THE EFFECTS OF FOOD QUALITY AND ABIOTIC HABITAT PARAMETERS ON METAL UPTAKE IN THE BLUE MUSSEL, *MYTILUS TROSSULUS*

by

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BSc. Simon Fraser University, 1999

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

In the Department
of
Biological Sciences

SIMON FRASER UNIVERSITY

Spring 2005

APPROVAL

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Degree: Doctor of Philosophy

Title of Thesis:

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ABSTRACT

The potential transfer of heavy metals via trophic transfer (bioaccumulation) in global aquatic ecosystems is of growing concern amongst members of today's society. Therefore it is important to examine contaminated ecosystems beginning with the organisms that have the highest potential for exposure. Bivalve species have traditionally been considered excellent biomonitors of metal levels in marine environments. My research examines the combined influences of abiotic (salinity, ecosystem quality) and biotic factors (food quality and selection) on heavy metal uptake and availability in the West coast blue mussel *Mytilus trossulus*.

Flow-through (48) and pulse-feeding (66) experiments were conducted using either single or combinations of marine bacteria, phytoplankton, natural sediments and clay food sources. Flow-through experiments involved feeding *M. trossulus* a marine bacterium containing non-radioactive Mn, Cd, or Pb oxides and varying amounts of carbon. Bacteria containing higher amounts of carbon had enhanced metal uptake due to an increased clearance rate of the organic rich particles. Pulse-feeding experiments involved feeding *M. trossulus* combinations of radiolabelled ^{109}Cd phytoplankton, clay and natural sediments containing varying amounts of carbon. To obtain natural, biologically-relevant sediments unique intertidal sediment traps were designed and deployed at two estuarine habitats. The sediment quality and salinity of these habitats (Boundary Bay and Deep Bay) differed significantly (24% C and 25 ppt vs.15% C and 15 ppt., respectively). Results indicated that lower salinity environments may result in

significantly lower dietary cadmium uptake and assimilation efficiencies in *M. trossulus* when compared to higher salinity environments.

My research demonstrated that the active selection of food particles by *M. trossulus* significantly affected the extent of metal uptake and assimilation. In addition, the bioaccumulation of metals by *M. trossulus* inhabiting different marine habitats varied depending on the natural abiotic conditions within that habitat, such as salinity. I conclude that using *M. trossulus* as a biomonitor of marine metal levels is useful so long as the feeding behaviour and abiotic habitat parameters are considered when comparing metal levels in *M. trossulus* between different habitats.

DEDICATION

For my husband Chris, who has provided me with unlimited support and guidance to set my goals, the confidence to achieve those goals, and the love and encouragement to never stop learning.

ACKNOWLEDGEMENTS

First and foremost I am extremely grateful to Leah Bendell-Young, my senior supervisor, for her encouragement, support, and friendship that began when I was an undergraduate student. Secondly I would like to thank Margo Moore and Daryl Crozier for their contribution to my thesis as dedicated committee members. In addition, I am very pleased to have had Frank Gobas and Landis Hare present as public and external examiners for my thesis defense. Their insight and contribution made my defense a true challenge, allowing me to effectively demonstrate my knowledge and skills. In addition, Mark Saffari from Environment Canada contributed huge amounts of time and energy to my project for which I am very grateful.

The unlimited support of my family members has played an instrumental role in allowing me to achieve my goals. I would like to acknowledge my Grandma Gilbert and Grandma King, both whom passed away during my PhD research. I would like to thank my mother Shirley Forwell, my father Paul King and wife Sherrie King, my grandfather Art Gilbert, cousin Sue Forwell, aunt JoAnna Forwell and my sister Kari King for their continuous love and support. The friendship and encouragement I received from my colleagues Kirsty Gurney, Christy Morrissey, Anna Gifford, Luba Vasiluk, and Linda Pinto is gratefully acknowledged. My Brazilian family Simone, Horacio, Ana Flavia and Priscila, muito obrigado. Finally I would like to thank and acknowledge the unwavering support from my husband Chris Widmeyer and my newborn son Wyatt, to the bright and exciting adventures we have yet to conquer.

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INTRODUCTION

Study Rationale

Contamination of Earth's ecosystems by potentially toxic metals is a growing concern in today's society. This will likely increase with our planet's increasing population and the requirements for natural resources and metals-based goods (Siegel 2002). Exposure to heavy metal pollution from air, food, and water sources is a chronic concern for all organisms within an ecosystem. Understanding and maintaining the current health of these ecosystems and all organisms within them may become more difficult as the human population continues to expand. It is therefore the role of ecotoxicologists to build upon current knowledge by developing future concepts and methodologies that preserve and sustain our global ecosystems.

The research presented in this thesis focuses on the uptake of heavy metals in bivalves being controlled by two types of influences. The first is an organism's behavioural traits such as food or digestive particle selectivity. The second is the effect of natural environmental characteristics within a habitat on the availability and uptake of heavy metals. The specific goal of this thesis is to examine how variations in natural food sources and abiotic marine habitat parameters affect heavy metal uptake in the suspension-feeding bivalve *Mytilus trossulus*. Understanding the role that both abiotic and biotic factors play in affecting suspension-feeding behaviour and heavy metal uptake in this species is critical because world regulatory agencies continue to use them as biomonitors of metal contamination in marine ecosystems. To develop realistic levels of metal contamination between marine environments, monitoring programs need to account for these natural variations when using suspension-feeding biomonitors.

Metal toxicology. Metals are perhaps one of the earliest medicines and poisons known to man. They range from compounds that are simple ionic salts to complicated structures consisting of a metal atom bound to an organic compound (Friberg et al. 1979). Metals are naturally-occurring elements found in the crust of the earth, within the rocks and processes by which they were formed (Siegel 2002). Metals are released into the environment from the natural weathering of soil and rock, making them available for uptake within the earth's waters, soil (terrestrial), sediment (aquatic), and vegetation. Natural metal sources can also be released into the atmosphere via volcanoes, geysers, thermal springs and forest fires (Chang 1996). The main anthropogenic source of metal introduction to the environment is through human activity. Sources of contamination and their magnitudes vary from large-scale industrial waste by-products and abandoned mines to small scale, casual neglect due to over usage and improper disposal of over-the-counter household products.

Metals fall into one of two categories, essential and non-essential. Essential metal micronutrients such as cobalt, chromium, copper, iron, manganese, selenium, and zinc are required for optimal functioning of biological and biochemical processes in humans in small quantities (a few mg or μg per day) (Siegel 2002). Essential metal macronutrients such as calcium, chlorine, magnesium, phosphorus, potassium, sodium and sulphur are also required for similar biological processes, but in larger quantities (100 mg or more per day) (Crounse et al. 1983). Non-essential metals such as cadmium, mercury, nickel, lead, and tin have no known biological function in the human body and therefore cause no adverse physiological effect if missing from a diet.

Regardless of the class of metal, it is the dose that makes the poison. The exposure to either an excess or deficient amount of metal causes various toxic effects within an organism. These effects vary depending upon whether the exposure is chronic versus acute, and whether they are mutagenic (induction of gene mutations), carcinogenic (induction of cancer), or teratogenic (developmental defects) in nature. In general, heavy metals produce their toxicity by forming complexes or "ligands" with organic compounds. These modified biological molecules lose their ability to function properly, and can result in the malfunctioning or death of the affected cells. The most common groups involved in ligand formation are oxygen, sulfur, and nitrogen (Merian 1991). The following paragraphs contain information on the toxicity, mode of action, and routes of uptake and elimination for the five metals studied in this thesis; cadmium, copper, lead, manganese and zinc.

Cadmium is a non-essential metal commonly used by industry for electroplating, as a paint stabilizer, colour pigment fixer in plastics and by the agricultural industry in superphosphate fertilizers. In non-polluted freshwaters it has a concentration lower than 1 μg/L while in seawater the concentration ranges from 0.04 to 3.0 μg/L (Chang 1996). The main route of aqueous cadmium uptake in aquatic organisms are via the gills or gut, after which it binds to low molecular weight proteins such as metallothioneins and is transported by the blood to the liver and kidneys. Once in these organs, cadmium stays complexed to the metallothionein making it difficult to excrete (Smith 1999). Cadmium may give rise to both acute and chronic types of poisoning. In chronic exposures the first signs of kidney damage, tubular proteinuria, can usually be diagnosed due to an increase in the excretion of low molecular weight proteins (Webb 1979). Over time, cadmium can

accelerate the osteoporotic process by substituting for elemental calcium, causing calcium deficiencies in the bones. In addition, cadmium is a known carcinogen and teratogen to vertebrates (Smith 1999).

Copper is an essential element, regulating and maintaining the functions of various metabolic enzymes as well as being a necessary component of haemoglobin. Industry most commonly combines copper with other metals to produce an alloy used in the manufacturing of electrical equipment and wires, or pipes. Background concentrations of copper in freshwater ranges from 0.83 to 105 μg/L and from 1-5 μg/L in seawater (Chang 1996). Copper is not a common particulate found in the air or atmosphere, therefore the most common route of uptake is via the stomach (Massaro 2002). After absorption has occurred, copper binds to albumin proteins and is stored in the liver. It is then transported to other tissues based on that tissues need for the metal. The main route of excretion is via the bile while a secondary route is the feces, it is not excreted via the urine. The normal concentration of copper in whole blood is approximately 1 mg/L (Massaro 2002). Copper is non-carcinogenic (Chang 1996).

Lead is a non-essential metal commonly used by the battery industry, as a gasoline additive, and by the pigment/paint and metal alloy industries. Background concentrations of lead in freshwater vary considerably, with values ranging from 34 to 300 μg/L while in saltwater they range from 0.3 to 20 μg/L. In countries where tetra-ethyl lead is added to gasoline (banned in North America but not third world countries) lead concentrations can reach 500μg/L (Chang 1996). The most common uptake routes are via the lungs or gills as it is not commonly absorbed by the GI tract/gut. Absorbed lead is bound to erythrocytes in the blood and initially distributed to the liver, kidney and

heart where it preferentially binds to cell membranes and mitochondria. Most forms of lead are then redistributed and stored in the bones. The primary route of elimination is via feces (90%) and the remaining via urine (10%) (Gilbert 2004). Lead is known to cause both acute and chronic adverse effects in the hematopoietic, nervous, gastrointestinal and renal systems. Acute poisoning causes gastrointestinal colic often resulting in mortality, while chronic poisoning causes anemia due to a fall in haemoglobin levels leading to organ damage in one or all of the four above systems (Gilbert 2004). Lead is non-carcinogenic (Chang 1996).

Manganese is an essential metal utilized by enzymes such as pyruvate carboxylase to control metabolic processes in vertebrates, as well as in the synthesis of chondroitin sulphate for cartilage formation (Klimis-Tavantzis 1994). It is usually combined with other metals to produce alloys that are used in the electrical and shipping industry, to produce dry cell batteries, and as a substitute for lead as the gasoline additive MMT (Merian 1991). Natural concentrations in freshwater vary from less than one to several hundred μg/L while in saltwater they are consistently around 2 μg/L (Chang 1996). The main route of uptake is via the gut, absorption across the gut wall is controlled by strict homeostatic mechanisms. Once absorbed into the blood it is distributed to tissues with high levels of mitochondria such as the liver, kidneys and endocrine glands. The main route of excretion for inorganic manganese is via the bile while organic Mn is via the urine. The reason for this difference is that the organic forms are biotransformed in the kidney and therefore water soluble upon excretion (Chang 1996). The most common route of exposure is via the gills or lungs, causing increased

incidences of pneumonia, bronchitis, and bacterial infections. Manganese is non-carcinogenic (Chang 1996).

Zinc is a trace element essential for growth, development and reproduction in vertebrates (Merian 1991). It is necessary for the function of various metabolic enzymes, and plays an integral role in the function of the enzyme DNA polymerase which allows for the synthesis of RNA, DNA and protein. Zinc is primarily mixed with copper to yield brass and commonly used by industry to produce non-corrosive alloys for automobile parts and household appliances. It is also used by the agricultural industry to produce carbamate pesticides, which commonly leach into the groundwater supply (Goyer and Cherian 1995). The main method of zinc extraction (blasting ore) causes a moderate loss to the atmosphere therefore industry often controls the concentration of airborne particles by employing a wet flotation treatment. This causes a large proportion of zinc emissions to enter the environment via the ground water supply. In both fresh, ground and saltwaters background levels of zinc are present at 10 μg/L (Chang 1996). This level increases significantly if the passage of water is via zinc or brass pipes. Zinc uptake is predominantly via the gut, upon which it is distributed to various organs depending on the organism of concern. The main mode of excretion (96%) is via the feces, while the remaining amount (4%) is via the urine and bile (Goyer and Cherian 1995). There is no known acute toxicity for zinc, and chronic toxicity is highly species specific. Zinc is a non-carcinogen (Chang 1996).

The above metal profiles demonstrate that the modes of uptake, distribution, excretion and toxic effects vary considerably depending on the species of metal. Before examining the toxic effects of a metal within the aquatic environment, it is important to

understand the basic characteristics controlling the distribution and availability of the metal of concern.

Factors controlling metal speciation. Metals are usually defined on the basis of their physical properties in the solid state. This type of classification is appropriate for industry, but provides little information regarding the potential for metal uptake in biological systems. A more useful definition of metals is one based on the properties of their ions in aqueous solutions. Hence, Friberg et al. (1979) modified the definition of a metal to "an element which under biologically-significant conditions may react by losing one or more electrons to form a cation".

The majority of metals are transported into the environment via three processes, atmospheric, aquatic, and biological transport (Friberg et al. 1979). The greater part of the metal load emitted into the environment is via water. Once a metal is discharged to an aquatic system, its distribution can be extremely difficult to predict as it can exist in various cationic states. Most metals commonly adsorb onto sediments and deposited particulate matter, or remain suspended in the water column either attached to suspended particles or detritus or as an aqueous cation that is easily absorbed by organisms (Selim and Amacher 1997). Specifically, sediments within aquatic ecosystems are increasingly becoming receptacles for a wide range of heavy metals generated by human activities (Tarradellas and Bitton 1997). It is therefore extremely important to examine the potential for interaction and uptake of metals by biota feeding on and living in contaminated substrates in these habitats.

The aquatic environment presents special challenges for quantifying metal speciation, sorption to particulate matter and subsequent uptake by suspension-feeding

bivalves. Metals can exist in both a dissolved or particulate form, thus the state of the metal can affect its uptake and assimilation potential within organisms. Water itself is a difficult medium to predict metal transport due to the constant fluctuation of environmental variables such as pH, redox potential (dissolved oxygen, ionic strength), salinity, alkalinity and hardness, the presence of organic and particulate matter, and biological activity (Goldschmidt 1937). Each of the above variables affect the individual speciation of the five metals examined in this thesis differently.

The salinity of an aquatic habitat affects metal speciation and thus availability to an organism, in addition to causing physiological modifications within an organism (Moore 1985; Hall and Anderson 1995; Nicholson 1999; Blackmore and Wang 2003). Previous studies have demonstrated that aqueous metals have higher uptake rates in bivalves inhabiting lower salinity habitats (Fisher 1986; Sadiq 1991; Hall and Anderson 1995). A decrease in salinity caused a decrease in the concentration of chloride anions. Divalent metal cations bind with chloride anions, therefore a lower concentration of anions allows for a higher concentration of aqueous metal cations to exist as a charged species in solution. This makes the charged metal cation more bioavailable for uptake via the gills in bivalve species. Changes in salinity can also cause cellular disruptions leading to a decrease in bivalve health and a higher incidence of mortality (Hauton et al. 1998; Ringwood et al. 1998; Nicholson 1999). At low salinities the adaptive capacity of the cellular processes in the oyster *Ostrea edulis* is reduced to such an extent that the haemocytes are unable to respond to favourable temperature regimes (Hauton et al. 1998).

Seasonal variations in food quality have also been shown to modify the distribution and uptake of metals in marine bivalves (Wright and Mason 1999; Cranford and Hill 1999; Rainbow et al. 2004; Sokolowski et al. 2004). In the winter, when the seas are heavy and erosion more prevalent, the sediment is coarser while in the summer, there is a build-up of fine particles that leads to a reduction in median grain size (Gray 1981). An increased potential exposure to heavy metals occurs in suspension-feeding bivalves feeding on metal rich small particles during the summer months (Mudroch and MacKnight 1991; Siegel 2002; Ibhadon et al. 2004). Organic content is an extremely important factor controlling food quality and quantity in the benthic environment. Many bivalve species maintain optimal feeding rates that are modified depending on the organic:inorganic ratio of available food particles (Luoma 1996; Arifin and Bendell-Young 2000; Widmeyer et al. 2004). The finest particle fraction of sediment (2-60 μm) is known to retain the highest proportion of metals due to an increase in the surface area to volume ratio. Therefore, bivalves selectively feeding on the smallest particles are also feeding on the most contaminated particles.

Metal sorption to particulate matter differs depending on the characteristics of the particle or seston. Studies demonstrate that metals bound to algal/phytoplankton cytoplasm had higher assimilation rates in bivalves than when bound to sediment, causing an increased metal uptake when feeding on phytoplankton. This is due to the binding of Cd and Zn to Mn and Fe oxides in sediments (Griscom et al. 2000; Bendell-Young et al. 2002), compared with the cytostolic bound metals in phytoplankton (Wang and Fisher 1996; Lee and Luoma 1998). The differential uptake of these metals is therefore partially controlled by the properties of the particle to which the metal is bound.

Additionally, the digestive efficiency of the bivalve gut also plays an important role in controlling metal speciation and availability. Two types of digestion occur in the bivalve gut, extracellular and intracellular digestion. When bivalves ingest food particles they enter the stomach and are then shuttled to either the intestine or digestive gland. Extracellular digestion occurs in the intestine and is less efficient in assimilating many types of carbon, as food particles are held for a very short (2-20) period of time, resulting in a short period of absorption and rapid egestion of food particles (Luoma 1996). Intracellular digestion occurs in the digestive gland and is much more selective, preferentially selecting higher quality food sources which undergo more intensive (4-72 hrs) digestion (Decho and Luoma 1991; Penry 2000). Intracellular digestion is a much slower, glandular process, allowing for a more efficient assimilation of particles higher in organic carbon. Digestive efficiency is therefore highly variable in its effects on metal uptake and gut passage times in bivalves as it is dependent on the bivalve species, food quantity, and quality (Wang et al. 1995).

Biology of Mytilus trossulus. The Pacific blue mussel *Mytilus trossulus*, also called the "foolish mussel" is an invertebrate organism belonging to the Phylum Mollusca, Class Bivalvia (Harbo 2001). With over 10,000 living species worldwide, bivalves (two shells) are exclusively aquatic and found in a variety of environments ranging from rivers and lakes to the depths of the oceans (Harbo 2001). In British Columbia, 180 bivalve species have been identified, with 70 inhabiting the intertidal zone (or to depths of 50 m). *M. trossulus* has a smooth bluish black shell which is elongated and somewhat pear shaped. It inhabits the temperate intertidal marine waters of Western North American coastlines from Alaska to central California (Harbo 2001). Its preferred

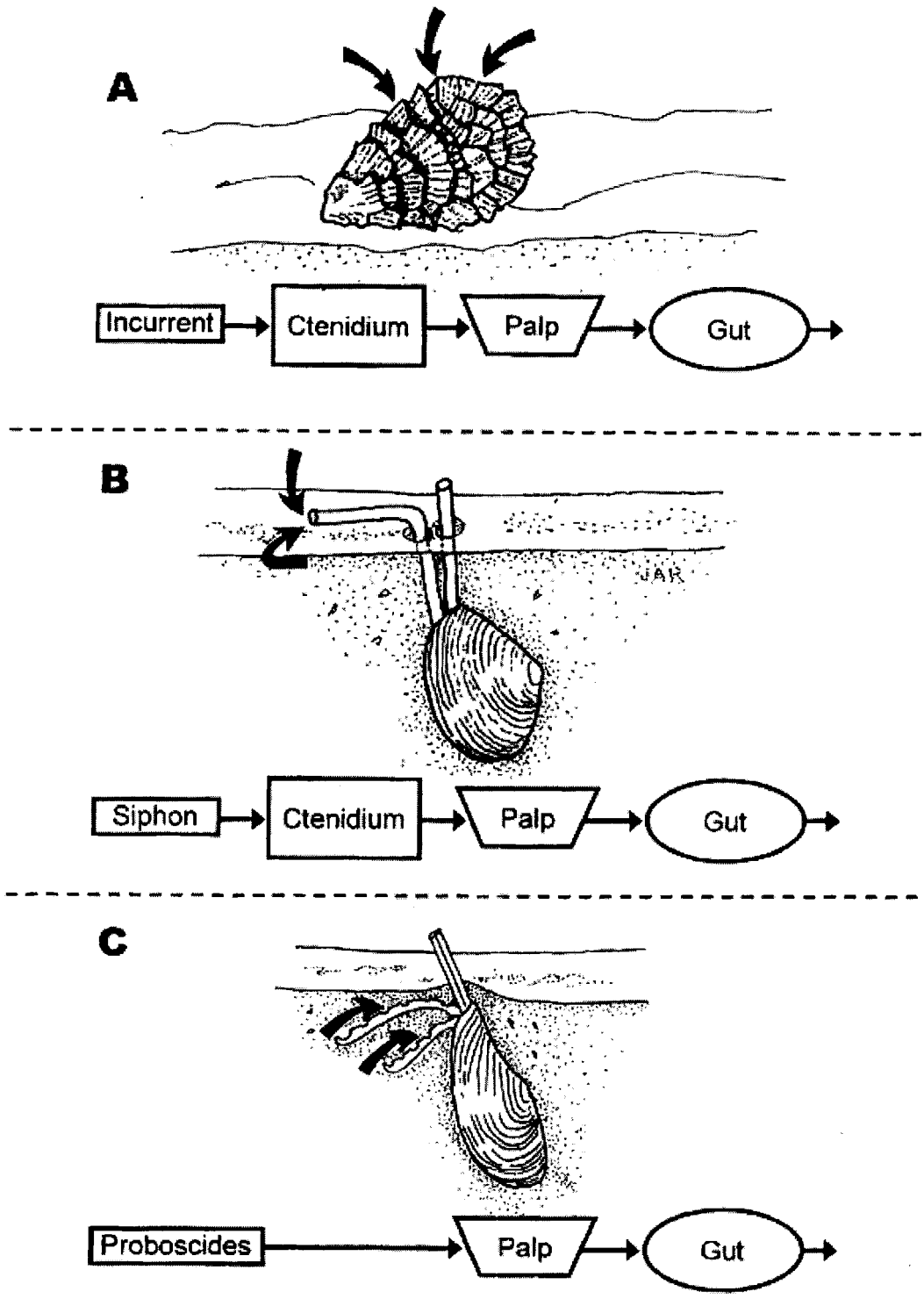
habitat is to anchor to rocks, pilings and other mussels to form "mussel beds" (Harbo 2001). *M. trossulus* is a suspension-feeding organism, filtering up to 40 L of water a day and selectively ingesting a variety of phytoplankton, zooplankton, bacteria, sediment and detritus suspended in the water column (Bayne 1976). Its main predators are starfish, whelks, moonsnails, crabs, seabirds, otters and humans (Harbo 2001).

M. trossulus has two dominant stages in its life cycle, a sessile stage and a mobile larval stage (Stasek 1965). As sessile adults, females release eggs and males release sperm into the water column. After fertilization, which usually occurs in mid-May to late June, the fertilized egg develops into a free floating larval form of the mussel known as a trochophore. These partially developed mussels float around in the ocean for a period of three to four weeks, allowing for its shell to mineralize and major body parts and systems to develop (Stasek 1965). Upon maturation the floating larvae attach themselves to a suitable growing surface by excreting a protein which hardens upon contact with sea water to form byssal threads. These filaments are incredibly tough, with a tensile strength of 20 - 30 pounds, and latch the mussel fiercely to its substrate (Bayne 1976). From this point on, the mussel is predominantly sessile, and will grow and mature into a fully developed adult (maximum 7.5 cm) (Bayne 1976). In the Pacific Northwest, the Pacific blue mussel lives only 1-2 years, but in other parts of the world blue mussels can live up to 17 years (*Mytilus edulis*, Sweden) (Harbo 2001).

Within aquatic benthic environments there are three main classes of bivalve feeders; (A) suspension or filter feeders, (B) deposit feeders using an inhalant siphon and ctenidia and (C) deposit feeders using palp proboscides (Figure 1.1, Ward and Shumway 2004, copyright permission was obtained by these authors for use in this thesis).

