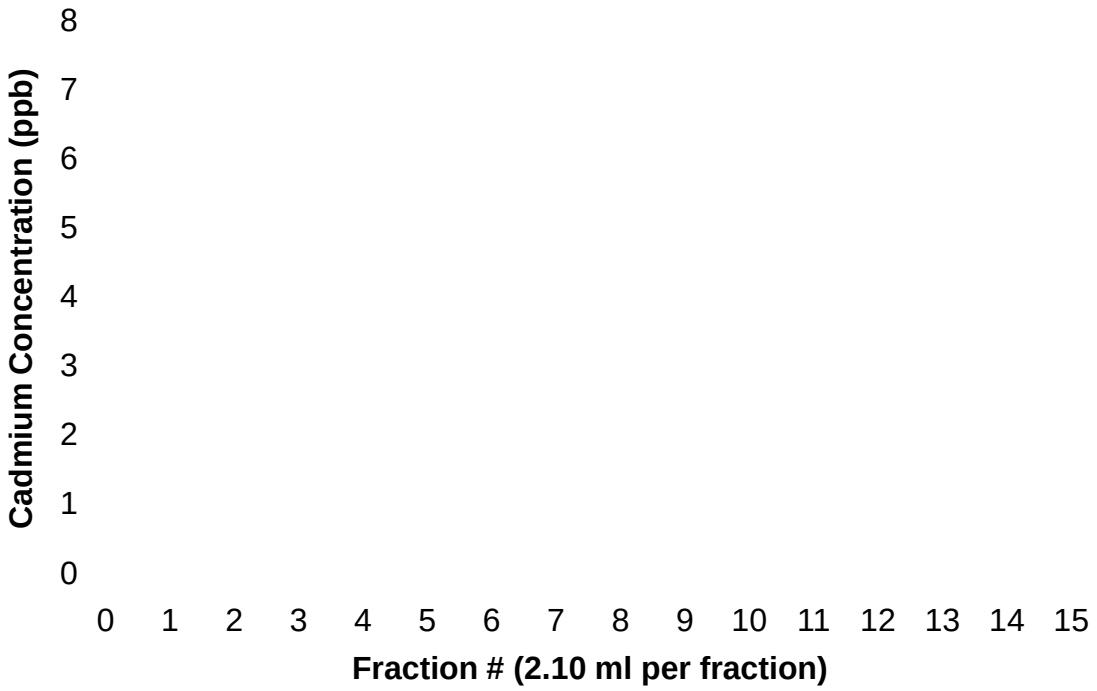


Figure A45: Elution profile for the size chromatographic separation (Sephadex G-15) of metallothionein-bound cadmium and free metal found in gill cytosol (~0.5 ml) from specimen #18 of *Orconectes propinquus* collected from the Laughing Whitefish River.

APPENDIX B

THE CONCENTRATION OF Cd^{2+} MEASURED IN FRACTIONS COLLECTED FROM A SEPHADEX G-15 COLUMN LOADED WITH GILL CYTOSOL FROM SPECIMENS OF *Orconectes propinquus* FROM COLLECTION SITES

Table B1: The concentration of Cd^{2+} measured in fractions 1-10 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #1 of *Orconectes propinquus* collected from the Dead River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
1	1.355
2	0.78
3	0.654
4	0.934
5	0.864
6	0.92
7	5.018
8	26.21
9	8.167
10	3.526
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**42.921**
Total Mass Of Cadmium In MT Fractions (ng)	**85.842**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**7.64E-10**
Total Moles of MT In Fractions (moles)	**1.27E-10**
Mass Of Gills (g)	**0.1076**
Moles MT/g Gills	**1.18E-09**
µMoles MT/g Gills	**1.18E-03**

Table B2: The concentration of Cd^{2+} measured in fractions 1-13 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #2 of *Orconectes propinquus* collected from the Dead River.