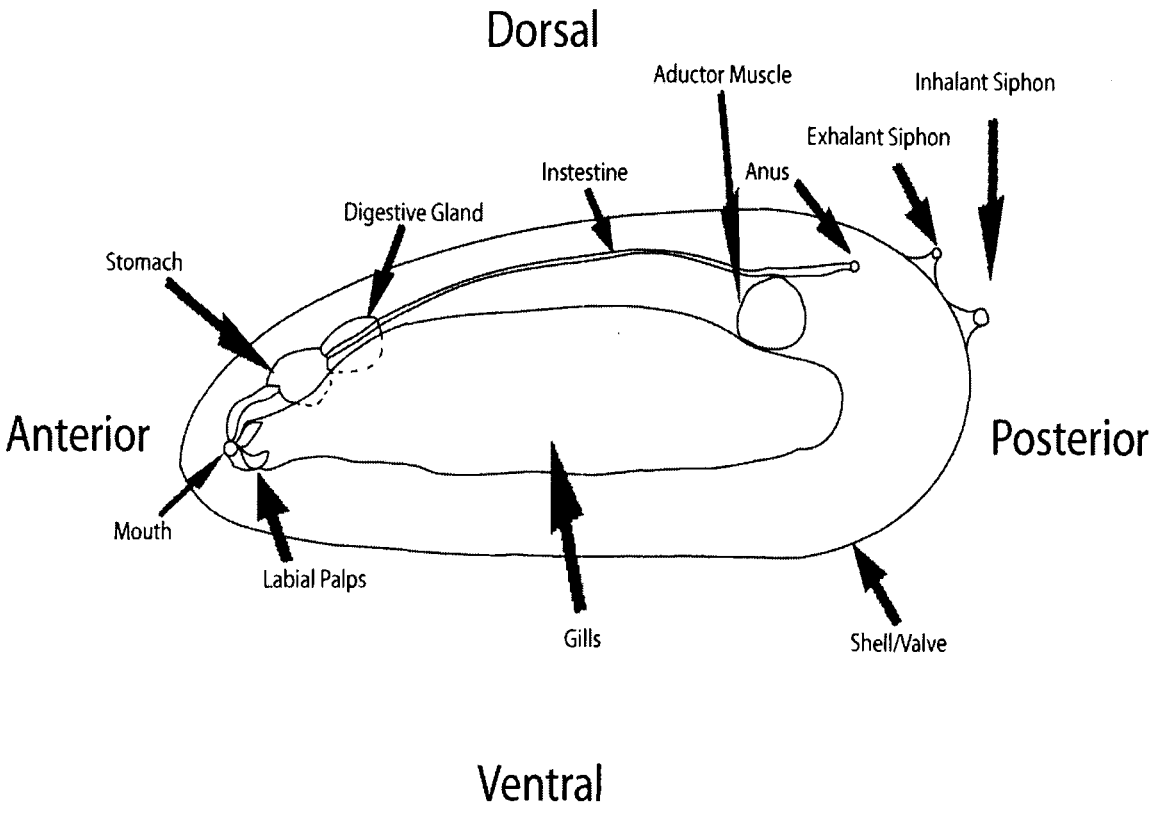
Suspension feeders (e.g. *Crassostrea* sp., *Mytilus* sp.) transport suspended particulate matter from the water column to their inhalant aperture, particles then enter the valves or body cavity and are captured by the ctenidium (gills) and transported toward the labial palps using either a mucous string or water currents produced by specialized cilia lining the surface of the gills (Figure 1.2). Particles are then sorted by the labial palps and either rejected or ingested via the mouth and passed onto the stomach/gut (Ward 1996, 1993). Deposit feeders (e.g. *Macoma* sp.) using either an inhalant aperture or siphon feed using the same process as suspension feeders, with the exception that they capture deposited food particles from the ocean floor. Deposit feeders (e.g. *Yolida* sp.) using palp proboscides capture deposited food particles using these appendages. Particles are then transferred to the labial palps, not the gills (which are quite reduced in size in these species), where they are sorted, passed onto the mouth or rejected, then digested in the gut (Jorgensen Barker 1966; Stasek 1965).

Figure 1.1. Major modes of food collection in the Bivalvia. (A) suspension feeding (B) deposit feeding via an inhalant siphon (C) deposit feeding via palp proboscides.



Source: Ward and Shumway 2004, by permission

Figure 1.2. General diagram of internal morphology for suspension feeding bivalves.



Suspension feeders such as *M. trossulus* are confronted with a wide range of living and non-living material commonly referred to as seston. Seston primarily consists of a wide range of plankton, material re-suspended from the ocean floor, sediments, and aggregates consisting of high molecular weight substances, detritus, fecal pellets and microorganisms (Alldredge and Gotschalk 1989). *M. trossulus* actively feeds on particles based on size, quantity, and quality (Arifin and Bendell-Young 1997, 2000). They prefer particles in the size range of 2-60μm and of varying organic content, with phytoplankton

containing the highest amount of carbon followed by bacteria then suspended sediments (Wang et al. 1995; Arifin and Bendell-Young 1997; Griscom et al. 2000). Metal uptake by *M. trossulus* can therefore vary significantly based on the selective feeding behaviour of this species.

Biomonitor species. Biomonitor species can serve as unique environmental indicators of the biological conditions within an ecosystem (Mandeville 2002). Many regulatory agencies utilize biomonitor species to calculate individual response metrics in an effort to assess water quality and overall habitat conditions (Mandeville 2002). In order to monitor potential adverse effects due to metal pollution, one needs to decide upon which species to monitor. Bivalves possess many attributes (listed below) that have led to their use as biomonitors of metal exposure in intertidal habitats (Dame 1996).

1. Bivalves are dominant members of coastal and estuarine systems and have wide geographical distributions.
2. These animals are sedentary and serve as integrators of metal contamination in a specific area.
3. They are relatively tolerant of (but not insensitive to) a wide range of environmental conditions.
4. Suspension-feeding bivalves pump large volumes of water and concentrate metals by several orders of magnitude over concentrations in seawater.
5. Concentrations of metals in bivalve tissues provide an assessment of bioavailable metal concentrations.
6. Commercially-important bivalve populations are relatively stable.
7. Bivalves can be readily transplanted and maintained in specific sites.

8. Several bivalves are commercially important as seafood, and their chemical contamination is of interest to public health.
9. Bivalves have several behavioural and physiological responses to stress that are easily and quickly measured.

Currently, bivalves are viewed as one of the best functional indicators of metal contamination due to human activity in marine intertidal ecosystems. In 1984, the American National Oceanic and Atmospheric Administration (NOAA) initiated the "Mussel Watch" program to monitor trends in chemical contamination in coastal and estuarine waters (Goldberg and Bertine 2000). The program monitors organic and metal contaminants within these ecosystems by collecting surface sediments and mussel (*M. trossulus and M. edulis*) and oyster tissues from over 200 sites on a seasonal basis (Sericano 2000). Sites are chosen to be representative of large areas rather than small-scale patches of contamination. The results are used to describe the spatial distribution and temporal trends in coastal contamination, and to help differentiate the effects of human activity (anthropogenic) from those of natural input (Sericano 2000).

Over the past two decades, the use of biological monitors has become a standard practice in national and international monitoring programs based on Mussel Watch. Several complicating factors have become apparent in the application of these programs, and need to be taken into account when sampling over large geographical areas. Species availability, physiological conditions, and climatic variables encountered over large areas of study must be considered in order to produce data that are meaningful and comparable between sites (Goldberg and Bertine 2000; Sericano 2000). Thus, the applicability of the Mussel Watch concept to global chemical contamination monitoring programs requires

careful planning to minimize the effects of these factors. Past studies demonstrate that changes in the rates of particle feeding, including the degree to which particles are captured, ingested or rejected, can potentially affect contaminant availability within and between ecosystems (Desous-Paoli et al. 1992; Smaal 1997; Vanderploeg et al. 1996; Prins et al. 1998; Baker et al. 1998). The physiological and behavioural traits that regulate metal uptake in bivalve biomonitors must be considered when interpreting tissue metal levels between sites. In addition, recent studies examining metal uptake in mussels (*M. trossulus*) and barnacles demonstrate significant geographical and seasonal variations within a large single site (Baltic Sea) (Rainbow et al 2004; Sokolowski et al. 2004). Monitoring programs using bivalve biomonitors that do not account for the above factors may risk over-generalizing in the interpretation of their results between and among sites.

Research Objectives

The main objectives of this research were:

1) to examine the role that organic carbon in food particles plays in the selective feeding behaviour of the blue mussel, *Mytilus trossulus* ingesting single and combination natural food sources,
2) to determine whether the type of natural food sources to which metals are sorbed affects the extent of metal uptake and assimilation by *M. trossulus*,
3) to determine whether the natural abiotic differences in two intertidal habitats affect metal uptake and assimilation in *M. trossulus*,
4) to develop an efficient sampling device to collect relatively large amounts of intertidal marine suspended sediments,

5) to examine seasonal changes in sediment metal levels (Cd, Cu, Fe and Zn) and organic carbon content in sediments collected from two intertidal marine habitats.

Outline of Thesis

This thesis contains three main data chapters that examine the above research objectives. In the second chapter, a marine bacterium *Leptothrix discophora (SP-6)* that naturally accumulates manganese oxides on its outer cell sheath was cultured and fed to *M. trossulus*. The manganese sheath acted as a sportive surface to which various other metals such as cadmium and lead can bind. In principle, the bacterium acted as an enhanced source of Cd and Pb to the blue mussel. In addition, the organic carbon content of the bacterium varied depending upon whether the primary metal (Mn) had secondary metals (Cd, Pb) associated with it. A total of forty-eight feeding experiments were conducted under controlled laboratory conditions using a continuous flow through system that distributed a single food source to *M. trossulus* over a four hour exposure period. The overall objective was to examine whether *M. trossulus* was selectively feeding upon food particles based on their quality, and as a result ingesting varying levels of Mn, Cd or Pb that were naturally sorbed to the single food source.

In the third chapter, a marine phytoplankton *Thalassiosira pseudonana* was combined with natural sediments from two distinct marine intertidal habitats and pulse-fed to *M. trossulus*. Sediments were collected from a mudflat estuary with a salinity of 15 ppt and organic carbon content of 15% (Boundary Bay) and a cobble-pebble estuary with a salinity of 25 ppt and organic carbon content of 24% (Deep Bay). Both sites were located within the Strait of Georgia, BC. A total of sixty-six feeding experiments were

conducted under controlled laboratory conditions using a static pulse-fed system that exposed *M. trossulus* to a radiolabelled food source (^{109}Cd) over a sixty minute time period. Metal assimilation was determined after a four-day purging period where the mussels were held in a continuous flow-through tank and fed unlabelled phytoplankton. The objectives of this chapter were twofold: 1) to determine whether *M. trossulus* was selectively feeding upon food particles based on their quality and as a result assimilating varying levels of Cd from the combination food sources, and 2) to determine whether abiotic variations in the two intertidal habitats (salinity, organic content) had any effect on metal assimilation in *M. trossulus* feeding on natural combination food sources.

The fourth chapter describes a novel intertidal sediment trap designed and tested by the author (JRW). To minimize the amount of time spent collecting and sieving the fine suspended sediment upon which suspension feeders such as *M. trossulus* feed, a sediment trap was designed and deployed for eleven months at both Boundary Bay and Deep Bay. Sediment was collected from the traps at each site (average of 10 to 12 per site) every three to four weeks. The primary purpose of these traps was to collect sufficient quantities of sediment so that a total of nine radiotracer feeding experiments could be conducted subsequently with *M. trossulus* in the laboratory. In addition to testing the efficacy of these traps, we monitored the seasonal changes in the organic carbon content and heavy metal levels in the two sites over an eleven month period.

The final chapter includes a discussion of the experimental results and their implications to the field of aquatic ecotoxicology. I include both general and specific conclusions regarding the effectiveness of suspension-feeding bivalves as biomonitors.

In addition, I make suggestions for future research based on key questions and criticisms that arose out of the present study.

Authorship

The research contained in this thesis is a compilation of 5 years of field and laboratory work conducted by the primary author (JRW). Each main chapter is written in a manuscript style for submission to selected journals. The first data chapter has my supervisory committee and Dr. Astrid Jurgensen as co-authors, the second data chapter has my primary supervisor Dr. Leah Bendell-Young as co-author, and for the third chapter, I retain sole authorship. In addition, there is a fourth manuscript contained in the appendix for which I hold secondary authorship. All study and equipment design, laboratory experiments, field research, sample collection and preparation, data analysis, and writing of the thesis were conducted by the primary author. My supervisor Dr. Leah Bendell-Young played a critical role in providing research funding, advice on experimental study design, mentoring the interpretation of experimental results, and reviewing drafts of the manuscripts written by the primary author. My supervisory committee contributed valuable time and advice towards the experimental study design and in the reviewing of drafts of the main data manuscripts. Several individuals also played important roles in assisting with laboratory analyses. These individuals are acknowledged at the end of each chapter.

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CHAPTER 2:

THE ROLE OF *LEPTOTHRIX DISCOPHORA* IN MEDIATING METAL UPTAKE IN THE FILTER-FEEDING BIVALVE *MYTILUS TROSSULUS (EDULIS).*

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This chapter has been substantially re-written from the original publication in Environmental Science and Technology, 2004, 38, 769-774.

Abstract

The potential for filter-feeding bivalves to accumulate metals from a wide range of food sources is an important consideration when examining trophic transfer of metals within food chains. The objective of this study was to determine the role of *Leptothrix discophora* in mediating metal uptake in the filter-feeding bivalve, *Mytilus trossulus*. The bacterium, *Leptothrix discophora* SP-6 was cultured in the absence or presence of Mn, allowing for a naturally formed Mn oxide sheath to develop. Secondary metals (Cd and Pb) were then added to the cultures, allowing for potential Cd and Pb adsorption to the Mn oxide sheath. Resulting bacterial aggregates of known diameter were then fed to the bivalve *M. trossulus* using a flow-through system. Initial concentrations of both Pb and Cd on the bacterium did not differ significantly in the presence or absence of the Mn oxide, conversely both Pb ($F=7.39$, $p<0.0001$) and Cd ($F=33.65$, $p<0.0001$) were found at lower concentrations in the mussel tissue when the Mn oxide was present.

To determine whether these differences in metal uptake could be attributed to sorting by the mussel based on food quality, nutritional analysis was performed. Bacterial food matrices containing Mn oxides were found to have significantly lower proportions of carbon ($F= 256$, $p<0.0001$). Particle clearance rates for the various food matrices were positively correlated with organic carbon content ($R^2=0.852$, $p>0.008$). The results of our study suggest that Cd and Pb uptake in *M. trossulus* was significantly decreased for Cd with a similar trend for Pb when the SP-6 sheath contained Mn oxides. The mechanism mediating this differential uptake is best explained by food quality, in

that a higher-quality food source enhanced metal uptake due to an increased clearance rate of organic rich particles by *M. trossulus*.

Introduction

Within marine ecosystems, bivalves occupy important intermediary food chain positions, linking lower to higher trophic level organisms. Thus, bivalve molluscs are considered key organisms when assessing levels of contamination in marine ecosystems (Paez-Osuna et al.1993)). Heavy metal contamination due to both natural and anthropogenic metal sources has been of increased interest to scientists examining the uptake and bioavailability of metals in bivalves (Stecko and Bendell-Young 2000; Griscom et al. 2000; Blackmore and Wang 2002). Anthropogenic metals introduced into the aquatic environment predominantly exist in a soluble phase complexed with an anion or neutral ligand, or in the particulate phase sorbed to organic or inorganic particles, depending on the pH of the environment (Kessick 1975; Jenne and Zachara 1984; Regnier and Wollast 1993). Many heavy metals associate with iron and manganese oxyhydroxides that precipitate close to the sediment-water interface in aquatic environments (Davison et al.1991, 1993). For filter-feeding bivalves, metals can be taken up from both soluble and particulate sources. Several studies have recently examined the uptake of aqueous metals in bivalves (Thomann et al. 1995; Lares and Orians 2001). Aqueous metal contaminants are known to contribute to less than 4% of metal uptake in the genus *Mytilus* (Chong and Wang 2000; Blackmore and Wang 2002). In contrast, the processes that control both the uptake and bioavailability of metals sorbed to food matrices are currently under research. Recent studies by our laboratory and others, have demonstrated the existence of preferential pre- and post-selective feeding behaviours

exhibited by bivalves when presented with metal sources sorbed to organic versus inorganic matter (Gagnon and Fisher 1997; Arifin and Bendell-Young 1997; Griscom et al. 2000).

Bacteria are key components of aquatic ecosystems, and are thus potential food sources for filter-feeding organisms (Sorokin 1971; Birbeck and McHenery 1982). *M. trossulus* actively feeds upon bacteria aggregates that fall within their particle size preference of 2.0 to 20.0 μm, principally when phytoplankton stocks are low relative to bacterioplankton (Lucas et al. 1987). In the past 10 years, researchers have examined the potential for bacterially-generated Mn and Fe oxides to serve as sorption surfaces for soluble toxic heavy metals found in polluted intertidal environments (Santschi et al. 1990; Emerson and Ghiorse 1993; Yee and Fein 2001). Bacteria in the genus *Leptothrix* actively oxidize both Mn and Fe on the surface of their extracellular polysaccharide sheath (Holt et al.1994). Oxidation is catalyzed by a secreted manganese oxidase (Corstjens et al. 1991). It is presently unknown whether these bacterially-mediated oxides can influence heavy metal uptake in filter-feeding organisms. Thus, the objective of our research was to examine the extent of Pb and Cd uptake in *M. trossulus* feeding on the aquatic bacterium SP-6, in the presence or absence of Mn oxides.

Methods

Eight different groups of *M. trossulus* (collected from April to December, 2001) with shell lengths ranging from 42.5 to 49.5 mm were collected from an intertidal rock cliff in Porteau Cove Provincial Park, located in southwestern BC, Canada. The mussels were acclimated in the laboratory to 13 °C, 25 ppt salinity, and held in 200 L fully aerated tanks. They were fed a diet of blue-green algae (*Spirulina pacifica*, 2% dry tissue weight

per day) for a minimum of 2 weeks prior to experimentation. Feeding ceased 24 hours prior to experimentation when mussels were separated into 30 L aerated tanks. To determine that all mussels were fully acclimated prior to the feeding experiments, a gravimetric condition index was performed the summer and fall of 2000 on 200 *M. trossulus*. The gravimetric index is the recommended standard method for determining bivalve condition indices (Crosby and Gale 1990).

Leptothrix discophora SP-6 (Minus Metal). The strain of *Leptothrix discophora* used was the sheath-forming SP-6 strain obtained from the American Type Culture Collection (ATCC # 51168). Pre-cultures (10 mL) were grown to mid log phase growth on a 125 rpm rotary shaker, at 19-22 °C for 48 hrs, then transferred to large scale (100 mL) cultures. After 72 hrs of large scale growth, late logarithmic phase cell growth was reached, at which time the cells were harvested for the *M. trossulus* feeding experiments. Approximately 70 mL of media was aseptically removed, samples were spun at 280 g for 20 minutes, and all but 5 mL of the supernatant was removed. Cells were washed with double distilled H_2O, resuspended and then re-centrifuged at 280 g. The supernatant was removed and the cells were re-suspended in 950 mL of 1.0 μm filtered seawater. Potential cell lysis caused by changes in osmotic pressures were accounted for by measuring particle concentrations prior to (<5% below 3 μm, N=15) and post cell washing (<7% below 3 μm, N=15) using a Z1 Coulter Counter with an aperture of 100 μm and counting window between 2-20 μm. The SP-6 solution was kept mixing at a low speed using a magnetic stirrer for a maximum of 30 minutes until commencement of the feeding experiment.

SP-6 cells naturally form filamentous aggregates, therefore to ensure that the majority of particles were within 2-20 μm, aggregates were passed through a 21-gauge needle. Average particle size (9 μm, SD=1, n=25) was determined using a Z1 Coulter Counter with a 100 μm aperture. The particle concentration fed to *M. trossulus* over the 4 hour exposure period was 17×10^6 cells L^{-1}(6.7 mg dry wt per L). In a flow-through experimental system, food particles falling below 2 and above 20 μm at a concentration of $17x10^6$ cells per L are virtually ignored by the mussels (Arifin and Bendell-Young 1997). In addition, particles below 2 and above 20 μm constituted less than 5% of the SP-6 food matrix once aggregates were syringed and re-suspended in 1.0 μm filtered seawater. Elemental analysis was performed to obtain % C, N and H for all bacterial food matrices fed to *M. trossulus*.

SP-6 with One Metal (Mn/Pb/Cd). SP-6 was cultured as noted above with the following additions. $MnSO_4$ (11.2 mg L^{-1}), lead acetate (1.83 mg L^{-1}) and cadmium acetate (0.112 mg L^{-1}) solutions were added to the flask cultures at the same time that cells were transferred to large scale growth. The molarity of each metal added to the cultures were: Mn, 7.5×10^{-6} M, Cd , 4.9×10^{-8} M, and Pb, 5.6×10^{-7} M. After the 72 hours, late logarithmic phase was reached, at which time the cells with sorbed metals were harvested for the *M. trossulus* feeding experiments.

SP-6 with Mn oxide Plus Pb or Cd. The previous protocol for culturing SP-6 food matrices without metals was followed with the following additions: $MnSO_4$ (11.2 mg L^{-1}) was added at the time of the transfer to large-scale cultures. After 24 hrs, either lead (1.83 mg L^{-1}) or cadmium acetate (0.112 mg L^{-1}) was added. This 24 hr delay ensured that the Mn oxide had adequately formed before the addition of Pb and Cd

occurred. Formation of the oxide was confirmed microscopically using a Vanox light microscope at 400x magnification. Following the addition of the second metal solution, cells were incubated 48 hours, and harvested for the *M. trossulus* feeding experiments.

Natural Sediments. Sediment at the sediment:water interface (floc) was collected for feeding and used as a control point of contrast, representing naturally occurring background levels of metal contamination. Sediments were wet sieved to 150 μm and kept at 4 °C for a maximum of 5 d in their native seawater. Particle size was confirmed using a single threshold Z1 Coulter Counter (100μm aperture). The particle concentration fed to *M. trossulus* was derived counting only the particles between 2 to 20 μm, with a final concentration of $19x10^6$ particles L^{-1} (5.6 mg dry wt per L).

Feeding Experiments. Seven different food sources/exposures were fed to *M. trossulus*. Each exposure was replicated five times, with each replicate lasting 4 hours in duration (n=9 per replicate). The food matrices were as follows; SP-6 alone, SP-6+Mn, SP-6+Pb, SP-6+Cd, SP-6+Mn+Pb, SP-6+Mn+Cd, and sediment alone. All experimental equipment used was acid washed for 24 hrs in a 10% HNO_3 bath to ensure no background or carry-over metal contamination. The experimental food matrix (1 L) was diluted to 15 L in filtered seawater and continually mixed with a submerged metal-free aquarium pump. Over the four hour experimental dosing, the food matrix was diluted with an additional 105 L supply of filtered seawater in a 2 L plexiglass mixing chamber (Arifin and Bendell-Young 1997, 2000). The mixing chamber was constantly mixed with a magnetic stirrer and the food matrix was distributed to four 920 mL tanks by a peristaltic pump at a controlled flow rate of 110 mL min^{-1}. The pH of the diluted food matrix was maintained at 7.1-7.3 for all food sources and the pH of the seawater was 6.9.

Three of the four exposure tanks were randomly assigned 3 *M. trossulus* with the fourth tank acting as a control (no mussels) to monitor the flow rate of the food matrix. Mussel viability was confirmed by leaving mussels out of the water for 15 minutes and then re-submerging so that air exiting the exhalent siphon could be observed. At the end of the 4 hour exposure period, mussels were held a further 24 hr and fed *Spirulina pacifica* blue-green algae to allow for gut contents to be purged. Previous research has shown that 80% of Cd and Pb are depurated within a 24 hr time period (Arifin and Bendell-Young 1997). Desorption of Mn, Cd and Pb from either the sediment or SP-6 food matrices constituted less than 4% of total available metals (Arifin and Bendell-Young 1997, 2000). Aqueous Mn, Cd and Pb uptake was therefore considered negligible. Finally, all mussels were rinsed with 0.05 M EDTA, filtered seawater, then removed from their shell using metal free tools, weighed, and dried in a 60 °C oven until a constant dry weight was obtained (average of 72 hrs).

Clearance Rates. Water samples were collected from each of the four 920 mL tanks every 30 minutes over the four hour exposure period. Particle concentrations for individual mussels in each exposure were determined using a Coulter counter (100 µm aperture, 2 to 20 µm window). Particle clearance rates (L/hr) of the various food matrices by *M. trossulus* were calculated using the following equation; CR = flow rate (L/hr) × [1-(concentration of particles in treatment / concentration of particles in control)].

Atomic Absorption Spectroscopy. Mussel tissues were prepared for metal analysis according to the U.S. Environmental Protection Agency Protocol (McDaniel 1991). Dried mussel tissues were weighed as noted above, homogenized, and a sub-sample of

approximately 0.2-0.4 g was used for metal analysis. Bacterial, control and NBS (TORT-2 lobster hepatopancreas and MESS-2 marine sediment) standard samples were digested in 5 mL of 70% environmental grade HNO_3 (<1 ppb of any metal) at 80 °C until a clear solution of 1 mL was obtained. Sediment samples had 10 mL of HNO_3 added 24 hrs prior to digestion and were acid extracted at 60 °C until a 3 mL volume was obtained. One mL of each sample was then removed and diluted to 10 mL and held at 4 °C until analysis could be completed. Digested tissue and acid extracted sediment samples were analyzed for Mn, Pb, and Cd using an AA spectrophotometer. NBS standards TORT-2 (with mussel tissues, 44 total) and MESS-2 (with sediment samples, 6 total) were used for every 9 samples digested, less than a 10% mean variance from the standard NBS values was achieved.