Fraction # (4.30 ml per fraction)	**Cadmium Concentration** (ng/ml)
1	4.626
2	1.098
3	1.721
4	38.99
5	16.45
6	4.75
7	5.183
8	2.633
9	2.222
10	3.014
11	1.911
12	2.661
13	1.754
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**61.911**
Total Mass Of Cadmium In MT Fractions (ng)	**266.2173**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**2.37E-09**
Total Moles of MT In Fractions (moles)	**3.95E-10**
Mass Of Gills (g)	**0.1676**
Moles MT/g Gills	**2.36E-09**
µMoles MT/g Gills	**2.36E-03**

Table B3: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #3 of *Orconectes propinquus* collected from the Dead River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.517
5	0.275
6	0.257
7	27.73
8	40.71
9	12.26
10	5.853
11	7.167
12	5.386
13	2.906
14	1.466
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**86.553**
Total Mass Of Cadmium In MT Fractions (ng)	**173.106**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**1.54E-09**
Total Moles of MT In Fractions (moles)	**2.57E-10**
Mass Of Gills (g)	**0.1026**
Moles MT/g Gills	**2.50E-09**
µMoles MT/g Gills	**2.50E-03**

Table B4: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #4 of *Orconectes propinquus* collected from the Dead River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	1.151
5	1.013
6	1.143
7	12.01
8	30.25
9	9.4
10	5.455
11	4.696
12	6.078
13	6.132
14	4.387
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**58.258**
Total Mass Of Cadmium In MT Fractions (ng)	**116.516**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**1.04E-09**
Total Moles of MT In Fractions (moles)	**1.73E-10**
Mass Of Gills (g)	**0.1333**
Moles MT/g Gills	**1.30E-09**
µMoles MT/g Gills	**1.30E-03**

Table B5: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #5 of *Orconectes propinquus* collected from the Dead River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.534
5	0.509
6	0.513
7	62.36
8	92.89
9	20.92
10	9.089
11	16.41
12	13.26
13	6.404
14	2.966
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**185.772**
Total Mass Of Cadmium In MT Fractions (ng)	**371.544**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**3.31E-09**
Total Moles of MT In Fractions (moles)	**5.51E-10**
Mass Of Gills (g)	**0.0911**
Moles MT/g Gills	**6.05E-09**
µMoles MT/g Gills	**6.05E-03**

Table B6: The concentration of Cd^{2+} measured in fractions 6-16 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #6 of *Orconectes propinquus* collected from the Dead River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
6	1.783
7	1.768
8	2.015
9	174
10	20.88
11	16.99
12	6.178
13	5.955
14	6.613
15	6.521
16	4.47
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**196.895**
Total Mass Of Cadmium In MT Fractions (ng)	**393.79**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**3.50E-09**
Total Moles of MT In Fractions (moles)	**5.84E-10**
Mass Of Gills (g)	**0.0808**
Moles MT/g Gills	**7.23E-09**
µMoles MT/g Gills	**7.23E-03**

Table B7: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #7 of *Orconectes propinquus* collected from the Dead River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	2.932
5	3.204
6	3.427
7	10.3
8	16.97
9	9.71
10	4.981
11	4.97
12	6.718
13	7.137
14	2.743
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**36.98**
Total Mass Of Cadmium In MT Fractions (ng)	**73.96**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**6.58E-10**
Total Moles of MT In Fractions (moles)	**1.10E-10**
Mass Of Gills (g)	**0.1279**
Moles MT/g Gills	**8.57E-10**
µMoles MT/g Gills	**8.57E-04**

Table B8: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #8 of *Orconectes propinquus* collected from the Dead River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.113
5	0.135
6	0.312
7	3.564
8	19.89
9	7.269
10	5.268
11	8.214
12	4.414
13	1.89
14	0.954
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**30.723**
Total Mass Of Cadmium In MT Fractions (ng)	**61.446**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**5.47E-10**
Total Moles of MT In Fractions (moles)	**9.11E-11**
Mass Of Gills (g)	**0.2051**
Moles MT/g Gills	**4.44E-10**
µMoles MT/g Gills	**4.44E-04**

Table B9: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #9 of *Orconectes propinquus* collected from the Dead River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.436
5	0.484
6	0.419
7	5.373
8	3.947
9	1.493
10	3.998
11	5.367
12	2.817
13	1.164
14	0.775
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**11.232**
Total Mass Of Cadmium In MT Fractions (ng)	**22.464**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**2.00E-10**
Total Moles of MT In Fractions (moles)	**3.33E-11**
Mass Of Gills (g)	**0.1046**
Moles MT/g Gills	**3.18E-10**
µMoles MT/g Gills	**3.18E-04**

Table B10: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #10 of *Orconectes propinquus* collected from the Dead River.