Kinetic Model of Uptake. A modified general model of metal uptake was used to compare observed with potential dietary metal uptake in *M. trossulus* (Thomann et al.1995):

$$\text{Dietary Uptake}_{\text{mussel}} = [\text{Metal}]_{\text{food}} \times \text{IR} \times \text{AE} / \text{BW} \qquad \text{(Paez-Osuna et al. 1993)}$$

Dietary metal uptake (μg/g) is the product of the concentration of metal in the food source (μg/g dry weight), ingestion rate (IR, g of particles dry wt per L ingested by *M. trossulus* multiplied by the clearance rate (CR) (L/hr), and the measured metal assimilation efficiency (%AE) divided by the dry body weight (bw) (g) of the mussel. Previously reported AE's (0.40 or 40%) for *M. trossulus* feeding upon a single food source labelled with Cd^{109} were used (Wang 1995; Arifin and Bendell-Young 1997). We were unable to find Pb AE's for any filter-feeding bivalve; therefore, for comparative purposes only, and to demonstrate the relative importance of CR versus AE in

influencing the amount of metal taken up by the bivalve, we used AE as determined for Cd. AE values were modified (0.15 to 0.40) and CR values held constant (1.5 L/hr) to determine whether AE or CR was driving the differences in Pb and Cd uptake by *M. trossulus*.

Results

Metal Concentrations in Sp-6 Samples. Metal concentrations in bacterial food matrices are presented in Table 2.1. Supernatant levels were measured to account for any metal that had not been sorbed to the SP-6 sheath or had been oxidized and re-released into solution. Complete sorption of metals to SP-6 cell sheaths did not occur in any of the six preparations therefore the amount of metal in solution was not limiting. Bacterial cells containing the Mn oxide and either Pb/Cd had higher concentrations of Pb (29%) and Cd (23%) sorbed to their sheaths than cells with just Pb or Cd and no Mn oxide. Differences noted were not significant at $p=0.05$ (ANOVA, $p>0.16$).

Table 2.1. Mass balance of metal concentrations in food matrices fed to *M. trossulus*. Metal concentrations (n=6, ± SD) fed to *M. trossulus* were characterized as the amount of metal sorbed to SP-6 cells. Initial metal concentrations are based on the amount of metal provided to SP-6 cells in their growth media. Concentrations of metals not sorbed to SP-6 can be obtained by subtracting % metal sorbed to SP-6 from 100. Predicted metal concentrations were derived using a kinetic model of uptake while actual metal concentrations were obtained experimentally. Both values are shown as µg of metal per g of dry body weight per four hour experiment.

Metal	SP-6 matrix fed to *M. trossulus*	[metal] present in SP-6 cultures (mg/L)	[metal] sorbed to SP-6 (mg/L)	% total metal sorbed to SP-6	Predicted [metal] with Kinetic Model (µg/g dry wt)	Actual [metal] in *M. trossulus* (µg/g dry wt)
Mn	SP-6+Mn	40.8	18.4 ± 0.5	45	1.65	9.08
	SP-6+Mn+Pb	40.8	17.0 ± 1.0	42	0.86	5.59
	SP-6+Mn+Cd	40.8	13.2 ± 0.9	32	0.54	7.76
Pb	SP-6+Pb	11.6	4.1 ± 0.3	35	0.51	8.17
	SP-6+Mn+Pb	11.6	5.8 ± 0.4	50	0.29	6.27
Cd	SP-6+Cd	0.54	0.13 ± 0.02	24	0.02	11.26
	SP-6+Mn+Cd	0.54	0.17± 0.02	31	0.01	8.40

Metal Concentrations in Sediment Samples. The concentrations of metals as determined by AAS were 19.8 µg/g dry wt Mn, 5.22 µg/g dry wt Pb, and 1.41µg/g dry wt Cd. Tissue metal concentrations in *M. trossulus* fed natural sediments were always greater than control *M. trossulus* concentrations (Figures 2.1.1, 2.1.2, and 2.1.3).

Figure 2.1.1. Concentrations of Mn (2.1.1) in *M. trossulus* tissue after 4 hours of exposure to SP-6 plus or minus metal oxides (n=15 ± SE for each food matrix. An ANOVA was conducted for each metal to determine if significant differences existed between exposures. F and p values are reported along with degrees of freedom.

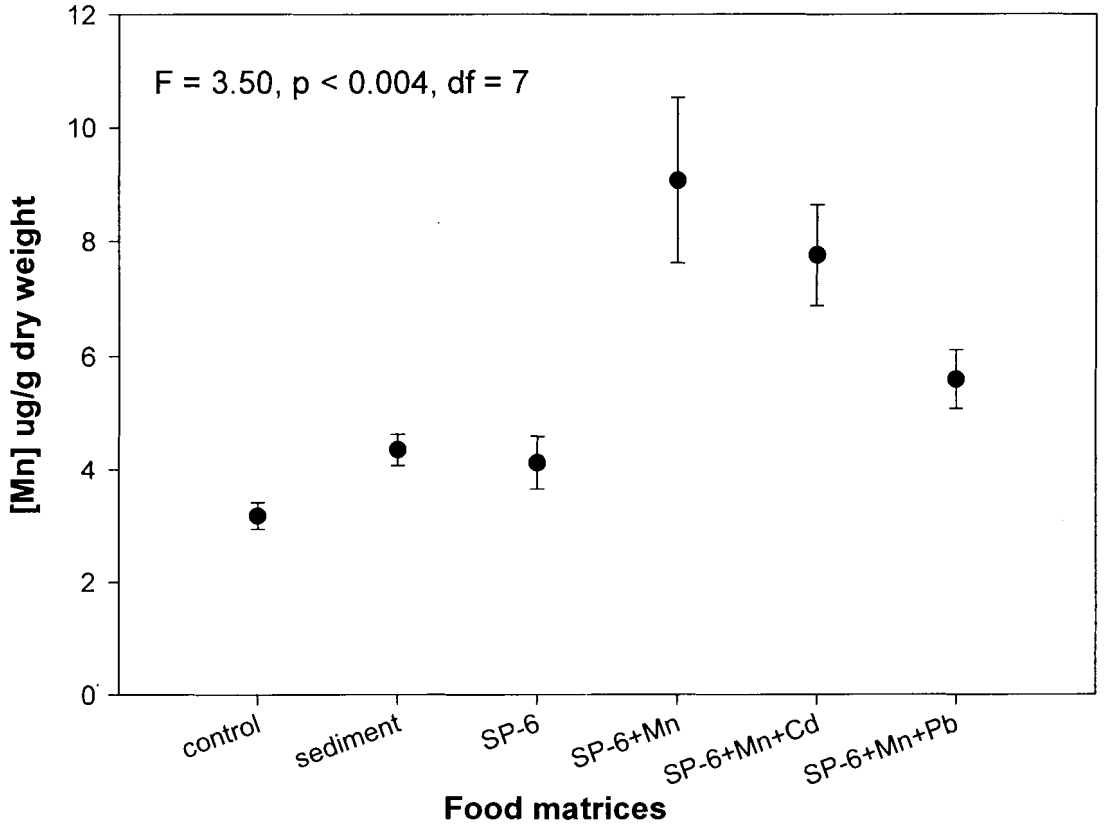


Figure 2.1.2. Concentrations of Pb in *M. trossulus* tissue after 4 hours of exposure to SP-6 plus or minus metal oxides (n=15 ± SE for each food matrix). An ANOVA was conducted for each metal to determine if significant differences existed between exposures. F and p values are reported along with degrees of freedom.

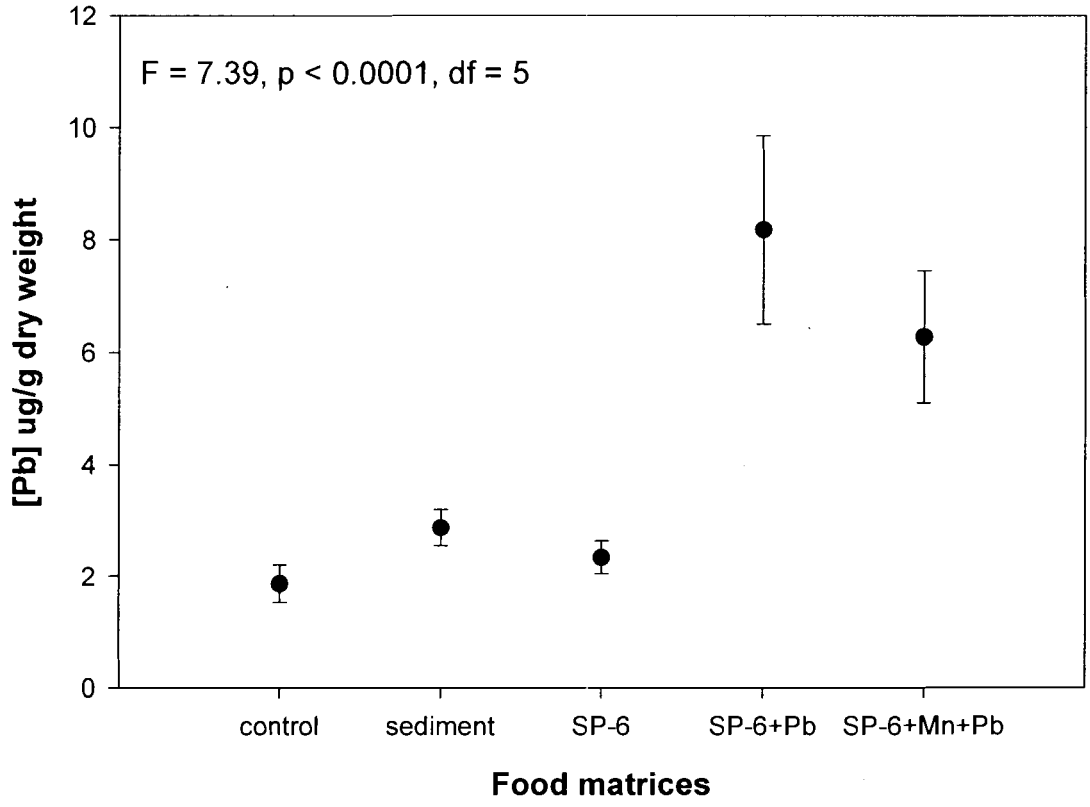
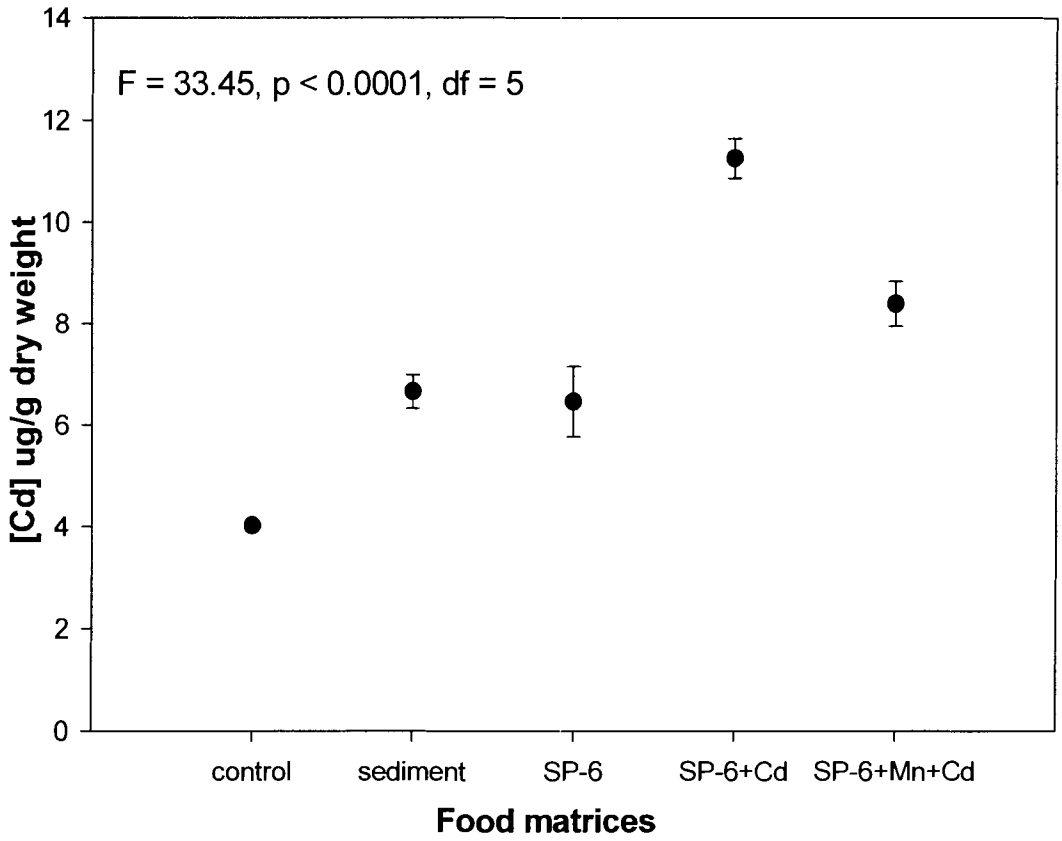


Figure 2.1.3. Concentrations of Cd in *M. trossulus* tissue after 4 hours of exposure to SP-6 plus or minus metal oxides (n=15 ± SE for each food matrix). An ANOVA was conducted for each metal to determine if significant differences existed between exposures. F and p values are reported along with degrees of freedom.



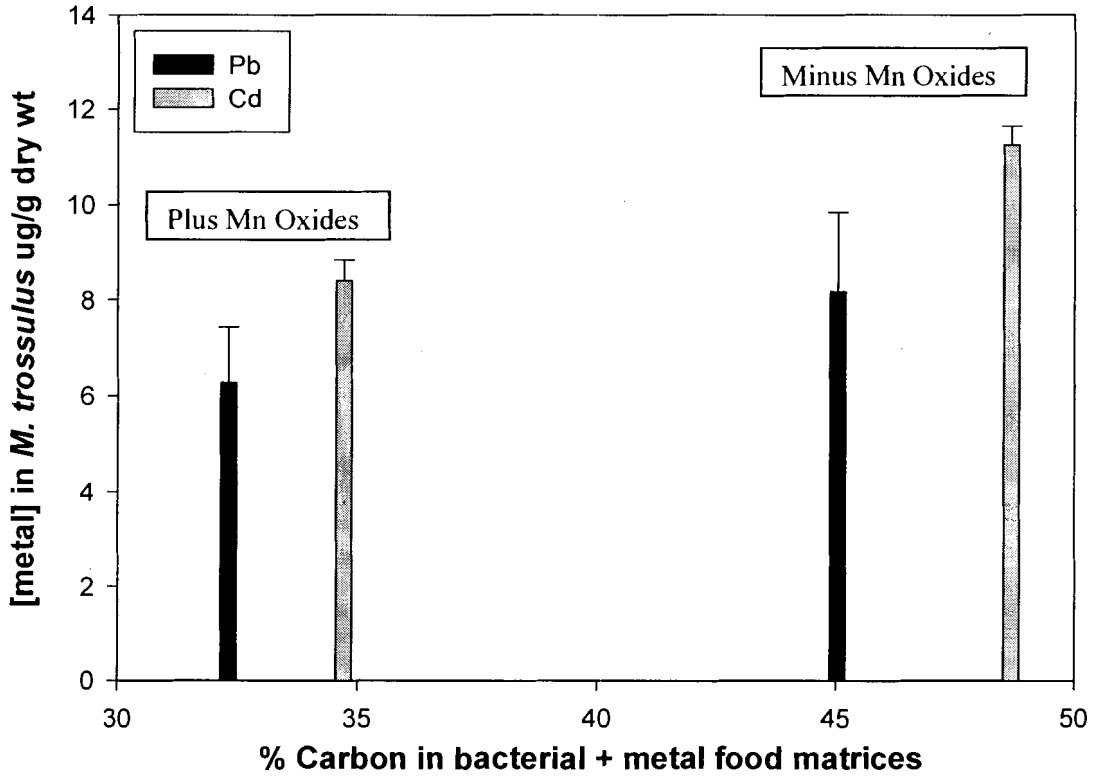
Organic Carbon Analysis. The nutritional composition of the bacterial food matrices is presented as %C, %H and %N for each treatment in Table 2.2. A one way ANOVA was used to determine any differences between food matrices containing Mn versus no Mn (n=9 per exposure). Food matrices with Mn were significantly lower in %C, N and H ($p<0.0001$) as compared to matrices without the Mn oxides (Table 2.1). Specifically, samples containing Pb in the presence of the Mn oxide were significantly lower in carbon as compared to samples with Pb alone ($F=365$, $p<0.0001$). This same

trend was apparent for bacteria samples containing Cd in the presence of the Mn oxide versus Cd alone ($F=1189$, $p<0.0001$).

Table 2.2. Elemental analysis of SP-6 samples fed to *M. trossulus* (n=9 ± SD). ANOVA values for %C were $F=256$, $p<0.0001$, %H, $F=252$, $p<0.0001$), and %N, $F=31.7$, $p<0.0001$.

Treatment	%C	%H	% N
SP-6	48.57 ± 0.34	7.05 ± 0.02	9.02 ± 0.10
SP-6+Pb	45.31 ± 0.40	6.70 ± 0.06	9.10 ± 0.34
SP-6+Cd	48.41 ± 0.72	6.95 ± 0.16	8.13 ± 0.59
SP-6+Mn	38.99 ± 0.22	5.88 ± 0.08	7.56 ± 0.02
SP-6+Mn+Pb	32.60 ± 1.53	5.15 ± 0.08	6.63 ± 0.24
SP-6+Mn+Cd	34.44 ± 0.66	5.42 ± 0.04	7.06 ± 0.26

Figure 2.2. A comparison of % carbon versus mussel tissue metal content in mussels fed SP-6 food matrices with or without Mn oxides present. The results of a one way ANOVA are F=365, p<0.0001 for Pb, and F=1189, p<0.0001 for Cd.

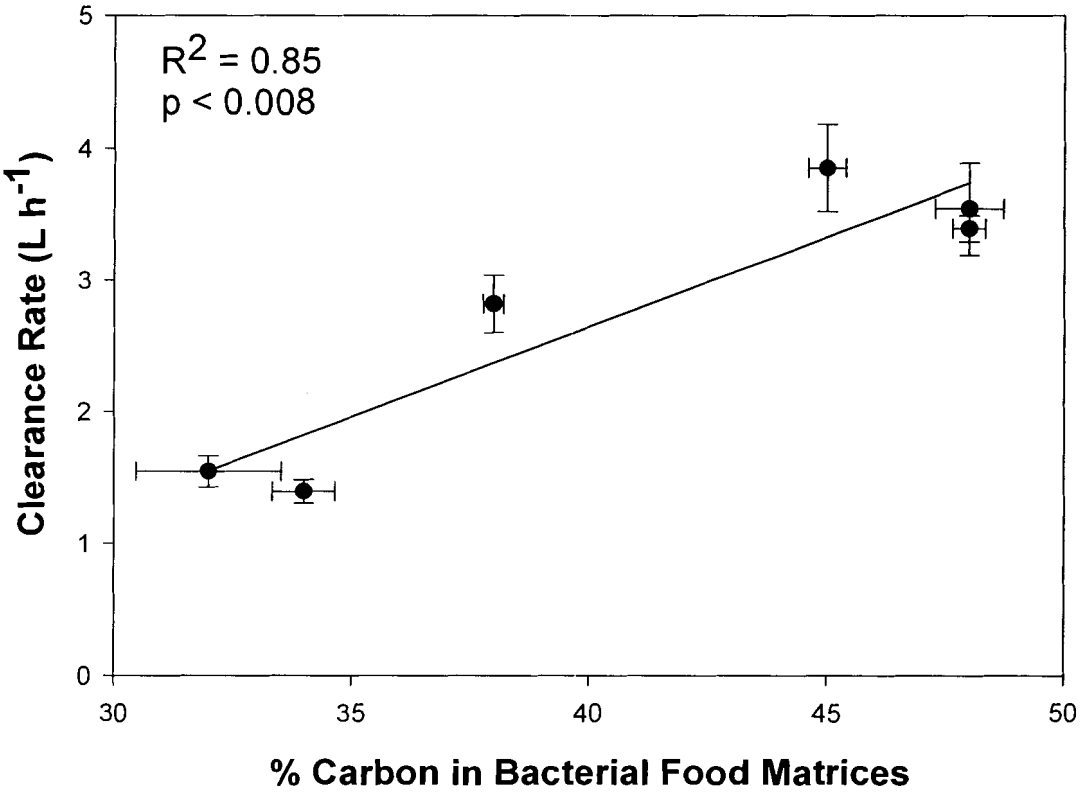


Metal Uptake by M. trossulus. Prior to experimentation, mussels were held in the laboratory and acclimated for a minimum of 2 weeks. To confirm proper acclimation, a gravimetric condition index monitoring the overall nutritional status of *M. trossulus* during the 4-6 week holding period was performed (F= 1.41, p >0.236). A total of thirty-five feeding experiments (n=9 per experiment) were conducted to determine metal uptake from seven various food matrices/exposures (5 replicates per exposure) by *M. trossulus*. Metal levels detected in *M. trossulus* tissues are presented in Figure 2.1.1, 2.1.2 and 2.13. No significant differences existed among tanks within each experiment (p >0.05, n=9,

each experiment had 3 tanks with 3 mussels per tank), therefore tank values were pooled to determine differences among exposures using an ANOVA. This changed each exposure n value from 45 to 15, avoiding possible pseudoreplication. Exposures containing Pb ($F= 7.39$, $p<0.0001$) and Cd ($F= 33.65$, $p< 0.0001$) had significantly different tissue concentrations in *M. trossulus* versus sediment and SP-6 control exposures. Background levels of Mn, Pb and Cd in *M. trossulus* tissues are referred to as the controls and included in Figures 2.1.1-2.1.3 to act as natural reference levels.

Particle Clearance Rates. Particle clearance rates (L/hr per individual) were constant for each experiment with standard errors below 10% for all replicates within each exposure. Clearance rates were highest for the SP-6+Pb matrix (3.85), followed by SP-6+Cd (3.54), SP-6 (3.39), SP-6+Mn (2.81), SP-6+Mn+Pb (1.54), and SP-6+Mn+Cd (1.39). Figure 2.3 presents the relationship between clearance rates and % carbon in the bacterial food matrices using a linear regression model. A positive correlation ($R^2= 0.852$, $p>0.008$) existed between % carbon and particle clearance rate by *M. trossulus*.

Figure 2.3. A comparison of individual *M. trossulus* particle clearance rates versus % carbon in SP-6 food matrices using a linear regression model.



Kinetic Model of Uptake. The predicted and observed levels of metal uptake over the four hour exposure period are presented in Table 2.1. Mussel metal concentrations were significantly higher for both Pb and Cd when *M. trossulus* was fed SP-6 without the Mn oxide (higher % carbon source, higher CR) versus SP-6 with the Mn oxide (lower % carbon source, lower CR) for both the predicted and observed values. When an AE of 0.40 was used, the predicted values demonstrated a 43% higher Pb and 56% higher Cd uptake by *M. trossulus* fed SP-6 sorbed Pb and Cd minus the Mn oxide. A lowering of

the AE to 0.15 maintained the same magnitude of difference for Pb and a slightly lower magnitude (50%) for Cd.

When CR/IR were held constant at 1.5 L/hr (the average CR for our 7 exposures) and an AE of 0.15 and 0.40 applied, the predicted Pb and Cd tissue concentrations were a complete reversal of the observed values. When an AE of 0.40 was used, the predicted values demonstrated a 30% lower Pb and 10% lower Cd uptake by *M. trossulus* fed SP-6 sorbed Pb and Cd minus the Mn oxide. A lowering of the AE to 0.15 lowered the magnitude of difference for Pb to 10% and eliminated any differences in magnitude for Cd.

Discussion

For toxicological studies to be ecologically relevant, both biotic and abiotic factors that influence metal uptake in aquatic organisms need to be considered. The research presented herein contributes to our understanding of how bacterial food matrices influence metal uptake in the filter-feeding bivalve, *M. trossulus*.

The bacterium used in this study is a gram-negative, filamentous, marine bacterium that forms aggregates in the range of 2-300 μm (Zobell 1949; Corstjens et al.1991). Not all bacteria are equally degraded by the bivalve gut, as degradation is dependent on the structure of the bacterial cell wall. In *M. edulis*, gram-negative but not gram-positive bacteria are degraded by the bacteriolytic lysozyme secreted by the digestive gland and crystalline style of the gut (Birbeck and McHenery 1982). A main assumption of our research is that the complete breakdown and digestion of the gram-negative *L. discophora* SP-6 occurs in *M. trossulus*.

Previous studies involving the characterization of Mn oxides associated with the SP-6 bacterial sheath were performed using light microscopy and transmission electron microscopy (Emerson and Ghiorse 1993). In these studies, the sheath was attached to the outer layer of the gram-negative cell wall and showed an affinity for cationic colloidal iron and polycationic ferritin, indicating that a negative charge was dominant In another study in our laboratory, we used X-ray absorption fine structure (XAFS) spectroscopy to obtain information on the oxidation state, co-ordination number and crystalline state of the Mn oxides on SP-6 (Jurgensen et al. 2004). The Mn K-edge of the XAFS indicated the presence of single layered micro Mn crystallites in the SP-6 sheath (not Mn colloids), with the layers having a structure similar to those of birnessite (Manceau 1988). Based on the results of the XAFS study, we believed that the outer bacterial electronegative sheath would form Mn oxides, and that these oxides would in turn provide an enhanced number of sorption sites for other cationic heavy metals such as Pb and Cd.

The results of our current research did not show significant evidence to support this previous hypothesis. When the Mn oxide was present on the *L. discophora* sheath, the amount of Pb and Cd that sorbed to the surface was not higher ($p>0.05$) when compared to the amount of either metal on the Mn deficient sheath (Table 2.1). More importantly, when we examined both the predicted (kinetic model) and experimentally derived metal concentrations in the exposed tissues of *M. trossulus*, lower levels of Pb and Cd were found when the Mn oxide was present and higher levels of both metals when the Mn oxide was absent (Figure 2.1.2, 2.1.3 and Table 2.1).

These data support the hypothesis that diet quality and consequently, M. trossulus behaviour, influenced metal uptake. Previous studies have shown that when *M. trossulus*

was fed an organic rich diet, a linear relationship existed between diet quality and Cd uptake (Brown 1986). The results of our study also confirm this linear relationship. The bacterium used in our study was found to differ significantly in terms of its carbon content when cultured in the presence or absence of Mn. SP-6 samples without the Mn oxide had a significantly higher proportion of carbon compared to samples containing the Mn oxide ($F=256$, $p<0.0001$). When *M. trossulus* were fed SP-6 with a high carbon content (without the Mn oxide), higher tissue concentrations of Pb/Cd were found (Figure 2.2).

Considering that there were no significant differences in Pb and Cd concentrations in the initial SP-6 food matrices, it is possible that if *M. trossulus* preferentially selected a higher quality food source based on % carbon, and any metals sorbed to the higher carbon food source were ingested at higher concentrations. Analyses of particle clearance rates support this alternate hypothesis. Using a linear regression model (Figure 2.3), we were demonstrated that SP-6 containing Cd without the Mn oxide had significantly higher carbon content and particle clearance rates, resulting in a higher concentration of Cd in the exposed *M. trossulus* tissues. Conversely, SP-6 containing Cd with the Mn oxide had significantly lower carbon content and particle clearance rates, resulting in a lower concentration of Cd in *M. trossulus*. The same trend was observed for Pb with one exception. The high level of variation observed for the Pb exposures caused a weaker, but significant relationship to be present for Pb tissue levels in *M. trossulus*. It is possible that the characteristics of Pb itself and not the properties of the SP-6 Mn oxide sheath are responsible for this observed variation. Unfortunately, the results of this study do not allow us to confirm this possibility.

To confirm the possible correlation of Pb and Cd tissue levels with clearance rates, we re-ran the uptake model while holding the CR constant. If the predicted values demonstrated the same trend as our experimental values, then our experimental tissue levels would not be a result of differences in CR. The predicted Pb and Cd uptake values demonstrated a reversed trend from the experimental values. Tissue concentrations were higher for Pb and Cd when *M. trossulus* was fed SP-6 with the Mn oxide. These model modifications suggest that the experimental differences in Pb and Cd uptake rates are a result of particle clearance rates. To rule out AE as a possible correlative factor modifying metal uptake, two AE values were used in the uptake model and the predicted metal values compared with the experimentally derived values. In both cases, the differences in Pb and Cd uptake from SP-6 with or without the Mn oxide versus without were almost identical and demonstrated the same trend as the observed values. Since the predicted and observed values followed the same trend regardless of the AE value, we believe that AE is not controlling Pb and Cd uptake in this study.

The preferential selection of organic over inorganic particles has previously been demonstrated in studies using benthic feeding organisms (Brown 1986; Maloney 1996). These studies reported a preferential organic particle uptake determined by the examination of gastropod and bivalve feces. Positive correlations existed between Cd, Cu, Ni and Zn uptake and increasing organic content in fecal products as well as within the organism (Brown 1986). Food quality and organic content were also directly correlated with Cd uptake in marine polychaetes fed different organic enrichment regimes of cellulose fibres, sewage and benthic algae (Maloney 1996). It is important to mention that some previous studies did not show a preferential selection of food sources

based on organic content using deposit-feeding bivalves (Wang et al. 1995; Griscom et al. 2000). The main difference in these studies from ours is that the previous studies used food sources that consisted primarily of sediment or laboratory synthesized matrices, not natural food sources with the same base (i.e.; bacteria), and therefore may not be ideal representations of food matrices naturally encountered and fed upon by benthic dwelling organisms.

Scientists have only recently begun to explore the mechanisms controlling bacterially-mediated metal oxides and their potential to act as dietary metal sources in aquatic organisms (Bargar et al. 2000). In this report, we have demonstrated that higher quality bacterial food sources are ingested at increased rates by *M. trossulus*, and that this resulted in significantly higher levels of Cd and moderately higher levels of Pb uptake when the metal is associated with the higher quality food source.

Acknowledgements

We would like to thank Linda Pinto for her guidance and assistance with the bacterial culturing techniques and Josephine Chow for her help with sample digests. This work was funded by a TSRI (Toxic Substance Research Initiative) grant through Health Canada.

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CHAPTER 3:

CONTRASTING DIETARY CADMIUM AVAILABILITY IN *MYTILUS TROSSULUS*: INFLUENCE OF FOOD QUALITY AND SALINITY

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Abstract

Natural sediments were collected from two distinct marine intertidal ecosystems. Sediments were of high organic carbon content collected from a 25 ppt salinity beach (Deep Bay, DE) or of low organic carbon content and collected from a 15 ppt salinity estuary (Boundary Bay, BB). Feeding experiments were conducted that simulated the conditions in the two ecosystems. Eleven different combinations of natural phytoplankton, collected suspended sediments and control clay were radiolabelled with ^{109}Cd and pulse-fed to the pacific blue mussel *Mytilus trossulus* for both high (25) and low salinities (15). The assimilation efficiencies (AEs) and [^{109}Cd] in *Mytilus trossulus* were significantly higher for 10 of 11 and 8 of 11 feeding experiments at the high salinity exposures. Gut passage times (GPTs) were significantly shorter in 10 of 11 experiments at the high salinity versus the low salinity exposures. A positive correlation existed for [^{109}Cd] in *M. trossulus* tissues vs. AE when combined ^{109}Cd-labelled phytoplankton food matrices from both salinities were examined ($R^2 = 0.53$) while no correlations existed for ^{109}Cd-labelled clay or sediment matrices ($R^2 = 0.04$). We found no significant correlations between % carbon vs. GPT, AE or [^{109}Cd] within each salinity even though the DE sediments were significantly higher than BB in carbon content ($p < 0.05$). In *M. trossulus,* changes in salinity had a greater effect on dietary Cd assimilation efficiency and gut passage times than food quality.

Introduction

Of increasing concern to society is the potential for heavy metals to bioaccumulate in aquatic food webs (Wang 2002a; Krapiel et al. 2003). Extensive research has been

carried out on both aqueous and dietary exposure pathways of metals in marine bivalves (Luoma et al. 1992; Wang and Fisher 1996; Arifin and Bendell-Young 1997). It is now accepted that dietary accumulation of metals is at least as important as metal uptake from the aqueous phase and in many cases dominates metal accumulation in bivalves from marine environments (Wang and Fisher 1996; Chong and Wang 2001; Ke and Wang 2001).

Marine bivalves feed on seston which is known to contain high amounts of trace metals, typically orders of magnitude greater than concentrations in overlying waters (Luoma 1989; Ward and Shumway 2004). In urban estuaries and coastal waters, particulate bound metals are found in concentrations that are well above historical background levels (Siegel 2002). Effluent from pulp and paper mills, mines and sewage treatment plants produce particulates with bound metals that increase the level of both metals and suspended sediments within marine systems (Sekela et al. 1995). Phytoplankton has also been shown to accumulate dissolved free ion forms of various metals for metabolic functions (Lee and Morel 1995). To assess the potential impact of metal contamination in marine ecosystems, it is important to determine the mechanisms by which metals are consumed and assimilated by animals.

Metal assimilation efficiencies (AE) are influenced by food quantity and quality as well as various abiotic and biological factors. Laboratory derived AEs are determined experimentally by pulse-feeding marine bivalves radioactive food particles and measuring the amount of ingested metal retained in the tissues after the gut has been purged with non-radioactive food. Using this technique, it has been demonstrated that food quality, rather than quantity, can have a greater effect on the amount of metal

assimilated (Decho and Luoma 1991; Wang and Fisher 1996, 1997). Food quality (% organic carbon content) and food availability can also differ within and among marine habitats (Thomson 1981). Key environmental abiotic factors influencing food and water quality are climate, water levels, currents, pH, gas levels and salinity (Thurman and Trujillo 2001). Variation in these factors can have a profound influence on the plants and animals living in these habitats. These same abiotic factors can also influence metal speciation and availability within aquatic systems (Hall and Anderson 1995; Siegel 2002: Bendell-Young et al. 2002). The high variability found in these habitats presents major difficulties in determining the effect of specific field variables on metal assimilation efficiencies. As a result, there is very little information available on the influences of these variables on heavy metal assimilation efficiencies in marine bivalves

Mytilus trossulus is a common marine filter-feeding bivalve that has been widely used in water quality monitoring programs and has been considered a good indicator species for ecosystem health (Dame 1996). Filter-feeders ingest various amounts and combinations of phytoplankton, bacteria and re-suspended bottom sediments depending on food availability and the physiological state of the mussel. To predict the fate of metals in marine environments and the role of filter-feeding bivalves, environmental factors modifying habitat quality must be examined to accurately determine dietary metal uptake in these bivalves.

Significant differences in aqueous metal availability have been demonstrated among differing aquatic environments, but no studies exist that compare dietary metal uptake between marine habitats (Sadiq et al. 1991; Bendell-Young et al. 2002; Blackmore and Wang 2003a). The purpose of this study was to evaluate the AEs of radioactive

cadmium by *M. trossulus* when Cd was bound to natural food source combinations obtained from an estuary or a beach habitat. The sediment quality and salinity of these ecosystems differed significantly, allowing us to examine the combined role that food quality and salinity play on Cd assimilation in a temperate filter-feeding bivalve.

Methods

Test organisms. Laboratory cultured *Thalassiosira pseudonana* phytoplankton were combined in different amounts with natural sediments collected from two distinct habitats, and these were fed to the filter-feeding bivalve *Mytilus trossulus* using two different salinities. Depending on the feeding regime, either the phytoplankton or sediments were radiolabelled with ^{109}Cd; these food sources were then pulse-fed to *M. trossulus* for 45 minutes, after which the retention of ^{109}Cd in individual *M. trossulus* tissues was monitored over a four day period. *M. trossulus* with shell lengths ranging from 42.5 to 52.5 mm were collected from the same site, an intertidal rock cliff in Porteau Cove Provincial Park (June 2003 to April 2004) located in southwestern British Columbia, Canada. The salinity of these waters varied seasonally, ranging from summer highs of 26 ppt and winter lows of 10 ppt. The mussels were acclimated in the laboratory to 13 °C and to a salinity of either 15 or 25 ppt, and held in 200 L fully aerated tanks (seawater was changed every 3 days). Salinity acclimation was gradual with a change of 5 ppt every 7 days. *M. trossulus* were maintained on a diet of blue-green algae (*Spirulina pacifica*, 2% dry tissue weight per day) for a minimum of 2 weeks prior to experimentation, ensuring adequate acclimation to the above water quality conditions (Widmeyer et al. 2004).

Collection and Characterization of Sediments. Natural sediments were collected from two marine intertidal habitats off the west coast of British Columbia. Boundary Bay (BB) is located to the South of Vancouver (124°N, 49°W) while Deep Bay (DE) is on the Eastern coastline of Vancouver Island in Baynes Sound (123°N, 48°W). Both are located within the Strait of Georgia. Boundary Bay is an estuary mudflat dominated by fine silty sediment that is heavily influenced by municipal and agricultural runoff and three freshwater rivers, consequently lowering the salinity (15 ppt) in this shoreline zone (Shaw and El-Shaarawai 1995). Deep Bay is a cobble-stone estuary that is marginally influenced by one large freshwater river causing the salinity to be lower than normal ocean salinity, but higher than Boundary Bay (25ppt). It currently houses many clam and oyster aquaculture farms (Paynter 2002).

Intertidal sediment traps were specifically designed for easy deployment at the low tide line and collected on a monthly basis. Traps were constructed of a single PVC pipe that was capped at one end and left open at the other. The main pipe was 24 inches tall by 3.25 inches wide (internal diameter). To anchor the pipe in the sediment, two 12 inch long by 0.5 inch wide (i.d) schedule 40 grade PVC arms were inserted either 10 or 12 inches from the base of the main pipe and through the 3.25 inch width at a 180° angle from one other. The arms were anchored to the 3.25 inch pipe using two stainless steel screws that were drilled into the arm where it met the main pipe from the outside. The end of each arm was capped with a 0.5 inch PVC cap, preventing sediment from entering the arms. This resulted in four 4.35 inch arms at 90° angles from one another, with one 12 inch arm crossing 1.0 inch above the other inside the 3.25 inch main pipe. Each trap was submerged 15 inches into the sediment, open pipe end facing upwards at a 90° angle

from the sediment water interface, completely burying the four arms. This upright vertical position allowed the trap to retain all particles during daily tidal fluxes and withstand strong wind and current conditions over the three to four week period.

Sediments were collected in acid washed 500mL polypropylene bottles, transported to the lab, sieved to 53 μm, and held at 4 °C (to minimize microbial activity) in their native waters for a maximum of 2 weeks prior to radiolabelling. Wet sediment of 66-190 mg for BB and 83- 274 mg for DE (dry wt) was centrifuged at 800 g for 20 minutes, native water was removed and the pellet was resuspended in 20 mL of 1.0 μm filtered seawater of the same salinity as the native water. Sediment was then spiked with 61 kBq of ^{109}Cd, shaken three times a day, and held at room temperature (21 °C). After 5 days, the sediment was collected twice by centrifugation, and the supernatant was discarded. The sediment was then re-suspended in filtered seawater before being pulse-fed to *M. trossulus*.

Sediment carbon content was determined using 1-3 g of wet sediment that was dried in ceramic crucibles at 65 °C for 3 d, then ashed at 650 °C for 1 h (%LOI). DE sediment had 24.2% carbon (n=16, ± 1.0% SE) and BB had 15.2% carbon (n=22, ± 0.5% SE). The wet:dry weight ratio for DE was 7.54 ± 0.44 SE (n = 28) and 3.47 ± 0.06 SE (n = 32) for BB. Background HNO_3 digested Cd levels (μg g^{-1} dry wt) in BB sediment was 0.85 ± 0.04 SE (n = 9) and in DE sediment 0.97 ± 0.18 SE (n = 10). Native Cd and ^{109}Cd partitioning was measured in sediments (n = 7) from each site using the first three steps of a sequential extraction (Tessier et al. 1979). A Perkin Elmer Elan 6000 ICP-MS was used to detect native Cd levels and a Canberra Model 2030 small well gamma counter

equipped with a Na-iodide crystal detector was used to measure ^{109}Cd levels in each of the three sediment extracts.

Culturing of Phytoplankton. Non-axenic *Thalassiosira pseudonana* (clone 3H) cultures were obtained from the North East Pacific Culture Collection, University of British Columbia, Canada, and were maintained in *f*/2 culture medium (Guillard 1979). The basis of this media is nitrogen, phosphorus and silica sources augmented with a trace metal and vitamin solution specific for growing diatoms, a unicellular alga with a silica cell wall in the family Bacillariophyceae. Experimental cultures were grown in modified *f*/2 media without EDTA, Cu, and Zn and spiked with 61 kBq of ^{109}Cd (in HCl, reconstituted with dd H_2O). To maintain the pH of the media (8.0), 400 µl of 0.6 M HNO_3 was added. The initial cell density was approximately 40,000 cells mL^{-1}, and cultures were grown in 2.8 L flasks in 1 L of medium at 16 °C, under 14 h dark and 10 h light illumination (18,000 lux), and shaken twice per day. After 4 to 5 d growth, a final cell density of 4.0×10^6 cells mL^{-1} was obtained and diatoms were collected on a 44 mm 0.45 µm polycarbonate filter and re-suspended into filtered seawater (25 ppt) before being pulse-fed to *M. trossulus*.

Pulse Feeding. Eleven food matrices composed of clay (kaolin), sediment (BB or DE), phytoplankton, or combinations of these three were fed to *M. trossulus* at salinities of either 15 or 25 ppt (Table 3.1, the portion of the food matrix containing the radiolabel is written in italics), each matrix was replicated three times and contained 9 replicates each. Food matrices differed in organic carbon content whereas food quantity was maintained constant at 40,000 particles mL^{-1} (5-8 mg 600 mL^{-1} depending on the inorganic matrix, below pseudofeces production level). Particle size and concentration

was verified using a Z1 Coulter Counter (50 μm aperture, 2-20 μm window). Sediment controls consisted of clay (67 to 220 mg dry wt) which was prepared in the same manner as the sediments with one exception. The clay (Engel-hard Corp., Pigments and Additives Division, Edison, NJ) was pre-hydrated in 20 mL of filtered seawater for 24 hrs at 21°C prior to the addition of the radiolabel to facilitate binding of the label to clay particles.

Twenty-four hours prior to the pulse-feeding, *M. trossulus* were transferred to a 30 L holding tank and fed the experimental food matrix in a non-radiolabelled form. To confirm mussel viability, immediately prior to the experiment, mussels were removed from the holding tanks, left exposed to air for 15 minutes then re-submerged to verify siphon action. Nine mussels were placed into individual polypropylene beakers containing 600 mL of 1.0 μm filtered seawater (15 or 25 ppt salinity). The experimental food matrix was pulse-fed to each mussel at 1, 15 and 30 minutes, maintaining a constant particle concentration of 40,000 per mL^{-1}. The food matrix totalled a volume of 15 mL, with 1.5mL being fed to each beaker (10 beakers total, 9 treatment and 1 control). After 45 min exposure, each mussel was washed with 0.1 M EDTA to remove any ^{109}Cd sorbed to the shell, 3 of the mussels were immediately dissected from their shells and all soft tissue was gamma counted.

The remaining 6 mussels were placed into 1 L polypropylene chambers (2 mussels per chamber) containing 920 mL of filtered seawater (15 or 25 ppt salinity) within a re-circulating seawater system, and allowed to depurate the ^{109}Cd isotope for 4 days. Previous studies have demonstrated that it takes an average of 55-72 hours for *M. trossulus* to fully purge their gut contents (Wang et al. 1995). Each chamber contained a

1 mm polypropylene mesh hammock that suspended the two mussels 5 cm from the bottom of the container. Water was recycled by pumping from a 20 L aquarium through each depuration chamber and back into the aquarium, as described by Wang (1995). The flow rate through each chamber was maintained at 12.6 L h^{-1}, and the water within each chamber was changed on a daily basis. Over the four day period, mussels were maintained on a diet of *T. pseudonana* (2% dry tissue wt per day). All feces and pseudofeces were collected from the surface of the mesh hammock every 2-4 hours for the first 12 hours, then every 6-8 hours over the remaining 3 day period. Daily feces and pseudofeces production was determined by dry weight and ^{109}Cd concentrations were obtained for each chamber. In addition, daily water samples were taken from each chamber (2 mL) to monitor desorption of ^{109}Cd from feces and pseudofeces into the water. After 4 days of depuration, all 6 mussels were rinsed with 0.1 M EDTA to remove any ^{109}Cd sorbed to the shell and to avoid cross-contamination, dissected from their shells, and all soft tissues gamma counted.

A tenth beaker was included that contained only seawater and the food matrix, which acted as a control for monitoring clearance rates and isotope desorption into the water. Water samples (2 mL) were collected from the ten beakers every 15 minutes and counted for radioactivity and particle concentration to obtain food matrix clearance rates. All fecal, water and tissue samples were analyzed with a Canberra Model 2030 small-well gamma counter equipped with a Na-iodide crystal detector. Gamma emissions were detected at 22 and 88 keV. All measurements were corrected for background scattering and decay.

Desorption Experiments. To quantify the amount of isotope ingested from aqueous sources, ^{109}Cd desorption from the food matrix and subsequent uptake by *M. trossulus* was determined. The experimental water from the control beaker was filtered using a 0.45 µm filter-tip syringe into a clean 600 mL polypropylene beaker and three mussels placed into the filtered water. Following 45 minutes exposure, they were removed from the beaker, shells were rinsed with 0.1 M EDTA, and all soft tissue removed from the shells and gamma counted. Water samples (1 mL) were also taken prior to and post filtration and gamma counted to monitor concentrations of ^{109}Cd.

Estimates of ^{109}Cd assimilation. The amount of ^{109}Cd ingested and assimilated by *M. trossulus* from the combination food sources at both salinities was determined as follows:

% carbon content (dry wt basis) of food source,

= % carbon (mg per exposure) / [food] (mg per exposure) x 100

particle clearance rates per hour of exposure(CR),

$$= 1 - (C_{in} / C_{total})$$

with C_{in} representing the particle concentration ingested by mussels in exposed beakers and C_{total} representing the particle concentration in control beakers

particle ingestion rates (IR) (g per hr),

= [food] x CR

To correct for the possible contribution of aqueous ^{109}Cd uptake, ^{109}Cd activity determined for control mussels exposed only to ^{109}Cd in seawater was subtracted from mussel tissue levels after 96 hours purging.

Assimilation efficiencies (AE),

$= [^{109}Cd]$ mussel tissue at 96 hrs - $[^{109}Cd]$ water /

$[^{109}Cd]$ ingested x 100.

Total dietary cadmium uptake was determined using a modified version of the Thomann model (1995).

$$\text{Dietary uptake}_{\text{mussel}} = AE \times IR \times [^{109}Cd \text{ food source}] / BW$$

Dietary metal uptake (dps g^{-1}, dps = gamma isotope disintegrations per second) is the product of the AE, IR (g per hr), and $[^{109}Cd]$ in the food (dps g^{-1}) divided by the dry tissue weight (BW) (g) of the mussel. To quantify the amount of time it took for the isotope to be egested by the mussel as feces, we determined the % feces egestion over 96 hrs and the gut passage time (GPT) (hrs) at both 24 and 96 hrs.

$= ([^{109}Cd]$ feces per exposure replicate / $[^{109}Cd]$ food x 100).

Results

Aqueous Uptake of ^{109}Cd. Desorption of ^{109}Cd from food matrices occurred at both salinities. At 25 ppt, the only exposures with aqueous desorption and uptake of ^{109}Cd by *M. trossulus* occurred when the ^{109}Cd radiolabel was bound to either clay or sediment (4 exposures, 1-36% of total uptake), whereas 15 ppt had aqueous ^{109}Cd uptake in all eleven exposures (4-100% of total uptake). Three of the four highest aqueous uptake rates occurred when the ^{109}Cd radiolabel was bound to an inorganic matrix at the lower salinity. ^{109}Cd desorption appeared to increase with an increase in clay content for *phytoplankton*: clay combinations, but not for *phytoplankton*: sediment combinations at the lower salinity. For combination food matrices, the portion of the food matrix containing the ^{109}Cd isotope is always written in italics.

Egestion Rates. For each salinity, egestion rates were divided into two groups: food matrices containing natural sediments and matrices containing control clay. The egestion rate of feces produced by *M. trossulus* over the 96 hr depuration time varied depending upon whether the ^{109}Cd label was bound to phytoplankton or to sediment or clay (Figure 3.1 a-d). The four shortest overall GPTs were for clay or sediment-bound ^{109}Cd whereas the longest overall GPT was the 100% phytoplankton diet for both salinities. The six remaining phytoplankton bound ^{109}Cd food matrices had egestion rates ranging from 2-23% for 24 hrs and 5-94% for 96 hrs at the low salinity. At the high salinity, these values ranged from 16-100% for 24 hrs and 19-100% for 96 hrs. A one-way ANOVA (analysis of variance between groups) was used to test for significant differences among the four day depuration period for each exposure/food matrix per salinity. No significant differences existed among any of the low salinity exposures ($p > 0.05$). At the high salinity, two exposures were found to differ significantly over time, both containing inorganic radiolabels, *50% clay:* 50% phytoplankton ($p < 0.05$) and *50% sediment:* 50% phytoplankton ($p < 0.0004$). A fit model two-way ANOVA was used to test for significant differences in GPT between the two salinities over the four day depuration time. Ten of the eleven high salinity exposures had significantly shorter overall GPTs than the low salinity exposures ($p < 0.05$), with *100% sediment* having no significant differences between the two sites ($p = 0.55$). The total amount of feces produced by *M. trossulus* did not differ greatly between the two salinities. Pseudofeces were produced within the first 24 hrs and also did not differ greatly between the two salinities (data not shown).

Sediment Geochemistry. Sequential extractions were conducted on both ^{109}Cd radiolabelled and unlabelled sediments from Boundary (low salinity) and Deep Bay (high salinity) using a protocol modified from Tessier (1979). Sediments (with and without ^{109}Cd) from BB had been previously analyzed by sequential extraction and were shown to have minimal Cd concentrations in the organic fraction (Stecko and Bendell-Young 2000; Thomas et al. 2003). Therefore only the first three steps were performed to determine metals bound to the inorganic fraction; these yield the exchangeable bound metals, carbonate bound metals, and reducible metals bound to iron and manganese oxides. The extraction efficiency of ^{109}Cd averaged 51% for BB and 70% for DE. The unlabelled extraction efficiency was quantified using the NBS standard MESS-2. Less than a 10% mean variance from the standard value was achieved (n=3) and a 90% recovery achieved in sediments from both sites.

The majority of ^{109}Cd was bound to Fe/Mn oxides in the reducible fraction for both BB (97%) and DE (89%). The remainder of the isotope was found in the exchangeable fraction for BB (3%) and in the exchangeable (9%) and carbonate fractions (2.4%) for DE. Native Cd levels (unlabelled) paralleled this same trend for both sites. The majority of native Cd was found in the reducible fraction of sediment for both BB (93%) and DE (75%), with neither containing Cd in the carbonate fraction and the remainder being found in the exchangeable fraction (7% and 25%).

Sediments used in all feeding experiments were analyzed for carbon content. The results of a one-way ANOVA showed that no significant differences in carbon content occurred within each site over the experimental time frame ($p < 0.05$). There was

significantly greater carbon content in DE sediment compared to BB sediment when analyzed using a one-way ANOVA ($p < 0.0001$).