Fraction # (1.90 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	2.688
5	2.649
6	2.565
7	7.281
8	13.04
9	11.56
10	31.46
11	59.61
12	46.31
13	35.89
14	36.34
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**22.886**
Total Mass Of Cadmium In MT Fractions (ng)	**43.4834**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**3.87E-10**
Total Moles of MT In Fractions (moles)	**6.45E-11**
Mass Of Gills (g)	**0.1543**
Moles MT/g Gills	**4.18E-10**
µMoles MT/g Gills	**4.18E-04**

Table B11: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #12 of *Orconectes propinquus* collected from the Dead River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.583
5	0.453
6	2.464
7	15.06
8	10.53
9	4.294
10	7.508
11	5.845
12	4.248
13	1.932
14	1.439
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**32.348**
Total Mass Of Cadmium In MT Fractions (ng)	**64.696**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**5.76E-10**
Total Moles of MT In Fractions (moles)	**9.59E-11**
Mass Of Gills (g)	**0.0687**
Moles MT/g Gills	**1.40E-09**
µMoles MT/g Gills	**1.40E-03**

Table B12: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #1a of *Orconectes propinquus* collected from Deer Lake.

Fraction # (1.95 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.273
5	0.364
6	0.26
7	2.789
8	6.058
9	2.434
10	2.075
11	2.661
12	4.007
13	5.489
14	5.084
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**13.616**
Total Mass Of Cadmium In MT Fractions (ng)	**26.5512**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**2.36E-10**
Total Moles of MT In Fractions (moles)	**3.94E-11**
Mass Of Gills (g)	**0.2214**
Moles MT/g Gills	**1.78E-10**
µMoles MT/g Gills	**1.78E-04**

Table B13: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #1b of *Orconectes propinquus* collected from Deer Lake.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.043
5	0.052
6	0.032
7	0.556
8	9.917
9	9.21
10	6.192
11	7.049
12	11.53
13	9.05
14	4.119
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**25.875**
Total Mass Of Cadmium In MT Fractions (ng)	**51.75**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**4.60E-10**
Total Moles of MT In Fractions (moles)	**7.67E-11**
Mass Of Gills (g)	**0.216**
Moles MT/g Gills	**3.55E-10**
µMoles MT/g Gills	**3.55E-04**

Table B14: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #2 of *Orconectes propinquus* collected from Deer Lake.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.04
5	0.147
6	0.048
7	0.186
8	7.226
9	1.764
10	1.297
11	1.439
12	3.156
13	2.36
14	0.994
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**10.521**
Total Mass Of Cadmium In MT Fractions (ng)	**21.042**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**1.87E-10**
Total Moles of MT In Fractions (moles)	**3.12E-11**
Mass Of Gills (g)	**0.191**
Moles MT/g Gills	**1.63E-10**
µMoles MT/g Gills	**1.63E-04**

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Table B15: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #4a of *Orconectes propinquus* collected from Deer Lake.

Fraction # (1.90 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.484
5	0.513
6	0.398
7	1.163
8	4.901
9	3.715
10	1.158
11	0.873
12	1.315
13	2.064
14	3.766
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**12.208**
Total Mass Of Cadmium In MT Fractions (ng)	**23.1952**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**2.06E-10**
Total Moles of MT In Fractions (moles)	**3.44E-11**
Mass Of Gills (g)	**0.175**
Moles MT/g Gills	**1.97E-10**
µMoles MT/g Gills	**1.97E-04**

Table B16: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #4b of *Orconectes propinquus* collected from Deer Lake.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.326
5	0.272
6	0.294
7	5.336
8	3.416
9	1.529
10	1.004
11	1.723
12	2.741
13	2.879
14	1.665
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**11.579**
Total Mass Of Cadmium In MT Fractions (ng)	**23.158**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**2.06E-10**
Total Moles of MT In Fractions (moles)	**3.43E-11**
Mass Of Gills (g)	**0.2079**
Moles MT/g Gills	**1.65E-10**
µMoles MT/g Gills	**1.65E-04**

Table B17: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #5 of *Orconectes propinquus* collected from Deer Lake.