^{109}Cd uptake and assimilation between sites. Student t-tests were used to examine whether significant differences existed between AE and [^{109}Cd] in *M. trossulus* tissues between salinities for each of the eleven food matrices (Table 3.1). A Bonferroni correction was made to avoid the chance of a type I error, changing the α value to 0.0046 and $p < 0.0001$. In ten of the eleven exposures, AEs were significantly higher for the higher salinity, with the exception of 50 sediment:*50 phytoplankton.* In eight of the eleven exposures, the [^{109}Cd] in *M. trossulus* tissue was significantly higher in mussels fed food matrices at 25 ppt versus 15 ppt salinity. The three exceptions were the 50 sediment:*50 phytoplankton*, the *50 clay*: 50 phytoplankton, and the *100 sediment* exposures. Fit model two-way ANOVAs were used to determine whether GPTs differed significantly between salinities and between the 24 and 96 hr depuration period. Overall GPTs were significantly shorter for the higher salinity in ten of the eleven exposures; the *100 sediment* exposure was the exception (Table 3.1, $p < 0.05$). GPTs for individual exposures did not differ significantly between 24 and 95 hours in 9 of 11 cases (50 clay*: 50 phyto* and *50 sediment:* 50 phyto being the exceptions). We therefore used the 24 hr values in our analyses. Particle clearance rates did not appear to differ between sites for each food matrix. The clearance rates for 15 ppt ranged from 0.35 to 0.80, and from 0.26 to 0.86 for 25 ppt.

When linear regressions were examined, the results were quite varied. Data from both ecosystems (Deep Bay and Boundary Bay) were therefore combined. The strongest relationship occurred for [^{109}Cd] in *M. trossulus* tissues vs. AE (R^2=0.52). We also

investigated whether significant correlations existed between, % carbon vs. GPT 24 hr (R^2=0.26), % carbon vs. [^{109}Cd] (R^2=0.27), % carbon vs. AE (R^2=0.14), GPT 24 hr vs. AE (R^2=0.05), and [^{109}Cd] vs. GPT 24 hr (R^2=0.03), but no significant relationships were found when all eleven exposures from each site were combined.

The significantly higher AE, [^{109}Cd], and shorter GPT observed for the higher salinity exposures were not likely a result of the higher carbon content of the DE sediments. Clay control matrices (5 exposures) consisting of identical % carbon content also had higher AE, [^{109}Cd], and shorter GPT for the higher salinity site, as observed for food sources containing sediment (5 exposures) with varying % carbon. The above differences observed between salinity exposures may therefore be a result of physiological changes or adaptations to the different salinities found in these two distinct ecosystems, not a result of varying sediment organic carbon content.

Table 3.1. Food matrix composition, AE, GPT, [^{109}Cd] in *M. trossulus* tissues and % C values for each food matrix. Matrices in *italics* indicate the portion of the food mixture containing the ^{109}Cd. * indicates the site with the greater AE or [^{109}Cd] value ($\alpha = 0.0046$ and $p < 0.0001$, T-test) ± SE ($n = 36$) or greater overall GPT ($p < 0.05$, ANOVA) ± SE ($n = 18$). DE is a high salinity, high carbon content site and BB is a low salinity, low carbon content site.

Food Matrix (%)	**Salinity ppt**	**AE (%)**	**24 hr GPT (%)**	**[Cd] (dps/g)**	**% C (LOI)**
100 Phytoplankton	15	5.40 ± 0.02	2.19 ± 1.99	3169 ± 348	71
	25	*54.6 ± 2.21	*16.0 ± 5.50	*10225 ± 986	71
100 Sediment	15	0.24 ± 0.05	62.5 ± 19.2	1335 ± 288	15.2
	25	*0.63 ± 0.07	64.8 ± 19.59	1217 ± 205	24.2
100 Clay	15	0.20 ± 0.15	43.3 ± 28.50	3 ± 2	0
	25	*2.72 ± 0.18	*100 ± 0	*685 ± 38	0
30 phyto:70 sediment	15	0.84 ± 0.08	5.00 ± 1.48	191 ± 23	31.9
	25	*3.30 ± 0.11	*42.8 ± 11.8	*862 ± 86	38.2
30 phyto:70 clay	15	0.68 ± 0.15	23.5 ± 7.02	222 ± 51	21.3
	25	*31.4 ± 1.07	*61.0 ± 16.9	*3803 ± 327	21.3
50 phyto:50sediment	15	*14.2 ± 0.42	21.8 ± 4.97	*5230 ± 312	43.1
	25	9.41 ± 0.34	*63.8 ± 7.59	1875 ± 303	47.6
50 sediment:50 phyto	15	0.78 ± 0.10	51.8 ± 3.18	1166 ± 159	43.1
	25	*4.18 ± 0.17	*80.7 ± 1.45	*7907 ± 594	47.6
50 phyto:50 clay	15	5.31 ± 0.29	2.89 ± 0.87	2218 ± 177	35.5
	25	*18.9 ± 0.78	*47.8 ± 17.1	*5496 ± 555	35.5
50 clay:50 phyto	15	10.9 ± 0.27	55.4 ± 22.7	*3010 ± 161	35.5
	25	*14.8 ± 0.20	*61.1 ± 13.3	630 ± 120	35.5
70 phyto:30 sediment	15	0.77 ± 0.07	19.75 ± 5.74	128 ± 15	54.3
	25	*9.07 ± 0.42	*23.5 ± 13.1	*4541 ± 298	57.0
70 phyto:30 clay	15	3.87 ± 0.29	5.94 ± 3.41	1630 ± 173	49.7
	25	*20.8 ± 0.77	*50.7 ± 12.8	*9912 ± 723	49.7

^{109}Cd uptake and assimilation within each site. The clearest trend demonstrated within each site were the differences in GPT based on which portion of the food matrix

contained the ^{109}Cd radiolabel. Clay or sediment bound ^{109}Cd had the shortest GPT for both salinities vs. phytoplankton bound ^{109}Cd food matrices (Figure 3.1 a-d). Trends for AE, [^{109}Cd] in *M. trossulus* tissue, GPT 24 hr and % carbon were investigated using linear regression models. Data was divided into two groups depending on what portion of the food matrix contained the ^{109}Cd radiolabel, phytoplankton bound vs. sediment or clay bound. No significant trends existed for sediment or clay bound ^{109}Cd matrices within each site when GPT vs. AE (Figure 3.2 b), [^{109}Cd] vs. AE, or % carbon vs. GPT were examined. For phytoplankton bound ^{109}Cd matrices a significant trend was present for [^{109}Cd] vs. AE (Figure 3.2 a), with little to no trend being present for GPT vs. AE (Figure 3.3), and % carbon vs. GPT (Figure 3.4). Within each salinity the differences in food matrix carbon content (clay vs. sediment combinations) did not demonstrate significant correlations or trends with AE, GPT or [^{109}Cd].

Figure 3.1 (a-d). % ^{109}Cd in *M. trossulus* tissues over 96 hrs (± SE) exposed to ^{109}Cd bound food matrices at two salinities, a/ 15 ppt salinity exposures b/ 15 ppt salinity clay controls c/ 25 ppt salinity exposures d/ 25 ppt salinity clay controls. * indicates the portion of the food matrix containing the ^{109}Cd radiolabel. (p) = phytoplankton, (s) = sediment, (c) = clay

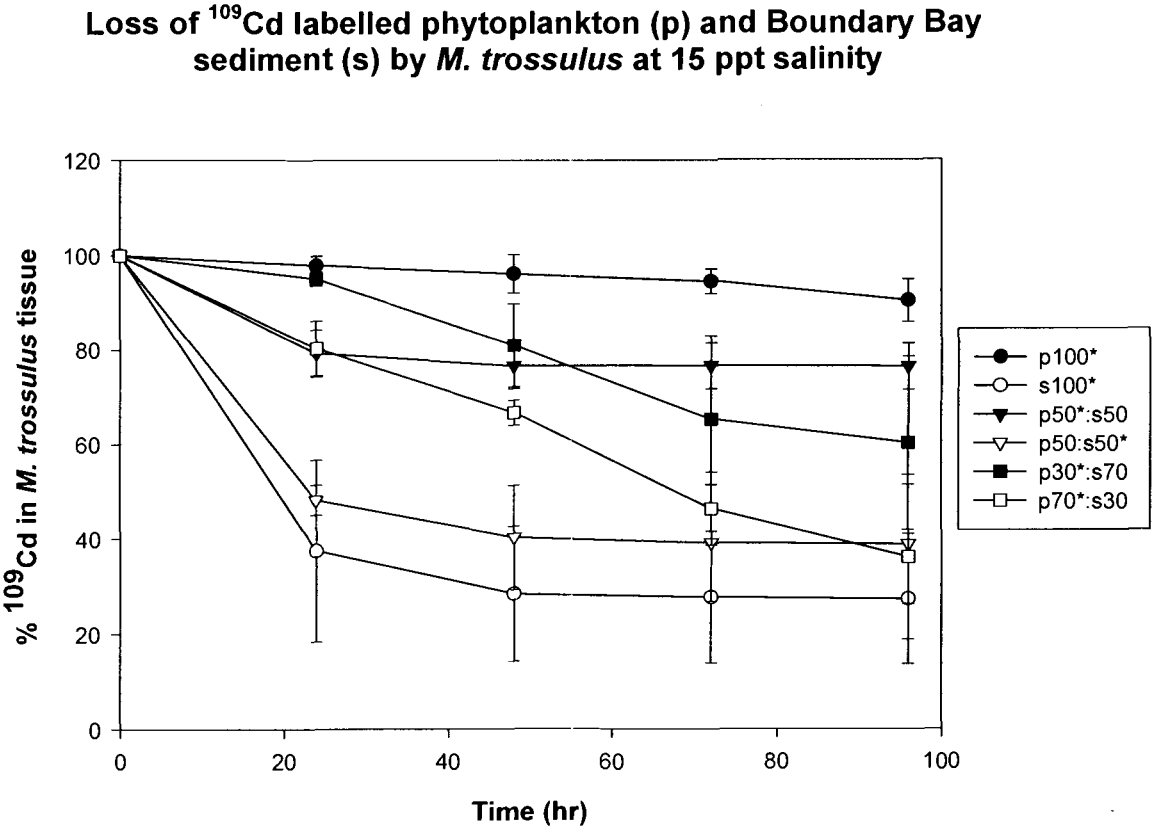


Figure 3.1.b. 15 ppt salinity clay controls

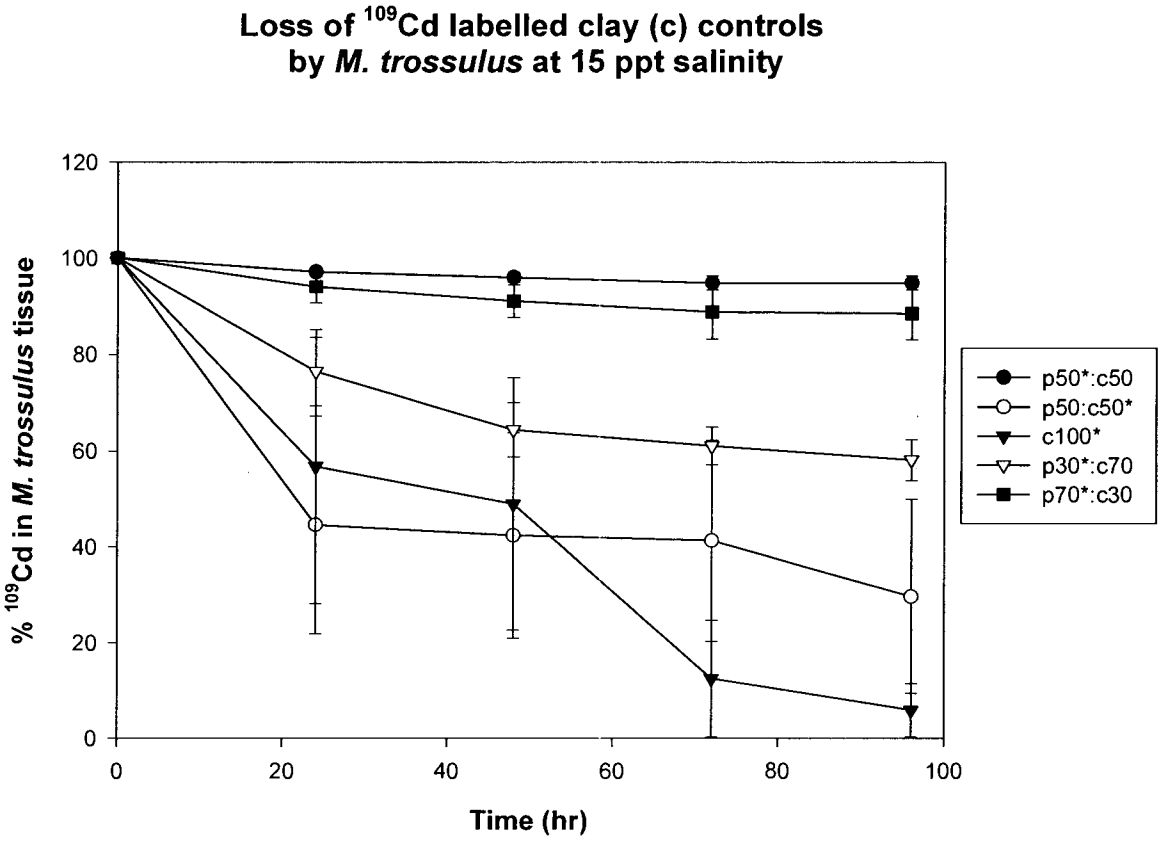


Figure 3.1.c. 25 ppt salinity exposures (phytoplankton and sediment)

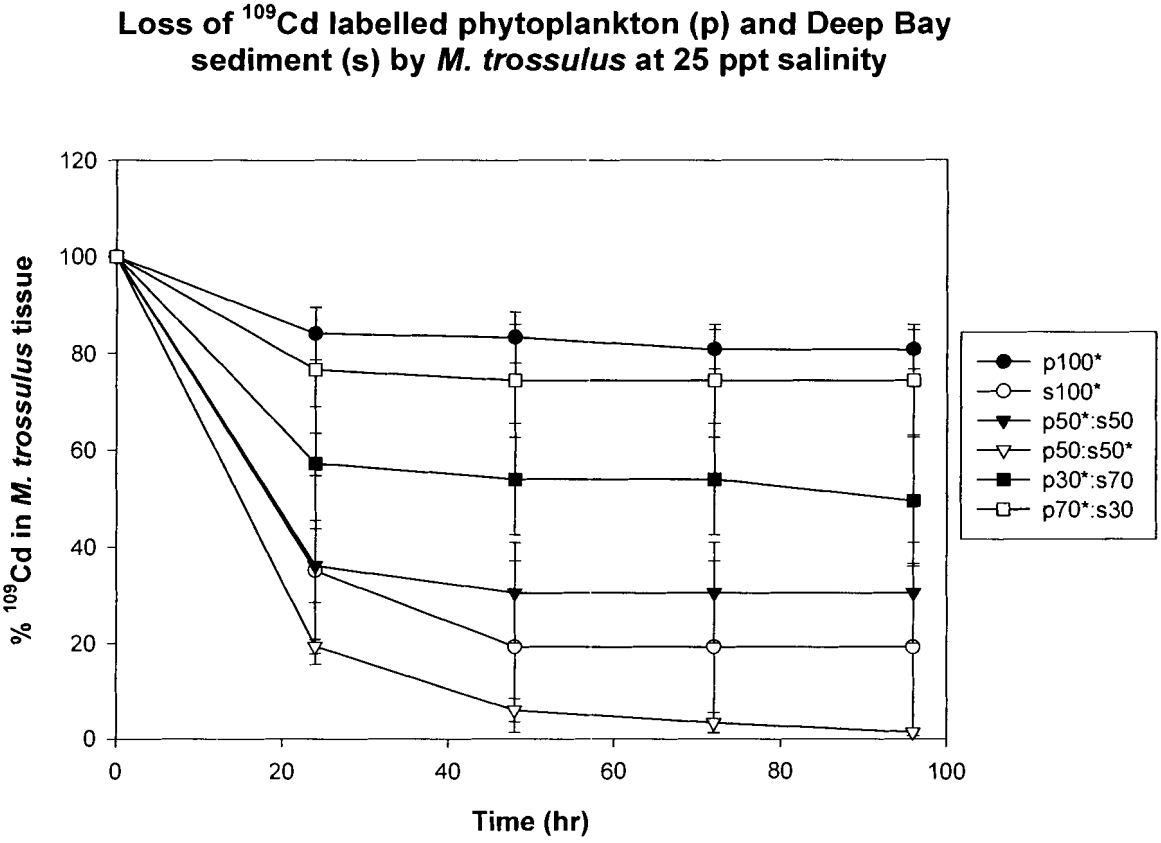


Figure 3.1.d. 25 ppt salinity clay controls

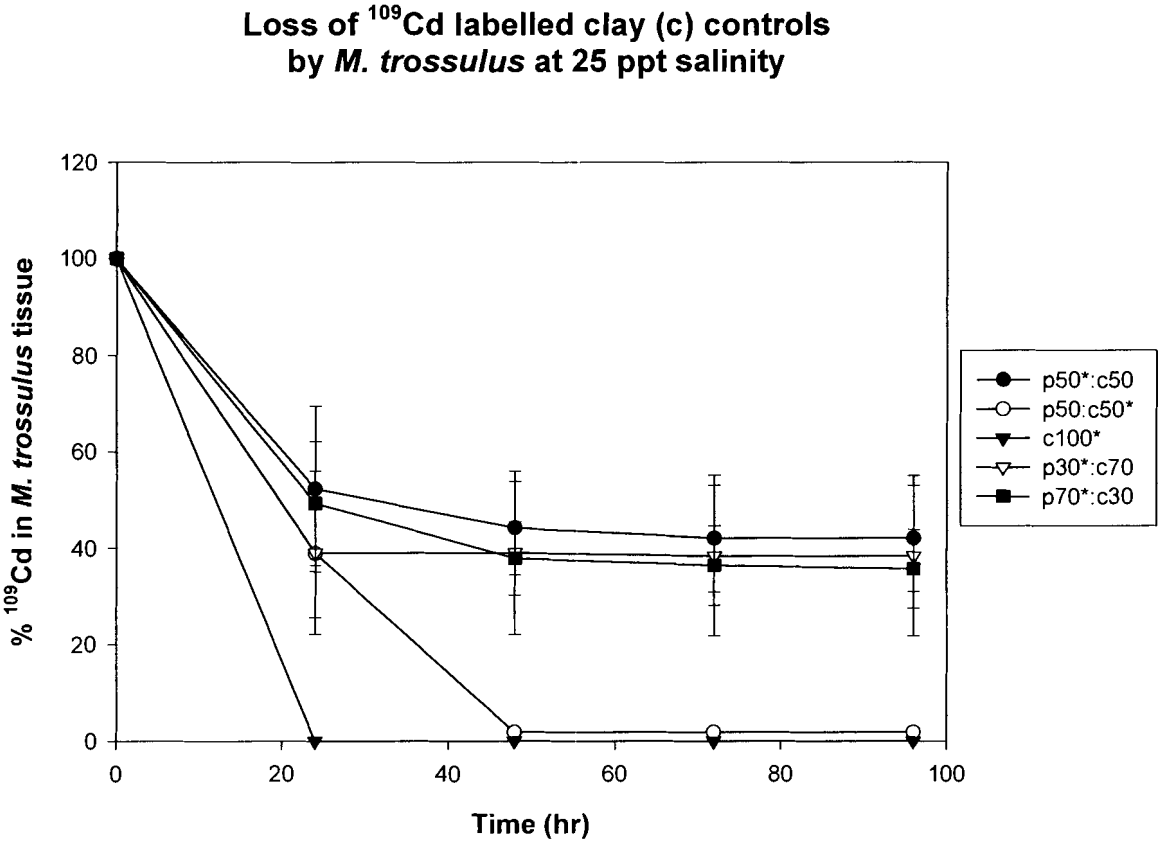


Figure 3.2 (a). Gut Passage Time at 24 hrs (±SE) of ^{109}Cd in *M. trossulus* versus the assimilation efficiency (AE) of ^{109}Cd after exposure to phytoplankton bound ^{109}Cd.

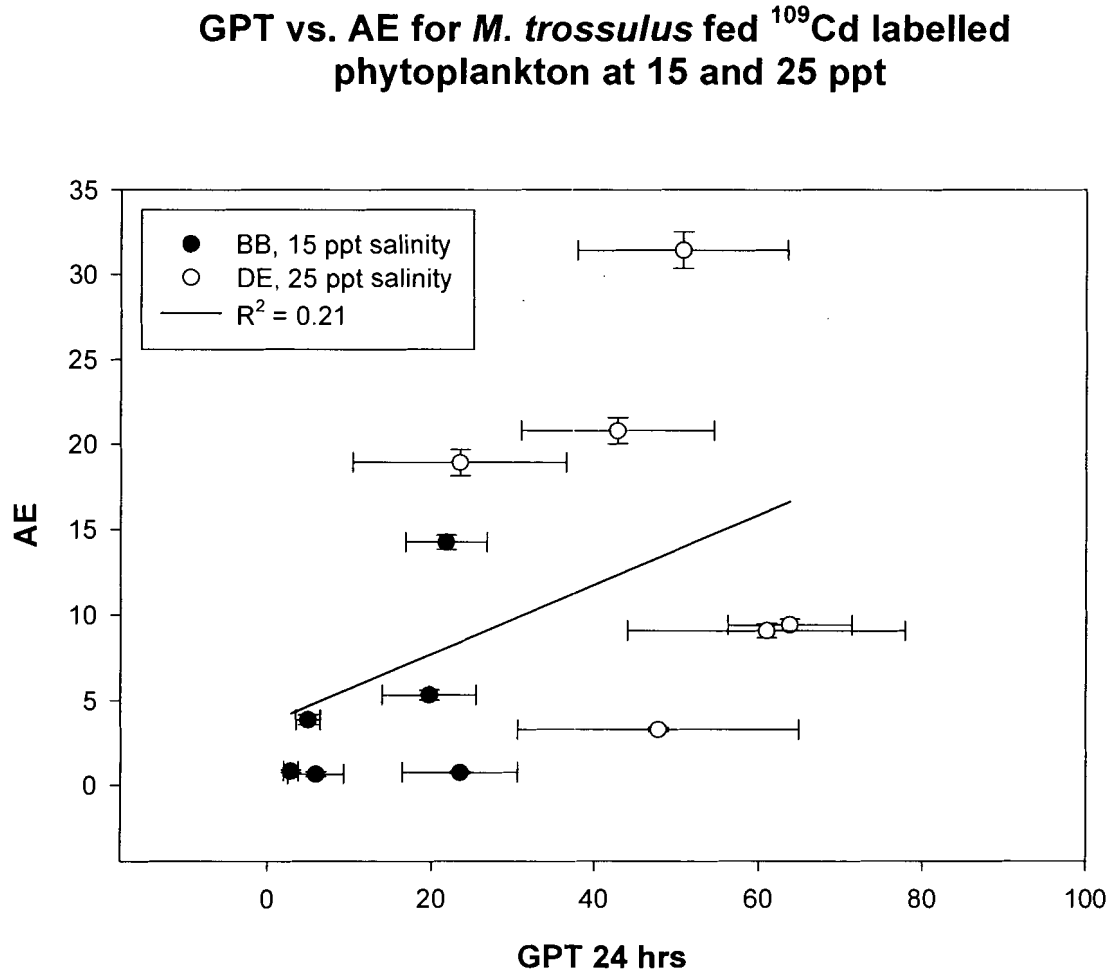


Figure 3.2.b. Gut Passage Time at 24 hrs (±SE) of ^{109}Cd in *M. trossulus* versus the assimilation efficiency (AE) of ^{109}Cd after exposure to clay or sediment bound ^{109}Cd exposures.