Fraction # (1.95 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.223
5	0.237
6	0.226
7	258.6
8	323.4
9	61.64
10	17.93
11	2.585
12	3.011
13	2.438
14	2.015
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**661.796**
Total Mass Of Cadmium In MT Fractions (ng)	**1290.5022**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**1.15E-08**
Total Moles of MT In Fractions (moles)	**1.91E-09**
Mass Of Gills (g)	**0.2704**
Moles MT/g Gills	**7.08E-09**
µMoles MT/g Gills	**7.08E-03**

Table B18: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #6 of *Orconectes propinquus* collected from Deer Lake.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.027
5	0.034
6	0
7	7.983
8	383.4
9	143
10	40.56
11	7.718
12	4.095
13	4.001
14	4.103
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**590.757**
Total Mass Of Cadmium In MT Fractions (ng)	**1181.514**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**1.05E-08**
Total Moles of MT In Fractions (moles)	**1.75E-09**
Mass Of Gills (g)	**0.2386**
Moles MT/g Gills	**7.34E-09**
µMoles MT/g Gills	**7.34E-03**

Table B19: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #8 of *Orconectes propinquus* collected from Deer Lake.

Fraction # (1.95 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.043
5	0.077
6	0.056
7	127.52
8	138.1
9	29.12
10	11.11
11	13.17
12	14.61
13	9.521
14	3.833
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**305.906**
Total Mass Of Cadmium In MT Fractions (ng)	**596.5167**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**5.31E-09**
Total Moles of MT In Fractions (moles)	**8.85E-10**
Mass Of Gills (g)	**0.2344**
Moles MT/g Gills	**3.77E-09**
µMoles MT/g Gills	**3.77E-03**

Table B20: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #10 of *Orconectes propinquus* collected from Deer Lake.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.089
5	0.096
6	0.116
7	102.06
8	173.2
9	27.23
10	6.619
11	3.1
12	4.075
13	6.477
14	4.868
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**312.325**
Total Mass Of Cadmium In MT Fractions (ng)	**624.65**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**5.56E-09**
Total Moles of MT In Fractions (moles)	**9.26E-10**
Mass Of Gills (g)	**0.2751**
Moles MT/g Gills	**3.37E-09**
µMoles MT/g Gills	**3.37E-03**

Table B21: The concentration of Cd^{2+} measured in fractions (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #11a of *Orconectes propinquus* collected from Deer Lake.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.329
5	0.263
6	0.275
7	0.205
8	2.13
9	0.996
10	1.195
11	3.952
12	3.136
13	3.128
14	0.754
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**3.331**
Total Mass Of Cadmium In MT Fractions (ng)	**6.662**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**5.93E-11**
Total Moles of MT In Fractions (moles)	**9.88E-12**
Mass Of Gills (g)	**0.3169**
Moles MT/g Gills	**3.12E-11**
µMoles MT/g Gills	**3.12E-05**

Table B22: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #11b of *Orconectes propinquus* collected from Deer Lake.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	1.725
5	1.552
6	1.449
7	3.686
8	5.05
9	2.634
10	2.485
11	4.946
12	3.899
13	2.883
14	2.595
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**12.819**
Total Mass Of Cadmium In MT Fractions (ng)	**25.638**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**2.28E-10**
Total Moles of MT In Fractions (moles)	**3.80E-11**
Mass Of Gills (g)	**0.2674**
Moles MT/g Gills	**1.42E-10**
µMoles MT/g Gills	**1.42E-04**

Table B23: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #12a of *Orconectes propinquus* collected from Deer Lake.

Fraction # (1.90 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.447
5	0.511
6	0.492
7	0.678
8	2.106
9	1.085
10	0.83
11	1.264
12	1.671
13	1.799
14	1.039
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**4.361**
Total Mass Of Cadmium In MT Fractions (ng)	**8.2859**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**7.37E-11**
Total Moles of MT In Fractions (moles)	**1.23E-11**
Mass Of Gills (g)	**0.2106**
Moles MT/g Gills	**5.83E-11**
µMoles MT/g Gills	**5.83E-05**

Table B24: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #12b of *Orconectes propinquus* collected from Deer Lake.

Fraction #	**Cadmium Concentration**
(2.00 ml per fraction)	(ng/ml)
4	0.273
5	0.084
6	0.035
7	1.952
8	2.893
9	1.883
10	1.286
11	2.894
12	1.981
13	1.563
14	1.347
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**8.049**
Total Mass Of Cadmium In MT Fractions (ng)	**16.098**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**1.43E-10**
Total Moles of MT In Fractions (moles)	**2.39E-11**
Mass Of Gills (g)	**0.1962**
Moles MT/g Gills	**1.22E-10**
µMoles MT/g Gills	**1.22E-04**

Table B25: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #13a of *Orconectes propinquus* collected from Deer Lake.

Fraction # (1.95 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.173
5	0.316
6	0.228
7	1.827
8	2.116
9	2.152
10	5.035
11	4.604
12	0.796
13	0.961
14	0.512
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**4.171**
Total Mass Of Cadmium In MT Fractions (ng)	**8.13345**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**7.24E-11**
Total Moles of MT In Fractions (moles)	**1.21E-11**
Mass Of Gills (g)	**0.1204**
Moles MT/g Gills	**1.00E-10**
µMoles MT/g Gills	**1.00E-04**

Table B26: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #13b of *Orconectes propinquus* collected from Deer Lake.

Fraction # (1.95 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.279
5	0.384
6	0.347
7	2.216
8	2.591
9	0.337
10	1.647
11	3.844
12	1.691
13	2.246
14	2.31
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**5.491**
Total Mass Of Cadmium In MT Fractions (ng)	**10.70745**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**9.53E-11**
Total Moles of MT In Fractions (moles)	**1.59E-11**
Mass Of Gills (g)	**0.1707**
Moles MT/g Gills	**9.30E-11**
µMoles MT/g Gills	**9.30E-05**

Table B27: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #1 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.178
5	0.205
6	0.2
7	2.21
8	7.993
9	2.688
10	8.284
11	8.027
12	0.37
13	0.282
14	0.245
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**13.091**
Total Mass Of Cadmium In MT Fractions (ng)	**26.182**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**2.33E-10**
Total Moles of MT In Fractions (moles)	**3.88E-11**
Mass Of Gills (g)	**0.14**
Moles MT/g Gills	**2.77E-10**
µMoles MT/g Gills	**2.77E-04**

Table B28: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #5 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.349
5	0.268
6	0.245
7	8.603
8	2.401
9	5.741
10	4.863
11	1.851
12	0.691
13	1.186
14	0.431
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**11.249**
Total Mass Of Cadmium In MT Fractions (ng)	**22.498**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**2.00E-10**
Total Moles of MT In Fractions (moles)	**3.34E-11**
Mass Of Gills (g)	**0.0911**
Moles MT/g Gills	**3.66E-10**
µMoles MT/g Gills	**3.66E-04**

Table B29: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #6 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

Fraction #	**Cadmium Concentration**
(2.00 ml per fraction)	(ng/ml)
4	0.096
5	0.092
6	0.204
7	1.291
8	11.63
9	4.22
10	1.863
11	1.225
12	3.248
13	1.919
14	0.999
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**20.433**
Total Mass Of Cadmium In MT Fractions (ng)	**40.866**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**3.64E-10**
Total Moles of MT In Fractions (moles)	**6.06E-11**
Mass Of Gills (g)	**0.0713**
Moles MT/g Gills	**8.50E-10**
µMoles MT/g Gills	**8.50E-04**

Table B30: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #7 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	2.747
5	1.441
6	1.496
7	12.28
8	17.68
9	4.621
10	1.58
11	1.069
12	1.094
13	2.285
14	3.416
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**38.726**
Total Mass Of Cadmium In MT Fractions (ng)	**77.452**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**6.89E-10**
Total Moles of MT In Fractions (moles)	**1.15E-10**
Mass Of Gills (g)	**0.0823**
Moles MT/g Gills	**1.40E-09**
µMoles MT/g Gills	**1.40E-03**

Table B31: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #8 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	1.321
5	1.962
6	1.389
7	5.69
8	26.924
9	5.529
10	0.529
11	1.197
12	1.909
13	3.943
14	5.249
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**40.061**
Total Mass Of Cadmium In MT Fractions (ng)	**80.122**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**7.13E-10**
Total Moles of MT In Fractions (moles)	**1.19E-10**
Mass Of Gills (g)	**0.1615**
Moles MT/g Gills	**7.36E-10**
µMoles MT/g Gills	**7.36E-04**