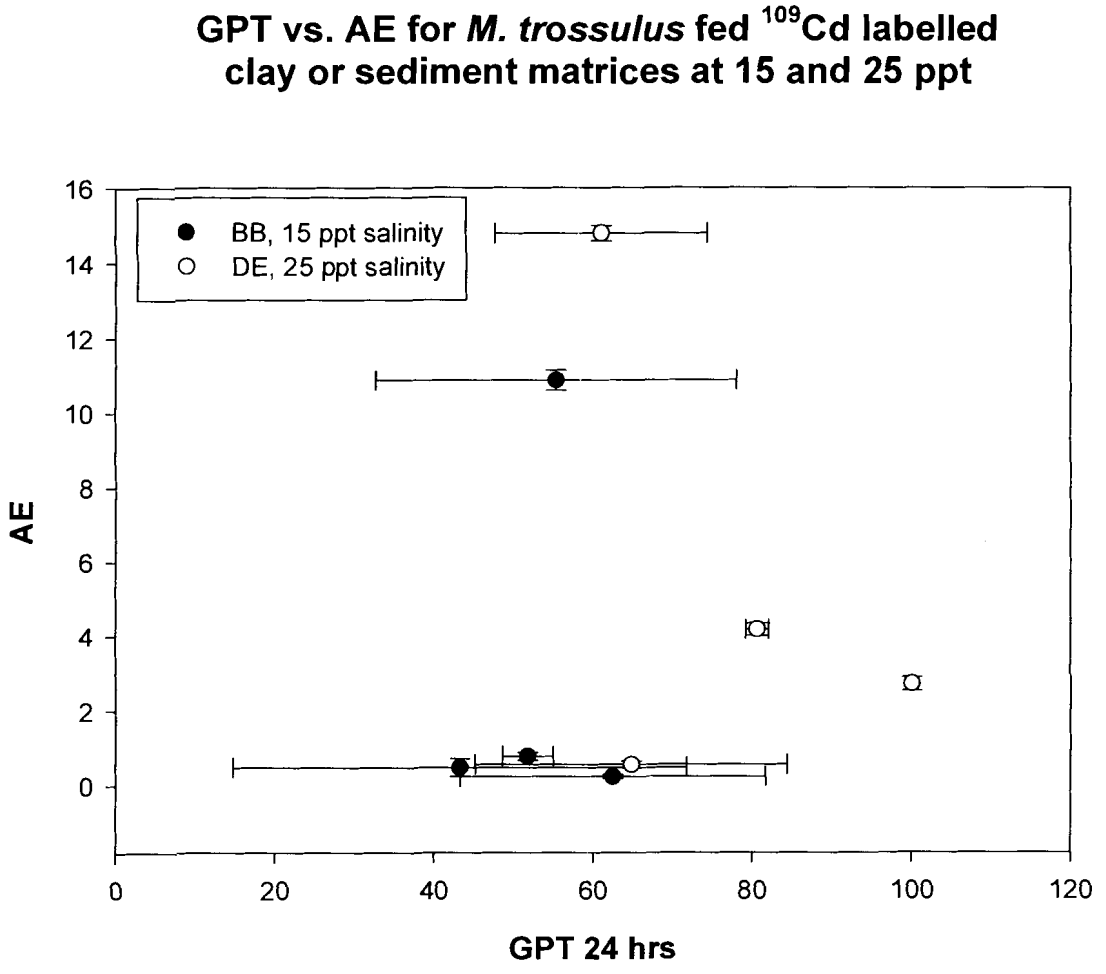


Figure 3.3. [^{109}Cd] in *M. trossulus* tissue vs. AE values (±SE) when feeding on phytoplankton bound ^{109}Cd combined with clay or sediment from BB and DE.

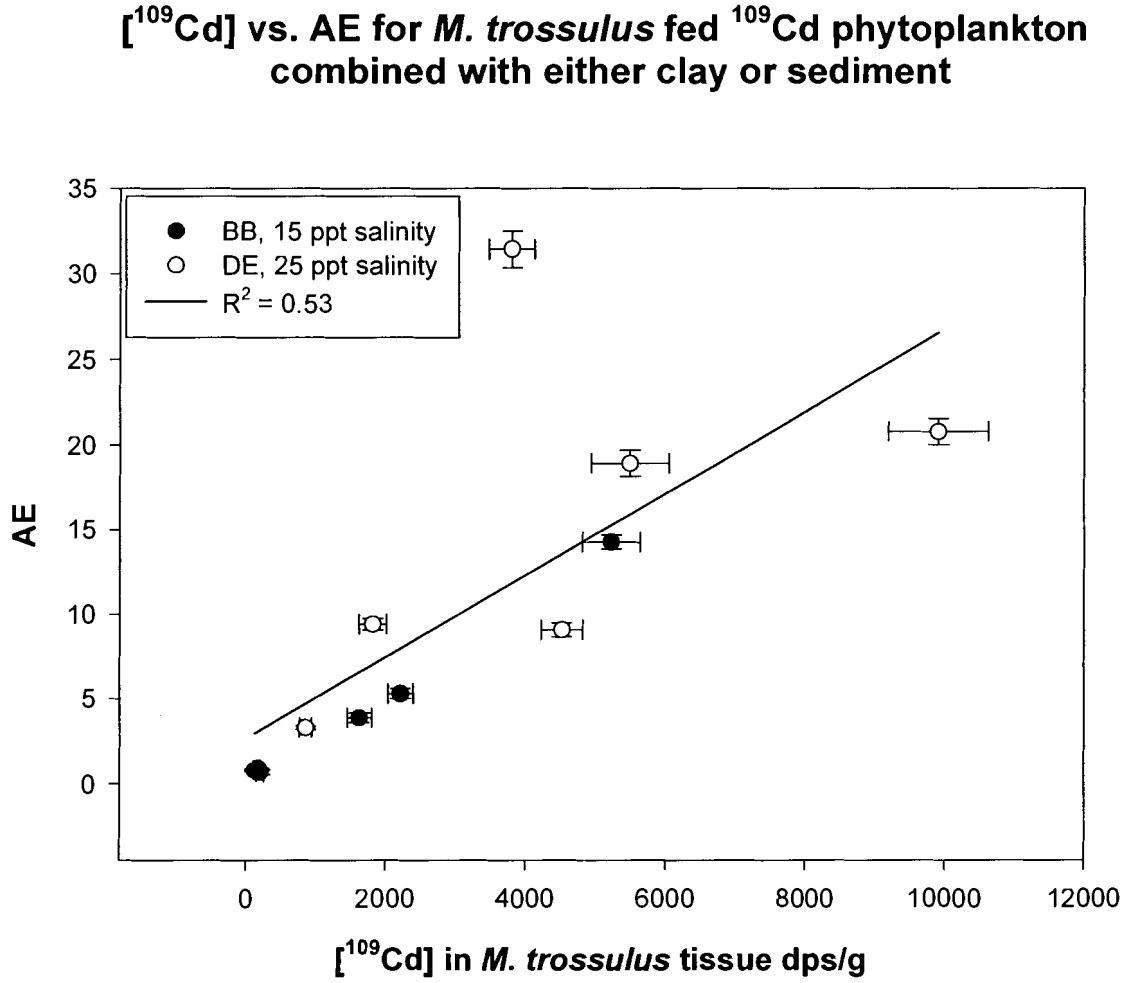
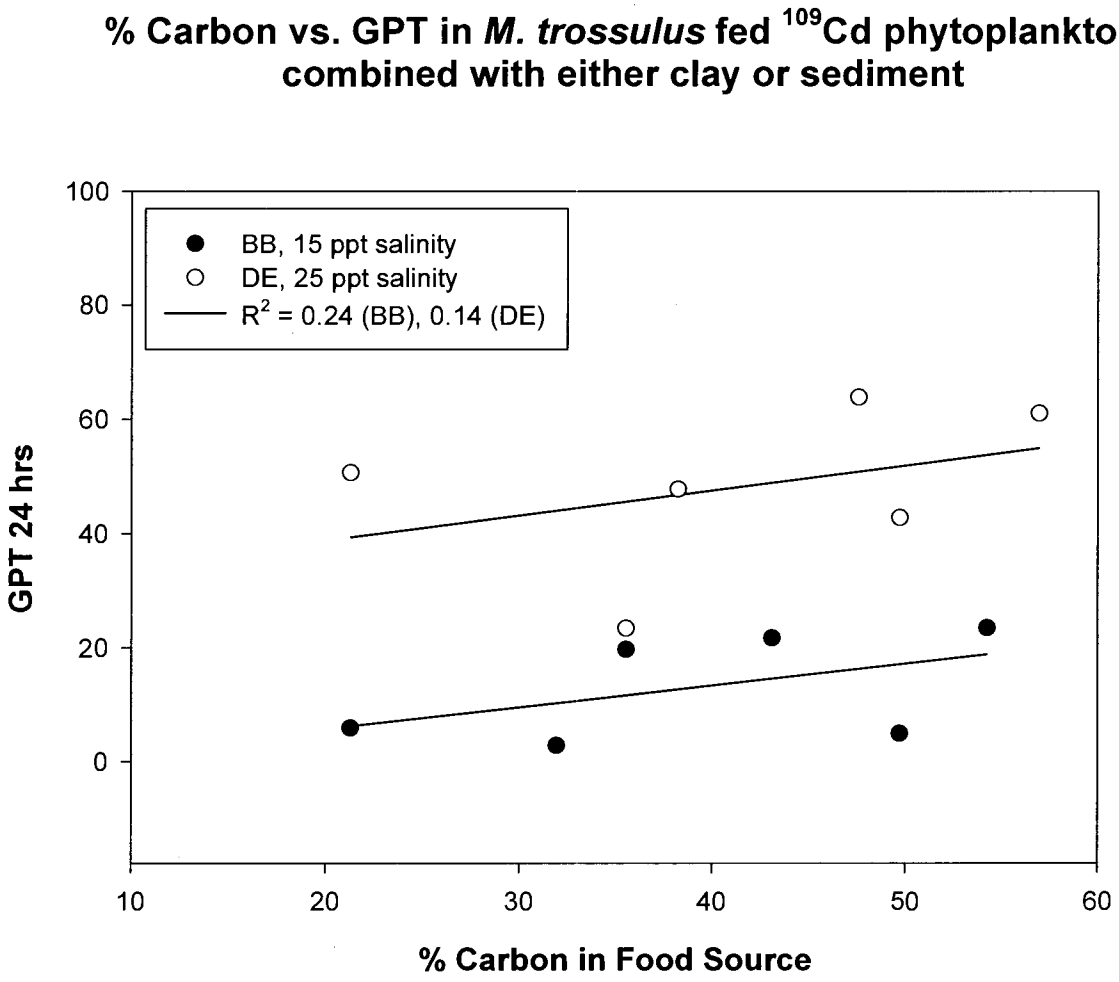


Figure 3.4. % Carbon in food sources vs. GPT at 24 hrs for *M. trossulus* feeding on phytoplankton bound ^{109}Cd combined with clay or sediment from BB and DE.



Discussion

In this study, the AEs, [^{109}Cd] in *M. trossulus* tissue and GPTs varied significantly between the two salinities but not for the various food matrices fed to *M. trossulus* within each salinity (Table 3.1, Figures 3.2, 3.3, 3.4). Certain relationships (low AE and fast GPT for inorganic labelled food sources) in AEs and GPTs existed within each salinity and paralleled one other as a result of gut desorption of the ^{109}Cd radiolabel within *M.*

trossulus's gut and the characteristics of the food matrix to which the ^{109}Cd radiolabel was bound. These relationships had no correlation with the very distinct abiotic characteristics naturally found in the two shoreline habitats used as models in this study, but are important mediating factors of metal uptake in both natural and laboratory systems.

Many studies have successfully determined metal assimilation efficiencies from ingested radiolabelled food sources in marine bivalves, revealing the significance of metal dietary exposure in marine invertebrates (Wang and Fisher 1999a, 1999b). Previous in vitro and in vivo studies have demonstrated one mechanism that partially accounts for varied metal AE values from single food sources: metal desorption within the bivalve gut (Wang 2002b; Yan and Wang 2002). The guts of bivalves are slightly acidic, typically with a pH value of about 5-6 (Owen 1974). Metal desorption due to a decrease in gut pH may be a critical step in affecting the assimilation of metals such as Cd which can be differentially bound to particles depending on the nature of the particle mixture ie: sediment vs. phytoplankton vs. clay (Wang 2001; Chong and Wang 2000).

When bivalves ingest food particles extracellular digestion is the first of two main digestive processes, and provides an opportunity for organically bound metals to desorb then readsorb onto inorganic particles within the food mixture. The second main digestive process is very selective and shuttles higher quality food sources to the digestive diverticula for a more intensive intracellular digestion process. Lower quality food sources skip intracellular digestion and are passed directly to the intestine where absorption and rapid egestion occur (Decho and Luoma 1991; Penry 2000). In our study *M. trossulus* had lower AEs and shorter GPTs when fed 5/6 low salinity, and 6/6 high

salinity combination (phytoplankton and sediment/clay) matrices versus when fed solely phytoplankton. Our results support the findings of lower AEs and shorter GPTs for mixture vs. pure phytoplankton food matrices. In addition, our results agree with those found by Wang (2001), where a lower assimilation of diatom-bound metals combined with sediment resulted in a shorter passage of sediment through the mussel gut.

The characteristics of the food particle to which a radiolabel is bound can also affect the AEs and GPTs controlling metal uptake in *M. trossulus*. Studies have shown that metals bound to algal cytoplasm are more bioavailable to bivalves, and have increased AEs and longer GPTs versus when bound to sediment (Wang and Fisher 1996; Lee and Luoma 1998). Our results confirmed this trend in both high and low salinity exposures because the seven food sources in which ^{109}Cd were bound to phytoplankton had longer 24 hr GPTs and higher AEs when compared to ^{109}Cd bound to sediment or clay. In addition, the four food matrices that contained sediment- or clay-bound ^{109}Cd had the highest egestion rates (shortest GPT) for both salinities (Figure 3.1 a-d). In addition, the sequential extractions performed in this study on both BB and DE sediments confirmed that the majority of naturally occurring Cd and ^{109}Cd were bound to the inorganic component (i.e., the reducible fraction comprised primarily of Mn and Fe oxides) and not the organic component within each sediment type.

The primary goal of this study was to determine if the natural environmental parameters present in beach and estuary habitats affected cadmium uptake and assimilation in the blue mussel, *M. trossulus*. There is very little information available on the influence of abiotic habitat conditions on dietary heavy metal AEs in marine bivalves. In our study, major differences in abiotic conditions between the two sites were salinity

and sediment organic carbon content. Deep Bay is a cobble-sand intertidal estuary with an average fall-winter salinity of 25 ppt and sediment organic carbon content of 24.2%, whereas Boundary Bay is a fine-silt estuary with an average fall-winter salinity of 15 ppt and sediment organic carbon content of 15.2%. As noted previously, the higher salinity exposures had significantly higher assimilation efficiencies, shorter GPTs and higher [^{109}Cd] tissue concentrations compared to the lower salinity exposures in 10/11, 10/11, and 8/11 of the food matrices, respectively. A positive correlation existed for [^{109}Cd] in *M. trossulus* tissues vs. AE when all eleven food matrices from each salinity were combined ($R^2 = 0.52$) whereas no significant correlations existed for % carbon with either AE, [^{109}Cd], or GPT. The significantly higher AEs, [^{109}Cd], and shorter GPTs observed for the higher salinity exposures were therefore not a result of DE sediments having a higher organic carbon content. The clay control matrices (5 exposures) in our experiments confirmed that sediment quality was not the driving force behind the higher AEs, [^{109}Cd], and shorter GPTs since the clay matrices had identical % carbon for each site and displayed the same trend as sediment matrices (5 exposures) with varying % carbon values. These observed differences may therefore be a result of possible physiological adaptations in *M. trossulus* to the lower versus. higher salinities used in our experiments.

To date, many studies have examined the effect of salinity on aqueous metal uptake in bivalves occupying different ecosystems but no study exists that examines the effect of salinity on dietary metal uptake (Fisher 1986; Lee and Luoma 1998; Blackmore and Wang 2003a, 2003b). It was reported that as salinity decreased, the influx of aqueous heavy metals (Cd, Zn and Cr) into the bivalve increased (Fisher 1986;

Blackmore and Wang 2003a, 2003b). This finding is likely related to the greater bioavailability of the free metal ion (toxic form) at lower salinity conditions (Hall and Anderson 1995). No significant trend has been demonstrated for AEs of aqueous Cd but significantly longer GPTs have been demonstrated at low (10 ppt) salinities, indicating that there is a possible effect of lowered salinity in the tropical bivalve *Perna virdis* gut physiology (Blackmore and Wang 2003b). Metal bioavailability from the aqueous phase has also been directly related to the physiological condition in three different species of bivalves (Wang 2001). Unfortunately neither study provided the details of how a decreased salinity modified bivalve physiology.

In our study, a lower salinity environment caused particles to remain longer in the bivalve gut and have a lower efficiency of ^{109}Cd tissue assimilation. This is unlike the situation observed for aqueous metals, where the metal remains in the gut longer and have higher tissue AEs at lower salinities (Hall and Anderson 1995). Based on the clearance rates measured in our study there was no reduction in food particle or ^{109}Cd uptake between the two salinities tested. The total production of feces and pseudofeces between the two salinities also did not vary, but the egestion rate or GPT did. We can therefore assume that the reduction in salinity did not reduce the filtration and uptake rate of food particles being ingested by *M. trossulus*, causing a reduction in AE for low salinity exposures. It is possible that salinity affected the physiological condition of *M. trossulus*. Specifically, a decrease in salinity may have caused an adverse cytological effect on *M. trossulus* digestive cells responsible for metal uptake.

Lysosomes are polymorphic, hydrolytic enzyme-containing, organelles with many intracellular and extracellular roles (de Duve and Wattiaux 1966). Their main function in

bivalves is food degradation, nutrient storage, and detoxification in the digestive diverticula cells (Owen 1972). Since lysosomal hydrolases are capable of degrading any biological material, membrane labilisation and subsequent hydrolase release can cause cell lysis within the organism (Goldman 1976). Many chemical, physical, and environmental stressors are known to destabilize or labilise lysosomal membranes, with injury being proportional to the magnitude of the stress (Moore 1985). In-vivo bivalve studies have demonstrated significant lysosomal membrane destabilisation due to extremes in temperature, salinity and copper exposure (Nicholson 1999; Hauton et al. 1998; Ringwood et al. 1998). Varying degrees of lysosomal destabilization have been observed depending on what species of bivalve was tested and whether the test was performed on field versus laboratory experiments. Nicholson (1999) demonstrated significant destabilization in *Perna viridis* gut haemocyte lysosomes when both temperature and salinity (35 vs. 25 and 10 ppt) were lowered in laboratory studies. Similar studies with *Crassostrea virginica* testing salinity and copper demonstrated no lysosomal destabilisation for variable salinity regimes but significant destabilisation for aqueous copper uptake at lower salinity exposures (Ringwood et al. 1998).

It is difficult to determine whether the combination of lower salinity and cadmium exposure can cause lysosomal destabilization in our experiments, leading to a decreased AE, decreased uptake of ^{109}Cd and a longer GPT in *M. trossulus*. If cellular injury due to lysosomal enzyme release is proportional to the magnitude of the stress, then two stresses would cause more severe effects in an organism versus one. Lysosomal destabilization due to a lower salinity (15 vs. 30 ppt) environment has been shown to cause a net solute loss leading to an increased energetic cost in cell volume regulation for *M. trossulus*

(Hawkins and Hilbish 1992). A reduction in the overall metabolic budget could lead to a reduction in the uptake of nutrients and ^{109}Cd by digestive diverticula cells. In response to a possible reduced nutrient uptake longer GPTs may result. In addition, increased metal uptake may not necessarily occur with a longer GPT as observed for aqueous metals due to the sorption properties of metals to the food source.

The results of our study demonstrate that lower salinity environments may cause significantly lower dietary cadmium uptake and assimilation efficiencies and longer gut passage times in *M. trossulus* when compared with higher salinity environments. Further emphasis needs to be placed on the potential contribution of natural environmental stressors from distinct habitats and their effects on heavy metal uptake from the diet of indicator species such as *M. trossulus*. In addition, the quality of a food source and the type of food source carrying a heavy metal contaminant also plays a significant role in determining metal uptake and assimilation in bivalve species.

Acknowledgements

I would like to thank the three anonymous reviewers for their thoroughness and insight regarding this manuscript. This research was made possible thanks to funding from L. I. Bendell-Young's NSERC discovery grant and Simon Fraser University Graduate Fellowships.

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CHAPTER 4:

A SIMPLE AND EFFICIENT MARINE INTERTIDAL SEDIMENT TRAP FOR STUDYING DIETARY METAL ASSIMILATION IN FILTER-FEEDING BIVALVES

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Abstract

To investigate the bioavailability of metal bound food sources in benthic organisms and their potential for metal trophic transfer within marine habitats, integrated studies accounting for varying abiotic habitat parameters coupled with laboratory feeding experiments, and stable isotope analysis are needed. Studies combining these techniques need a substantial amount (5-10 g wet wt) of very fine sediment, collected in a cost-effective and efficient manner, made possible by using a marine intertidal sediment trap. A new type of sediment trap was designed to be deployed in the marine intertidal to collect biologically relevant (<63 μm) suspended and re-suspended particulate matter for studies examining metal uptake and assimilation in filter-feeding bivalves. Sediments were collected monthly over four seasons from two intertidal estuaries, allowing us to investigate any seasonal or site effects in collection efficiency, sediment quality (%C, %N) and metal levels over time. The results of an ANOVA indicated that both C and N varied significantly between the two sites as well as over the four seasons sampled. Concentrations of Cu, Cd, Pb, and Zn were also determined using ICP-MS and compared to the current Canadian marine sediment quality guidelines. Significant differences (2-way ANOVA) were observed between the two sites for Cu, Cd, and Pb as well as among seasons for all four metals. Both sites had Cu, Cd, and Pb levels exceeding the Canadian sediment quality guidelines (SQG) at least 50% of the time while Cu had levels above the probable effect level (PEL) for all four seasons.

Introduction

Within the oceanic water column, sediment traps are used to accurately quantify the vertical flux and composition of sinking material. These traps are generically open cylinders or cones deployed in the water column like passive "rain gauges" (Knauer and Asper 1989). Since the advent of sediment traps, there have been a variety of studies evaluating trap designs and field conditions for which they can be used as unbiased collectors of sinking oceanic particles. These studies have focused primarily on hydrodynamical concerns (Gardner 1980a; Butman et al. 1986; Gust et al. 1992), artifacts related to the collection of non-sinking material (i.e. "swimmers" – Michaels et al. 1990; Lee et al. 1988; Steinberg et al. 1998), and the preservation of the sample once collected by a trap (Knauer et al. 1984; Wakeham et al. 1993; Kortzinger et al. 1994). To date, no sediment trap exists that collects relatively large amounts of fine (<200 μm) suspended sediments in the marine intertidal over an integrated period of time. This fine fraction of sediment, largely consisting of silt and clay constitutes the majority of food sleectedc from the water column by filter-feeding invertebrates, and is therefore the most biologically relevant A trap of this nature would provide scientists with the ability to examine the composition and possible contamination of biologically-relevant sediment food sources regularly ingested by benthic organisms.

Sediments within aquatic ecosystems are increasingly becoming receptacles for a wide range of heavy metals generated by human activities (Tarradellas and Bitton 1997; Selim and Amacher 1997). Metal inputs vary from large-scale industrial waste by-products and abandoned mines to small scale, improper disposal of over-the-counter

household products. It is therefore extremely important to properly assess the potential for sediments to act as a source of heavy metal exposure within an ecosystem.

Within aquatic benthic environments there are three main classes of invertebrate feeders: deposit, suspension or filter feeders, and combinations of the two (Ward and Shumway 2004). Suspension feeders feed on a wide range of plankton, material re-suspended from the ocean floor, sediments, and aggregates consisting of high molecular weight substances, detritus, fecal pellets, and microorganisms (Ward and Shumway 2004). As adults, suspension feeders are predominantly sessile and therefore need to filter large volumes of water in order to consume sufficient amounts of particulate matter to grow, reproduce and survive. Suspension feeders often inhabit the marine intertidal or subtidal zone, an area constantly undergoing change due to the dynamic nature of the tidal cycle. Sampling sediments from this constantly changing environment can be difficult, as both biological and physical re-suspension processes continually affect the quantity and quality of fine sediments accumulating at the sediment:water interface (Gray 1981; Thomson 1981).

Trophic transfer of metals is increasingly recognized as an important pathway for metal accumulation in marine invertebrates and fishes (Fisher and Reinfelder 1995; Wang and Fisher 1999). For many metals, dietary exposure represents a dominant route for metal accumulation, but the quantitative significance of dietary exposure is greatly dependent on the different aquatic species and the physico-chemical and ecological conditions within that ecosystem (Bendell-Young 1999; Wang 2002). Marine intertidal habitats are extremely diverse in composition, varying significantly in their geography, hydrology, and the physico-chemical conditions that define each environment.

Seasonal and spatial abiotic variations in salinity and temperature are important physico-chemical variables that modify the properties of suspended sediments and therefore the distribution of metals within these sediments (Moore 1985; Hall and Anderson 1995; Rainbow et al. 2004; Sokolowski et al. 2004). Organic content is also an extremely important abiotic factor controlling sediment quality in the benthic environment. Sediment geochemistry can vary drastically between and within an ecosystem, affecting the distribution and availability of metal contaminants within the sediments to benthic organisms (Stecko and Bendell-Young 2000a; Fan et al. 2002). Finally, the speciation and concentration of heavy metals within sediments can vary significantly among intertidal habitats (Griscom et al. 2000). The finest particle fraction of sediment (2-60 μm) is known to retain the highest proportion of metals. When there is an accumulation of finer particles in marine sediment an increased exposure to heavy metals has been shown to occur in invertebrates actively feeding on these sediments (Mudroch and MacKnight 1991; Siegel 2002; Ibhadon et al.2004).

Until recently, the majority of laboratory studies examining the effects of abiotic parameters on dietary sediment metal uptake have used artificial or deposited sediments in place of suspended sediments. These sediment types have demonstrated significant differences in their metal sorption abilities and geochemical properties and represent poor substitutes for suspended sediments (Stecko and Bendell-Young 2000b; Ke and Wang 2002; Yamaguchi et al 2003; Widmeyer and Bendell-Young 2004). The marine intertidal sediment trap described in this report is the first trap that collects large amounts of biologically-relevant suspended sediments from the intertidal zone in a simple and efficient manner.

Materials and Procedures

Study Sites. Sediment traps were deployed over an eleven month period at two intertidal estuaries located within the Straight of Georgia, BC, Canada. Deep Bay (DE) is on the eastern coastline of Vancouver Island in Baynes Sound (123°N, 48°W) while Boundary Bay (BB) is located directly south of the city of Vancouver in the Fraser River Estuary (124°N, 49°W). Deep Bay is a cobble-stone tidal estuary that is marginally influenced by one large freshwater river to its north, causing the salinity to be lower than normal ocean salinity (average 25 ppt), but higher than Boundary Bay (average 15ppt). It is located within Baynes Sound, a shallow coastal channel which comprises about 8,500 hectares, fringed by protected bays, open foreshore, intertidal mud and sand flats, low grade deltas, tidal estuaries, inshore marshes and rocky shorelines (Paynter 2002). These rich productive habitats are a result of the combination of sheltered water, low gradient tidal areas, fine substrates and nutrient-rich freshwater input. The area is a critical staging, breeding and wintering area for migratory birds and considered the most important waterfowl habitat in British Columbia after the Fraser River estuary. A number of native and exotic intertidal bivalves are found in the area, resulting in a large increase in foreshore shellfish aquaculture development, as this area produces approximately 50% of British Columbia's cultured shellfish (Paynter 2002). This region has also been subject to a substantial amount of mining activity. The main source of metal contamination is from effluent and run-off from the Mt. Washington copper mine.

Boundary Bay is an estuary mudflat dominated by fine silty sediment that is heavily influenced by outflows from three freshwater rivers. The areas surrounding Boundary Bay are zoned and utilized for agricultural purposes and drainage from these

areas enters Boundary Bay from five land pump stations (Swain and Walton 1990). Boundary Bay is within the lower southern end boundary of the Fraser River Estuary. The Fraser River is the largest river in British Columbia, extending 1,378 km in length, draining approximately 25% of the land mass and is home to approximately 63% of the province's total population (Millman 1980; Dorcey and Griggs 1991). Contaminant loading into this system is from a contribution of atmospheric, terrestrial and aquatic deposition due to agriculture, forestry, urban and industrial manufacturing and processing effluent (Shaw and El-Shaarawi 1995). As a result, the accumulation of PCB's, chlorinated phenolics, metals, dioxins and furans in water, sediment and biota have been well documented (Norecol 1993). Mining also contributes significant amounts of trace metals to receiving waters in the Fraser River Basin (Sekela et al. 1995). Although most mines have self-contained water treatment systems, acid mine drainage from tailings and waste rock from abandoned mines still enter the upper waters of the Fraser and remain a concern.

Sediment Traps. Intertidal sediment traps were designed for easy deployment at the low tide line to collect large volumes (5-10 g wet wt) of suspended sediments on a monthly basis. Traps were constructed of a single schedule 40 grade PVC pipe that was capped at one end and left open at the other. All PVC material was rinsed 3X with 10% HNO_3 then dd H_2O prior to assembly. The main pipe was 24 inches tall and 3.25 inches wide (internal diameter, i.d). To anchor the pipe in the sediment, two 12 inch long by 0.5 inch wide (i.d) schedule 40 grade PVC arms were inserted either 10 or 12 inches from the base of the main pipe and through the 3.25 inch width at a 180° angle from one other. The arms were anchored to the 3.25 inch pipe using two stainless steel screws that were

drilled into the arm where it met the main pipe from the outside. The end of each arm was capped with a 0.5 inch PVC cap, preventing sediment from entering the arms. This resulted in four 4.35 inch arms at 90° angles from one another, with one 12 inch arm crossing 1.0 inch above the other inside the 3.25 inch main pipe (Figure 4.1). Each trap was submerged 15 inches into the sediment, open pipe end facing upwards at a 90° angle from the sediment water interface, completely burying the four arms. This upright vertical position allowed the trap to retain all particles during daily tidal fluxes and withstand strong wind and current conditions over the three to four week period (Figures 4.2, 4.3).

Figure 4.1: Schematic of sediment trap.

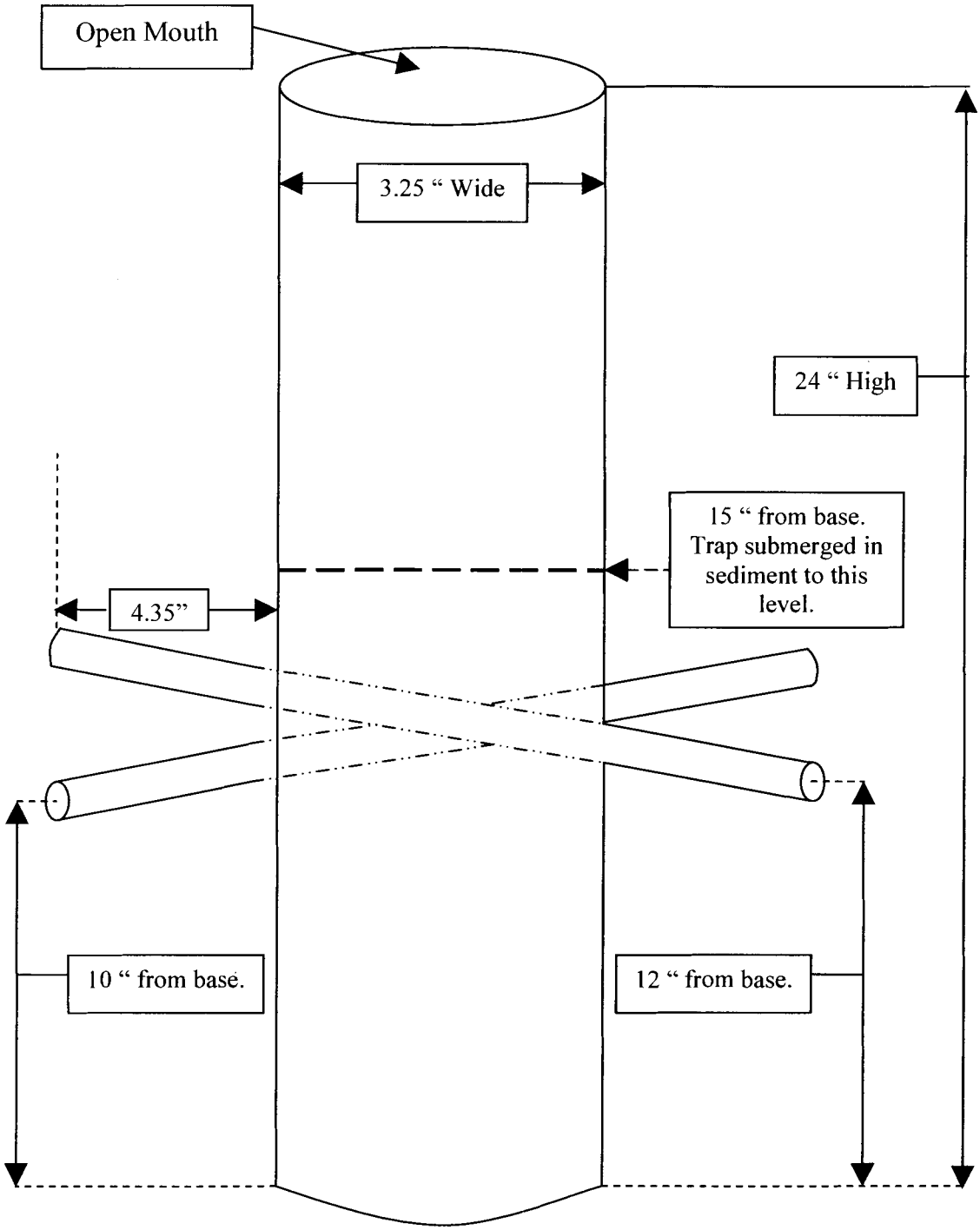


Figure 4.2: Sediment traps deployed at Boundary Bay.

Figure 4.3: Sediment traps deployed at Deep Bay.

From April until August the mouth of each trap was covered with large mesh polypropylene netting. The netting prevented the trapping of juvenile salmon, shrimp, and crab species in over 90% of the traps on a monthly basis. Any sediment that was contaminated with the above dead organisms was discarded and traps were thoroughly cleaned with seawater prior to being re-deployed. The width of the netting was 0.5mm and the mesh size was 1.7 mm by 1.7 mm. The netting was secured to the mouth of each trap with a 40 cm polypropylene cable tie woven through the mesh and around the outer pipe.

Traps were deployed over a single site within each bay, with a total of twelve traps per site during the fall and winter and ten during the spring and summer. They were positioned parallel to the shoreline, 3 m from one another, at the 0.9 m tidal line. In the Pacific Northwest tides are mixed semi-diurnal, and from October to March there is only one spring tidal cycle per month that reaches 0.9 m. This spring low tide occurs during the night and lasts approximately 4-6 days with 2-3 days extending below 0.9 m. From April to September two spring low tides occur per month reaching below 0.9 m. These occur in the morning or afternoon and are longer in duration (5-8 days) and lower in magnitude (0 m).

Sediment traps were sampled every 24-28 days from October 2003 to August 2004. Salinity and temperature readings were taken at each sampling period (Table 1). All collection and holding containers were acid-washed for 24 hrs in 10% HNO3 to remove any sorbed metals. Trap contents were emptied into 3 L polypropylene beakers, transferred to 500mL polypropylene bottles, and transported to the lab on ice within 6 hours (Trap yields in Table 4.1). US standard stainless steel testing sieves #30 mesh (600

μm), #70 mesh (212 μm), and #270 (53 μm) were used to wet sieve sediments in their native waters. All particles below 53 μm were held at 4 °C (to minimize microbial activity) for 24 hrs in clean 500 ml bottles. Supernatant waters were then removed by simple vacuum filtration, resulting in sediment in approximately 40 ml of native water. The slurry was transferred to acid-washed 50 ml polypropylene test tubes and centrifuged at 3500 rpm for 20 minutes. Following centrifugation, the sediment formed a semi-solid pellet, and the remaining native water was decanted. Wet sediments were weighed, placed in a 60°C oven for 48 hours, and a dry weight was obtained. Sediments were homogenized using an acid-washed ceramic mortar and pestle and stored at room temperature in 25 ml acid-washed polypropylene jars.

Analytical Methods. Total sediment organic content was determined for each sampling period using two techniques; 0.2 to 0.4 g dry wt sediment was ashed at 650 °C for 1 hr in ceramic crucibles to obtain a % carbon loss on ignition (%LOI) by muffle furnace and 10-30 mg for total organic carbon (TOC) by Carlo Erba NA-1500 Analyzer. Sediment nitrogen levels were also measured using the Carlo Erba Analyzer. To determine the amount of inorganic carbon contributing to the total carbon value, sediment carbonate content (50 mg per sample) was quantified seasonally using a Model 114 CO2 Coulometer (n = 6 per season).

Sediment metal levels (Cu, Cd, Pb, Zn) were measured using a Perkin Elmer Elan 6000 ICP-MS (inductively coupled plasma-mass spectroscopy). Sediment samples (0.50 g dry wt) were acid digested in 1.5 ml environmental grade HCl and 4.5 ml HNO3 at room temperature for 1 hr, then in a block digestor at 95°C for 2 hrs. Samples were cooled to room temperature, diluted to 50 ml with dd H2O and held at 4°C until analysis.

Analytical efficiency was checked using National Bureau of Standards marine sediment standard reference materials (MESS-3, PACS-4). Standards were digested and analyzed using the same procedures as our samples and measured values were always within 95% of the reported standard values.

Assessment

Efficiency of Sediment Traps. Several studies have defined certain criteria that a sediment trap must meet in order to minimize bias in estimates of collection rates. The sediment trap should have (1) a cylindrical collection vessel or tube (Gardner 1980a, b; Hargrave and Burns 1979) with (2) an inner tube diameter of $\geq$ 45 mm (Blomqvist and Lofoed 1981) and (3) an aspect ratio (height:diameter) of 3 to 5 or higher (Blomqvist and Hakanson 1981; Mudroch and MacKnight 1991). It is crucial that the collection tube maintains a steady vertical position, irrespective of water currents, internal waves, pulling, strumming and slanting of mooring lines (Gardner 1980a, 1985; Blomqvist and Lofoed 1981). The marine intertidal sediment trap conformed to the above three criteria; being tubular in shape, having an inner diameter of 8.19 cm and an aspect ratio of 7.4. This allowed for the reduction in any collection biases often observed with baffle and funnel shaped traps. The bottom half of the main pipe and the four 11.34 cm arms were completely buried, with the arms being approximately 8 cm below the sediment:water interface. This firmly anchored the traps, maintaining a vertical position without the need to utilize mooring lines, which decreased the amount of biofouling near the mouth of the traps. Biofouling on the main pipe of the trap was present at both sites from March to August. The majority of the material was barnacle spat which was removed from the

outside and inner mouth with a sharp knife, then rinsed with native seawater prior to being re-deployed.

Table 4.1 presents the amount of sediment below 53 μm caught per trap for the two sites over the four seasons. The results of a 2-way ANOVA indicated that trap collection efficiencies differed significantly both between the two sites and seasonally (Table 4.3). The amount of sediment collected was higher for Boundary Bay in all four seasons with less variance between the seasons while Deep Bay had much lower fall and winter collection efficiencies versus in the spring and summer.

The amount of nitrogen and carbon (TOC and %LOI) in Boundary Bay sediments was lower overall than in Deep Bay (Table 4.2). Both C and N varied significantly between the two sites as well as over the four seasons sampled, as indicated by the high F and low p values (<0.0001, Table 4.3). Inorganic carbon levels contributed minimally to overall total carbon levels in the sediments, with the average seasonal dry wt (g) values for carbonate in Boundary Bay sediments being (n = 6) 0.20 ± 0.01 for the fall, 0.18 ± 0.03 winter, 0.31 ± 0.05 spring, 0.42 ± 0.42 summer and for Deep Bay 0.92 ± 0.27 fall, 1.35 ± 0.35 winter, 1.49 ± 0.21 spring, 1.62 ● 0.29 summer.

Table 4.1. Total amount of suspended sediment (wet and dry wts) under 53 μm collected from traps at Boundary Bay (BB) and Deep Bay (DE) over four seasons.

Site/ Salinity/ Temp °C	Season	n	Wet:Dry Wt Ratio	SE	Wet Wt (g)	SE	Dry Wt (g)	SE
BB								
16.8 / 8.1	Fall	12	3.54	0.05	5.43	0.11	1.55	0.11
15.2 / 3.5	Fall	8	3.16	0.12	7.34	0.58	2.46	0.58
15.4 / 7.0	Fall	11	3.62	0.09	4.25	0.65	1.21	0.20
15.2 / 7.4	Winter	13	2.91	0.04	8.06	0.68	2.81	0.27
15.7 / 7.9	Winter	12	2.82	0.07	4.71	0.47	1.71	0.19
15.2 / 11.2	Winter	11	3.76	0.31	3.93	0.72	1.24	0.28
18.5 / 17.5	Spring	9	3.18	0.16	5.87	0.63	1.92	0.25
18.9 / 20.1	Spring	11	5.39	0.11	9.62	0.79	1.80	0.16
21.0 / 23.2	Spring	9	6.47	0.12	14.33	2.13	2.23	0.34
24.4 / 20.2	Summer	8	5.55	0.11	8.81	0.91	1.60	0.17
23.5 / 18.3	Summer	7	6.26	0.25	7.31	1.32	1.21	0.24
DE								
25.2 / 10.9	Fall	13	7.00	0.35	1.97	0.45	0.29	0.07
25.5 / 7.8	Fall	15	8.00	0.76	1.91	0.42	0.32	0.10
27.1 / 8.3	Winter	9	8.25	0.36	0.96	0.15	0.12	0.02
27.3 / 8.3	Winter	7	6.04	0.60	1.12	0.16	0.21	0.05
21.8 / 9.3	Winter	10	6.21	0.25	1.95	0.14	0.33	0.04
23.6 / 12.0	Spring	13	5.92	0.08	5.24	0.43	0.89	0.08
23.3 / 16.7	Spring	12	5.47	0.21	10.04	0.90	1.89	0.20
24.8 / 17.4	Summer	12	5.74	0.09	7.55	0.86	1.33	0.15
26.8 / 20.5	Summer	8	5.54	0.09	4.49	0.66	0.82	0.13

Table 4.2. %N and %C in suspended sediment collected from traps at Boundary Bay (BB) and Deep Bay (DE). Two methods were used to determine %C in sediments, TOC was determined by elemental analysis and LOI was determined using a muffle furnace.

			% TOC					% LOI	
Site	**Season**	**n**	**% N**	**SE**	**% C**	**SE**	**n**	**% C**	**SE**
BB	Fall	9	0.53	0.01	3.84	0.05	7	14.59	0.20
	Fall	9	0.41	0.03	3.19	0.20	4	11.42	0.36
	Fall	6	0.59	0.01	4.63	0.07	11	16.91	0.43
	Winter	9	0.40	0.02	3.21	0.11	13	10.78	0.18
	Winter	9	0.27	0.02	2.35	0.14	8	11.00	0.32
	Winter	9	0.53	0.02	3.77	0.12	7	14.05	0.44
	Spring	6	0.62	0.03	4.25	0.17	5	12.85	0.20
	Spring	6	0.73	0.01	5.22	0.06	6	22.46	0.66
	Spring	6	0.66	0.01	5.25	0.08	7	22.71	0.81
	Summer	6	0.79	0.01	5.84	0.07	8	22.72	0.60
	Summer	6	1.08	0.04	7.78	0.35	7	27.46	1.06
DE	Fall	9	1.04	0.11	7.07	0.72	8	28.83	1.60
	Fall	8	1.08	0.17	7.38	0.94	10	24.67	1.25
	Winter	4	0.67	0.03	5.92	0.11	6	21.13	0.77
	Winter	8	1.02	0.04	8.09	0.15	3	30.65	0.91
	Winter	7	1.03	0.03	7.72	0.14	7	23.95	0.31
	Spring	6	0.82	0.03	7.08	0.22	6	28.19	0.46
	Spring	6	0.66	0.01	5.84	0.07	7	24.26	0.30
	Summer	6	0.83	0.02	6.42	0.09	7	24.63	0.41
	Summer	6	1.18	0.05	8.87	0.20	8	28.58	0.72

Table 4.3. Results of 2-way ANOVAs with interaction (site vs. season) for total carbon (%LOI and TOC), nitrogen, and sediment collecting efficiency (g dry wt) for Boundary Bay versus Deep Bay over four seasons.

Parameter	Statistic	Site	Season	Site x Season
%C (LOI)	F	203	36.0	19.2
	p	0.0001	0.0001	0.0001
%C (TOC)	F	153	14.1	18.3
	p	0.0001	0.0001	0.0001
%N (TOC)	F	86.3	11.9	19.0
	p	0.0001	0.0001	0.0001
Dry wt (g)	F	86.4	8.92	9.41
	p	0.0001	0.0001	0.0001

Metal Levels in Sediments. All four metals (Cu, Cd, Pb, and Zn) differed significantly among the four seasons sampled per site, as well as between the two sites for Cu, Cd, and Pb (2-way ANOVA, Table 4.4). Zinc was the only exception; no significant differences in zinc levels were observed between BB and DE ($p>0.05$). Metal levels were compared to sediment quality guidelines (SQGL) and probable effect sediment quality guidelines (PEL) in order to evaluate the potential for biological adverse effects in benthic organisms inhabiting the two sites (Figures 4.4, 4.5, 4.6, 4.7). Copper levels exceeded the PEL for both sites in all four seasons. Cadmium had levels between the SQGL and PEL value for both sites in all four seasons. Lead either exceeded the PEL or SQGL values for both sites in the fall and winter, whereas spring and summer levels fell below the PEL for both sites. Zinc followed a similar seasonal trend as lead, Zn levels exceeded the PEL value for both fall and winter at BB and for the winter at DE, while spring and summer levels fell below the PEL for both sites.

Table 4.4. Results of 2-way ANOVAs with interaction (site vs. season) for Cu, Cd, Pb, and Zn in suspended sediments from Boundary Bay versus Deep Bay over four seasons.

Metal	Statistic	Site	Season	Site x Season
Cu	F	30.3	19.9	18.7
	p	0.0001	0.0001	0.0001
Cd	F	114	73.8	21.1
	p	0.0001	0.0001	0.0001
Pb	F	5.09	49.2	22.6
	p	0.0261	0.0001	0.0001
Zn	F	0.09	35.5	7.54
	p	0.7650	0.0001	0.0001

Figure 4.4. Copper levels (μg/g dry weight) in suspended sediments collected in traps from BB and DE. SQG is the current Canadian interim marine sediment quality guideline level (22% chance of an adverse biological effect above this level), PEL is the probable effect level (56% chance of an adverse biological effect above this level).

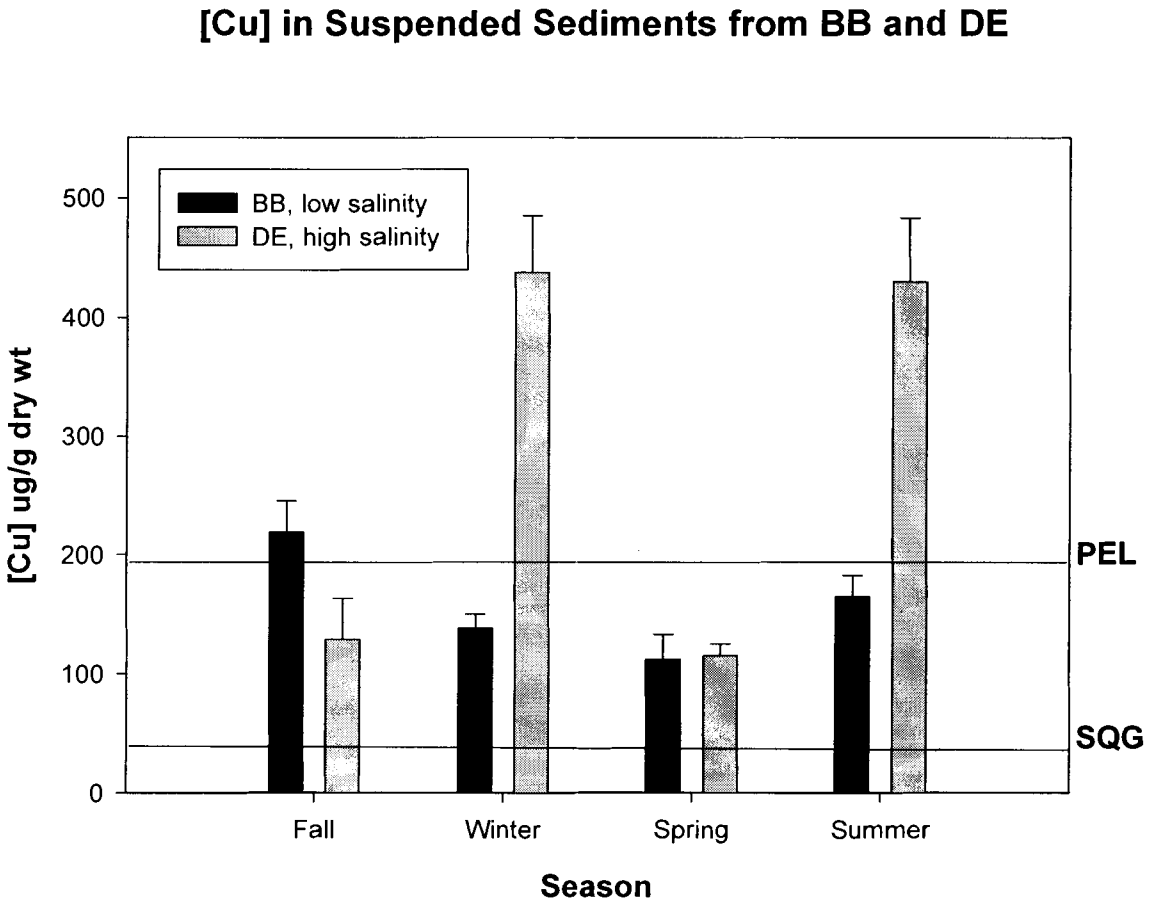


Figure 4.5. Cadmium levels (μg/g dry weight) in suspended sediments collected in traps from BB and DE. SQG is the current Canadian interim marine sediment quality guideline level (20% chance of an adverse biological effect above this level).