Table B32: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #10 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.701
5	0.338
6	0.351
7	8.496
8	5.026
9	2.087
10	1.261
11	4.142
12	5.014
13	4.825
14	2.35
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**17.221**
Total Mass Of Cadmium In MT Fractions (ng)	**34.442**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**3.06E-10**
Total Moles of MT In Fractions (moles)	**5.11E-11**
Mass Of Gills (g)	**0.0979**
Moles MT/g Gills	**5.22E-10**
µMoles MT/g Gills	**5.22E-04**

Table B33: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #11 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.277
5	0.131
6	0.186
7	0.778
8	13.98
9	6.136
10	6.366
11	10.43
12	9.044
13	4.464
14	1.874
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**21.080**
Total Mass Of Cadmium In MT Fractions (ng)	**42.16**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**3.75E-10**
Total Moles of MT In Fractions (moles)	**6.25E-11**
Mass Of Gills (g)	**0.1522**
Moles MT/g Gills	**4.11E-10**
µMoles MT/g Gills	**4.11E-04**

Table B34: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #17 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.267
5	0.502
6	0.487
7	1.448
8	1.203
9	0.655
10	0.567
11	0.658
12	0.876
13	1.482
14	1.011
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**4.360**
Total Mass Of Cadmium In MT Fractions (ng)	**8.72**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**7.76E-11**
Total Moles of MT In Fractions (moles)	**1.29E-11**
Mass Of Gills (g)	**0.1016**
Moles MT/g Gills	**1.27E-10**
µMoles MT/g Gills	**1.27E-04**

Table B35: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #18 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

Fraction #	**Cadmium Concentration**
(2.00 ml per fraction)	(ng/ml)
4	0.133
5	0.123
6	0.271
7	0.744
8	1.156
9	0.803
10	0.703
11	0.888
12	1.774
13	1.325
14	0.629
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**3.800**
Total Mass Of Cadmium In MT Fractions (ng)	**7.6**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**6.76E-11**
Total Moles of MT In Fractions (moles)	**1.13E-11**
Mass Of Gills (g)	**0.1204**
Moles MT/g Gills	**9.36E-11**
µMoles MT/g Gills	**9.36E-05**

Table B36: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #19 of *Orconectes propinquus* collected from the East Branch of the Escanaba River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.635
5	0.563
6	0.519
7	1.727
8	1.671
9	0.785
10	0.496
11	0.433
12	0.528
13	1.051
14	1.058
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**5.631**
Total Mass Of Cadmium In MT Fractions (ng)	**11.262**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**1.00E-10**
Total Moles of MT In Fractions (moles)	**1.67E-11**
Mass Of Gills (g)	**0.1809**
Moles MT/g Gills	**9.23E-11**
µMoles MT/g Gills	**9.23E-05**

Table B37: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #1 of *Orconectes propinquus* collected from the Laughing Whitefish River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.304
5	0.283
6	0.262
7	10.99
8	11.93
9	5.047
10	7.238
11	7.187
12	3.003
13	2.851
14	1.474
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**28.229**
Total Mass Of Cadmium In MT Fractions (ng)	**56.458**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**5.02E-10**
Total Moles of MT In Fractions (moles)	**8.37E-11**
Mass Of Gills (g)	**0.2328**
Moles MT/g Gills	**3.60E-10**
µMoles MT/g Gills	**3.60E-04**

Table B38: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #3 of *Orconectes propinquus* collected from the Laughing Whitefish River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.383
5	0.685
6	0.7
7	1.558
8	8.81
9	6.187
10	6.579
11	13.98
12	7.078
13	2.687
14	1.715
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**16.555**
Total Mass Of Cadmium In MT Fractions (ng)	**33.11**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**2.95E-10**
Total Moles of MT In Fractions (moles)	**4.91E-11**
Mass Of Gills (g)	**0.1522**
Moles MT/g Gills	**3.23E-10**
µMoles MT/g Gills	**3.23E-04**

Table B39: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #7 of *Orconectes propinquus* collected from the Laughing Whitefish River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.512
5	0.418
6	0.437
7	1.558
8	7.261
9	6.678
10	3.912
11	9.912
12	6.205
13	2.904
14	1.216
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**15.497**
Total Mass Of Cadmium In MT Fractions (ng)	**30.994**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**2.76E-10**
Total Moles of MT In Fractions (moles)	**4.60E-11**
Mass Of Gills (g)	**0.1234**
Moles MT/g Gills	**3.72E-10**
µMoles MT/g Gills	**3.72E-04**

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Table B40: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #9 of *Orconectes propinquus* collected from the Laughing Whitefish River.

Fraction # (1.90 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	10.74
5	1.584
6	1.61
7	5.369
8	4.663
9	3.088
10	2.672
11	4.508
12	4.893
13	5.351
14	3.303
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**13.120**
Total Mass Of Cadmium In MT Fractions (ng)	**24.928**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**2.22E-10**
Total Moles of MT In Fractions (moles)	**3.70E-11**
Mass Of Gills (g)	**0.2088**
Moles MT/g Gills	**1.77E-10**
µMoles MT/g Gills	**1.77E-04**

Table B41: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #10 of *Orconectes propinquus* collected from the Laughing Whitefish River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	1.098
5	1.149
6	2.648
7	6.105
8	3.554
9	2.33
10	3.613
11	5.077
12	5.086
13	2.297
14	1.946
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**14.637**
Total Mass Of Cadmium In MT Fractions (ng)	**29.274**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**2.60E-10**
Total Moles of MT In Fractions (moles)	**4.34E-11**
Mass Of Gills (g)	**0.1877**
Moles MT/g Gills	**2.31E-10**
µMoles MT/g Gills	**2.31E-04**

Table B42: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #14 of *Orconectes propinquus* collected from the Laughing Whitefish River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.977
5	0.168
6	0.107
7	0.26
8	1.129
9	0.78
10	1.576
11	0.807
12	2.439
13	0.942
14	0.486
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**2.276**
Total Mass Of Cadmium In MT Fractions (ng)	**4.552**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**4.05E-11**
Total Moles of MT In Fractions (moles)	**6.75E-12**
Mass Of Gills (g)	**0.1261**
Moles MT/g Gills	**5.35E-11**
µMoles MT/g Gills	**5.35E-05**

Table B43: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #16 of *Orconectes propinquus* collected from the Laughing Whitefish River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.251
5	0.172
6	0.233
7	6.912
8	9.571
9	3.676
10	2.119
11	3.788
12	3.301
13	2.646
14	1.015
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**22.511**
Total Mass Of Cadmium In MT Fractions (ng)	**45.022**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**4.01E-10**
Total Moles of MT In Fractions (moles)	**6.68E-11**
Mass Of Gills (g)	**0.1028**
Moles MT/g Gills	**6.49E-10**
µMoles MT/g Gills	**6.49E-04**

Table B44: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #17 of *Orconectes propinquus* collected from the Laughing Whitefish River.

Fraction # (2.00 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.042
5	0.08
6	0.539
7	0.502
8	6.948
9	3.974
10	0.788
11	1.249
12	0.292
13	0.756
14	0.479
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**12.212**
Total Mass Of Cadmium In MT Fractions (ng)	**24.424**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**2.17E-10**
Total Moles of MT In Fractions (moles)	**3.62E-11**
Mass Of Gills (g)	**0.1446**
Moles MT/g Gills	**2.50E-10**
µMoles MT/g Gills	**2.50E-04**

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Table B45: The concentration of Cd^{2+} measured in fractions 4-14 (highlighted fractions contain metallothionein-bound cadmium) collected from a Sephadex G-15 column loaded with ~0.5 ml of gill cytosol from specimen #18 of *Orconectes propinquus* collected from the Laughing Whitefish River.

Fraction # (2.10 ml per fraction)	**Cadmium Concentration** (ng/ml)
4	0.541
5	0.193
6	0.557
7	5.028
8	2.191
9	1.805
10	6.86
11	0.393
12	0.423
13	0.349
14	0.368
Total Concentration Of Cadmium In MT Fractions (ng/ml)	**7.776**
Total Mass Of Cadmium In MT Fractions (ng)	**16.3296**
Total Moles Of Cadmium In MT Fractions (moles Cd)	**1.45E-10**
Total Moles of MT In Fractions (moles)	**2.42E-11**
Mass Of Gills (g)	**0.1249**
Moles MT/g Gills	**1.94E-10**
µMoles MT/g Gills	**1.94E-04**