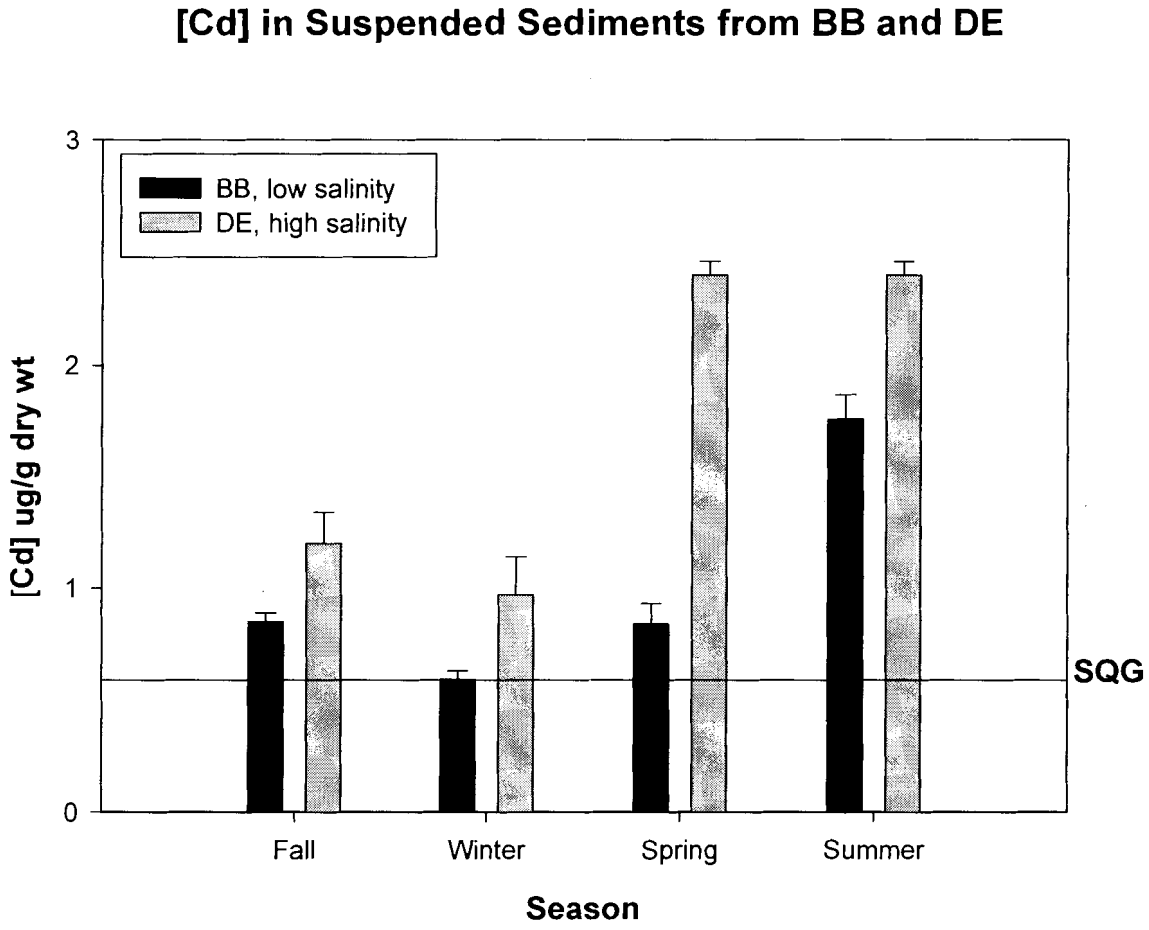


Figure 4.6. Lead levels (µg/g dry weight) in suspended sediments collected in traps from BB and DE. SQG is the current Canadian interim marine sediment quality guideline level (26% chance of an adverse biological effect above this level), PEL is the probable effect level (58% chance of an adverse biological effect above this level).

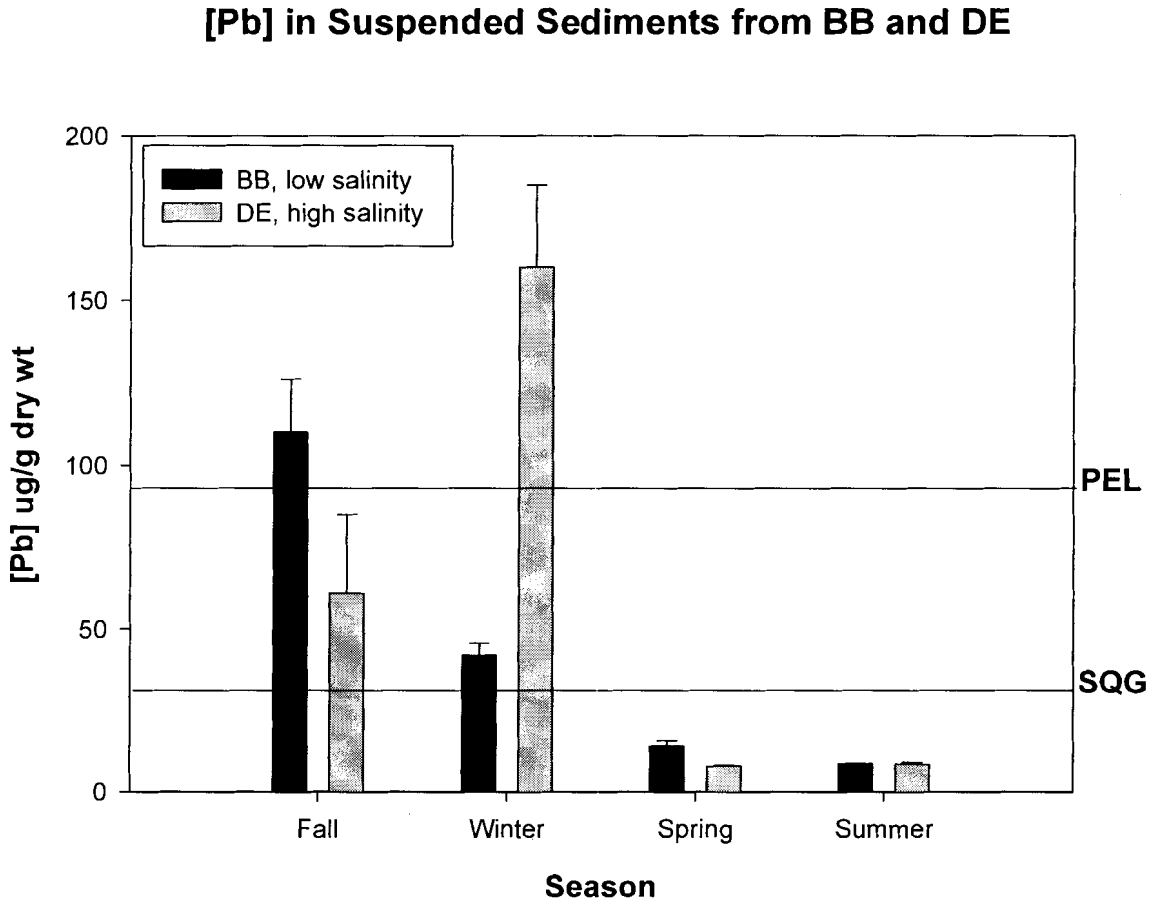
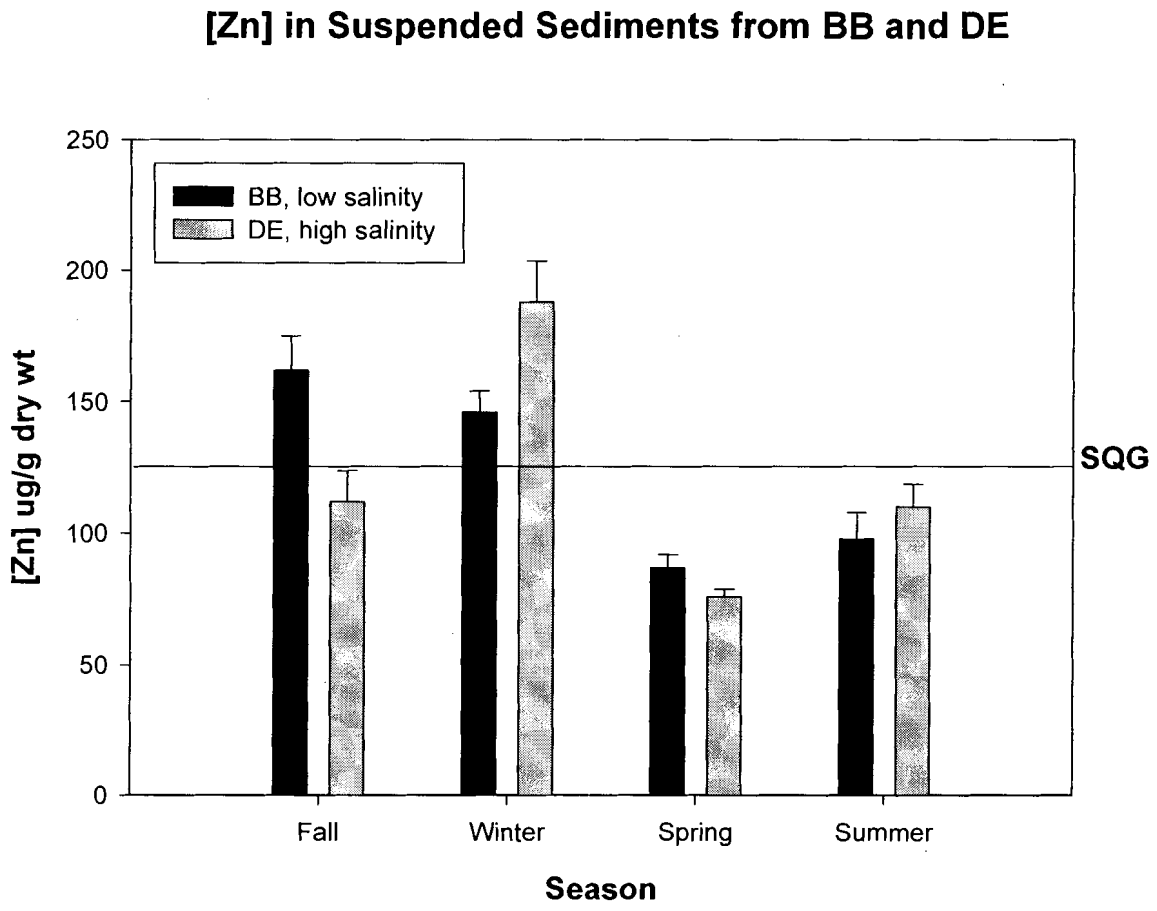


Figure 4.7. Zinc levels (μg/g dry weight) in suspended sediments collected in traps from BB and DE. SQG is the current Canadian interim marine sediment quality guideline level (27% chance of an adverse biological effect above this level).



Strengths and Limitations of Traps. The intertidal marine sediment trap was extremely successful over the eleven month deployment period at the two sites. Milligram quantities of dry sediment were collected from each site. This was sufficient to complete feeding experiments with intertidal bivalves, conduct sediment analysis using TOC and %LOI, and quantify sediment metal concentrations using both sequential extractions and ICP-MS. To date, it has been difficult to interpret metal levels in bulk collected sediments. Sediments were often too coarse (above 53 μm) and therefore not entirely biologically relevant, or not in sufficient quantities to run the numerous

toxicological tests needed to quantify metal concentrations in biota and sediments within a habitat.

Sediment diagenesis was not measured in this study, and it may have contributed to changes in %C and N levels and the mobility of metals within sediments collected using this trap. Broadly defined, diagenesis results from a set of conditions (change in redox potential and lowering of pH) causing physical and chemical changes in sediment after it has been deposited and buried under another layer of sediment (Siegel 2002). The occurrence of diagenesis could be tested by collecting sediment on a daily basis over a two week spring low tide period and comparing the metal distribution and sediment organic levels to samples left in the traps for the same time period. Seasonal changes would be extremely difficult to test, especially during the fall and winter months due to the short spring low tide periods making traps inaccessible for sampling. For bivalve feeding experiments, changes in the %C to N ratio are not problematic, as the overall trend in %C to N levels between sites would remain similar. Problems would result only if the %C to N ratio varied within each site.

The cost of building, deploying and maintaining the traps was minimal, making them both cheap and efficient. Each sampling period consisted of trap removal, sediment collection, trap re-deployment, and water parameter measurements, lasting approximately 2 hours in length. Access to the low tide line was restricted to a 2 to 4 hour window depending on the season, therefore only one site could be sampled over a 24 hour period. The only downfall to working on the west coast is that during October to March sediment traps must be deployed and sediments collected at night, when the lowest tide cycle occurs. The 1.7 mm by 1.7 mm mesh netting used to cover the mouth of the trap was

quite successful in keeping out juvenile invertebrate biota (90% of the traps per month). It is recommended that a minimum of 5 traps be deployed per site in order to allow for a n=3 per sampling period, as there were months that 10-20% of the traps were contaminated with these juvenile organisms and could not be used due to their influence on %C and %N in the sediments. Overall, the number of traps needed would be site- and region-specific, and would depend upon the needs of the individual researcher.

Discussion

The intertidal sediment trap presented in this study is a tool that facilitated an integrated level of research, and provided a sufficient amount of biologically relevant sediments for laboratory filter-feeding experiments. Within the field of ecotoxicology scientists are striving to develop more integrated levels of research, attempting to link contaminant levels with adverse biological effects within the food web of a community. Regulatory agencies are also recognizing the need for integrated levels of research that can be used to develop more realistic exposure guidelines. The current Canadian sediment quality guidelines are used by the Canadian government to control and monitor contaminant levels in aquatic sediments. The current guidelines are only interim values, due to the limited amount of laboratory and field research available for each aquatic contaminant. To develop more comprehensive guidelines, the Canadian government recognizes that at lease four studies are required on two or more sediment-resident invertebrate species that occur in North American waters, at least one of these must be a benthic amphipod species, and at least two of these studies must be partial or full life-cycle tests that consider ecologically relevant end points (i.e., growth, reproduction, developmental effects (CCME 2001). Sediment traps could be used by monitoring

agencies to collect biologically-relevant sediments at various intertidal sites to identify areas exceeding sediment quality PEL's. Once identified, collected sediments and benthic organism laboratory life-cycle tests could proceed, investigating the potential for adverse effects within these communities.

Ecotoxicologists have recognized the influence of environmental abiotic parameters on metal availability within food webs for aqueous metals. Investigation of these influences on dietary metal uptake (i.e.: sediments) within filter- and deposit-feeding invertebrates is currently deficient (Hall and Anderson 1995; Arifin and Bendell-Young 2000; DelValls et al. 2002). Integrated laboratory and field studies examining sediment metal availability in filter-feeding organisms need efficient collection techniques and large quantities of biologically relevant sediment. The intertidal sediment trap is a tool that can help identify habitats containing high levels of sediment bound metals (dietary), collecting biologically-relevant food sources for the investigation of potential adverse biological effects within filter-feeding bivalves ingesting these sediments.

Acknowledgements

Dr. L. I. Bendell-Young for providing me with the opportunity to develop and test the ideas contained in this paper, and for editing support. Mark Saffari from the Pacific Environmental Science Centre for generously analyzing sediment samples on the ICP-MS and for use of lab space. Chris Widmeyer for his help in the field and with the design and construction of the sediment traps. This research was made possible thanks to funding from L. I. Bendell-Young's NSERC discovery grant and Simon Fraser University Graduate Fellowships.

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CHAPTER 5:

DISCUSSION AND RECOMMENDATIONS

Discussion

Environmental stressors can modify the diversity within an ecosystem either by altering the abundance of a species and therefore modifying competition and predation relationships between that species, or by modifying the distribution of a species and its niche/habitat boundaries due to changes in the structural heterogeneity of the habitat. The goal of regulatory agencies using aquatic biomonitors is to monitor adverse effects within the biomonitor species, helping to provide an indication of potential stress and to sustain critical resources within that ecosystem before they are lost or adversely affected (USEPA 2004). Due to the robustness and stability common to biomonitor populations, any changes in their population distribution or abundance potentially represents an early warning of stressors due to metal contamination within that ecosystem (Dame 1996). Commonly observed changes in community relationships for aquatic bivalve biomonitors adversely affected by metal contamination are decreased competitiveness due to metabolic stress or a reduced reproductive capacity (Stewart et al. 2004), a weakened physiological state making them more prone to predation (Myrand et al. 2000), a reduction in overall population levels causing a decrease in prey availability or quality to predators (Jamil et al. 1999), and relocation or redefining habitat boundaries due to changing water quality conditions (McGreer 1981).

As stated in the introductory chapter, an effective metal biomonitor species should be a/ tolerant to a wide range of environmental conditions and b/ provide an assessment of bioavailable metal concentrations based on metal tissue levels (Dame 1996). In my

opinion, these two prerequisites can vary both inter- and intra-specifically for *M. trossulus* and for other filter-feeding invertebrates (Blackmore and Wang 2003a,b; Rainbow 2002). The research presented in this thesis demonstrated that food quality, salinity, and animal behaviour affect metal uptake and assimilation in laboratory studies using *M. trossulus*. Alterations in these biotic and abiotic parameters affected metal tissue levels, indicating the need to consider these variations when making comparisons between *M. trossulus* populations in different habitats.

The results obtained in Chapter 2 of this thesis demonstrate that *M. trossulus* can selectively feed on bacterial food particles containing Cd, Pb, and Mn by altering its ingestion rate of these particles based solely on food quality (as defined by organic carbon content). As a result of selectively feeding on higher carbon food sources, *M. trossulus* ingested a higher concentration of metals that were sorbed to the sheath of the higher carbon *L. discophora* cells. The preferential selection of organic over inorganic particles has previously been demonstrated in studies using benthic feeding organisms (Brown 1986; Maloney 1996). These studies confirmed a preferential organic particle uptake through the examination of gastropod and bivalve feces, and marine polychaete gut contents. Positive correlations existed between Cd, Cu, Ni and Zn uptake and increasing organic content in fecal products (Brown 1986) as well as with Cd uptake within the organism (Maloney 1996). It is important to mention that previous studies have also disproven the preferential selection of food sources based on organic content using deposit-feeding bivalves (Griscom et al. 2000; Wang et al. 1995). The main difference in these studies from ours is that the food sources consisted primarily of sediment or laboratory synthesized matrices, not natural food sources with the same base

(i.e.; bacteria), and therefore may not be ideal representations of food matrices naturally encountered and fed upon by benthic dwelling organisms.

To date, ours is the only study that has examined metal uptake from one food source (*L. discophora*) containing varying levels of organic carbon. Previous studies used multiple bacteria or phytoplankton species which not only differed in organic content, but also in cell size (Birbeck and McHenery 1982; Bougrier et al. 1997). Variations in particle size or quantity modify ingestion rates in bivalves fed phytoplankton or bacterial species alone or in combination with sediments (Bougrier et al.1997; Iglesias 1996; Ke and Wang 2002). The unique properties of *L. discophora* and its extracellular sheath allowed us to examine the uptake of multiple metals coupled with natural variations in organic carbon. The disadvantage to using this species was that only metal uptake, not assimilation could be examined, because lead and manganese do not have natural radioisotopes which are necessary to determine metal assimilation in bivalves. Alternative isotopes such as americium have been used in previous studies, but high desorption rates from food matrices occurred, causing inflated uptake and assimilation (Arifin and Bendell-Young 2000).

This is the first study indicating that secondary metals (Cd, Pb) attached to the Mn oxides on the cell sheath reduces the carbon proportion of the bacteria. When no metals or just Mn alone were sorbed to the cell sheath the organic carbon content of the cells was higher than when the Mn sheath plus one other metal were sorbed to the sheath. These changes in the proportion of organic carbon content for *L. discophora* were not previously examined or demonstrated (Corstjens et al. 1991; Emerson and Ghiorse 1993). The main conclusion from this research was that metal uptake was controlled by the

behaviour and selective criteria of *M. trossulus* when ingesting pure *L. discophora* cells. This research further emphasizes the importance of an organism's behaviour and it's potential to alter metal uptake and tissue levels in bivalve species. Regulatory agencies collecting bivalves and analyzing tissue metal levels to monitor metal contamination need to be aware of changing feeding conditions (phytoplankton, sediment, bacteria) and a bivalve's ability to selectively feed during these conditions. This is especially important for programs like Mussel Watch that compare contamination levels among various intertidal sites and among seasons.

In Chapter 3, we examined the influence of salinity and its ability to modify metal assimilation in *M. trossulus* feeding on combinations of phytoplankton, clay and suspended sediments from two distinct marine habitats (Deep Bay and Boundary Bay). To examine metal assimilation versus uptake (as in Chapter 2) we used a ^{109}Cd radiolabel that was attached to the various food sources fed to *M. trossulus*. Our results indicated that *M. trossulus* had lower amounts of dietary Cd assimilated in its tissues when fed both natural and control food sources under lower salinity conditions. This is the first in vivo study to demonstrate the effects of salinity on dietary metal uptake in filter feeders. Until now, only the assimilation of aqueous Cd has been examined in bivalve species inhabiting habitats with varying salinities (Hall and Anderson 1995; Fisher 1986; Lee and Luoma 1998; Blackmore and Wang 2003a, 2003b). Under laboratory conditions, these studies demonstrated an increase of aqueous heavy metals (Cd, Zn and Cr) in the bivalve with decreasing salinity (Fisher 1986; Blackmore and Wang 2003a, 2003b). This trend is the opposite of what our study demonstrated, and is likely related to the greater concentration of the free metal cation at lower salinity conditions (Hall and Anderson

1995). Metal bioavailability from the aqueous phase was also directly related to the physiological condition in three different species of bivalves (Wang 2001). No significant trend has been demonstrated for aqueous Cd AEs but significantly longer GPTs have been demonstrated at low (10 ppt) salinities, indicating a possible effect of lowered salinity on *P. virdis* gut physiology (Blackmore and Wang 2003b). In our study, the lower salinity environment had food particles remaining in the bivalve gut longer with ^{109}Cd being assimilated at a lower efficiency, unlike aqueous metals that remain in the gut longer and have higher AEs.

It is possible that salinity affected the physiological condition of *M. trossulus* as speculated by the authors of the above aqueous uptake studies. Past studies have demonstrated that chemical, physical and environmental stressors have adverse cytological effects on the digestive lysosomal cells responsible for metal uptake and assimilation in bivalves (Moore 1985; Nicholson 1999; Hauton et al. 1998; Ringwood et al. 1998). Significant lysosomal membrane destabilisation due to extremes in temperature, salinity and copper exposure were examined. Varying degrees of lysosomal destabilization occurred depending on what species of bivalve was tested and whether the test was performed on field collected versus laboratory run experiments. Nicholson (1999) demonstrated significant destabilization in *Perna viridis* gut haemocyte lysosomes when both temperature and salinity varied in laboratory studies. Similar studies with *Crassostrea virginica* testing salinity and copper demonstrated no lysosomal destabilisation for variable salinity regimes but significant destabilisation for aqueous copper uptake (Ringwood et al. 1998). Further adverse physiological effects such as decreased growth or inhibited reproduction due to increased lysosomal destabilisation

were not apparent or of noticeable significance. One study using field collected *M. edulis* from nine sites contaminated with metal and organic contaminants demonstrated significant gut lysosomal destabilization resulting in smaller sized and lower weight species (Krishnakumar et al 1994). Unfortunately, there are no further laboratory studies done by these researchers to single out what concentration or specific contaminant (or combination of) caused these adverse morphological effects.

Based on the above studies it is difficult to determine whether the combination of lower salinity and cadmium exposure caused lysosomal destabilization leading to a decreased AE, decreased uptake of ^{109}Cd and a longer GPT in *M. trossulus*. Lysosomal destabilization due to a lower salinity environment can cause a net solute loss leading to an increased energetic cost in cell volume regulation for *M. trossulus* (Hawkins and Hilbish 1992). It is possible that a reduction in the overall metabolic budget could lead to a reduction in the uptake of nutrients and ^{109}Cd by digestive diverticula cells. In response to a possible reduced nutrient uptake, longer GPTs may occur to compensate for this reduction. This is purely speculation, and would have to be tested using laboratory feeding tests combined with the neutral red cellular assay (used by previous studies testing lysosomal destabilisation) to determine whether a relationship exists for ^{109}Cd uptake, GPTs and lysosomal destabilization in *M. trossulus*.

Food quality also differed between the two marine habitats (Deep Bay and Boundary Bay) but did not have a statistically significant role in determining Cd assimilation in *M. trossulus* when compared to salinity. It is possible that food quality did play a role in controlling Cd assimilation, but the influence of salinity was so strong that these effects were not discernible. In addition, our experiments were conducted

using ideal food quantity conditions. It is possible that *M. trossulus* exhibits a decrease in selective feeding when food quantity is ideal. Previous studies demonstrate a dynamic interplay between clearance rates and carbon assimilation efficiency for *M. trossulus*, allowing it to maintain a constant rate of carbon assimilation (Arifin and Bendell-Young 1997, 2000). At low quantity conditions organic rich particles were preferentially selected by *M. trossulus* in order to fulfil its carbon budget. In that study, the difference between low versus high quantity food was between 10 and 20 fold (1.4 to 56 mg/L), whereas in the present study, food quantity was maintained at optimal levels for all exposures (5 - 8 mg/600ml).

The most important finding was the overall dominant effect that habitat salinity had over food quality. If variations in salinity greatly affect the extent of metal uptake by *M. trossulus*, then these effects must be accurately determined and accounted for when collecting samples and comparing metal levels between different marine habitats. If not, the potential for more sensitive species to suffer adverse effects may go unnoticed particularly if salinity increases leads to enhanced metal uptake. Adverse effects may also be exaggerated in habitats where dietary metal uptake is lower than expected (i.e. lower salinity). For governments to promote and maintain a high level of sustainability among marine habitats regulatory measures must be based on accurate and thorough research. Thus, an organism's behavior and the characteristics of its habitat must be considered in order to properly quantify the effects of metal contamination for the organism and the trophic levels directly linked to it.

In Chapter 4, unique sediment traps were designed and deployed at two sites over a four season period (ten months), collecting suspended sediments for Ch. 3 feeding

experiments and for geochemical analysis. Within the oceanic water column, sediment traps are used to accurately quantify the vertical flux and composition of sinking oceanic particles. As a result, most traps are designed and utilized to study oceanic hydrodynamics (Gardner 1980; Butman et al. 1986; Gust et al. 1994), and collect non-sinking material suspended within the top 100m of an oceanic environment (Lee et al. 1988; Michaels et al. 1990; Steinberg et al. 1998). To date, no sediment trap exists that collects relatively large amounts of fine (<200 μm) suspended sediments in the shallow marine intertidal. This fine fraction of sediment, largely consisting of silt and clay is what the majority of filter-feeding invertebrates actively select from the water column, and is therefore the most biologically relevant Our trap is the first to provide scientists with the ability to examine the composition and possible contamination of biologically relevant sediment food sources regularly ingested by intertidal benthic organisms.

The results of our study demonstrated that the sediment geochemistry between two marine intertidal sites varied significantly both on a spatial and on a seasonal basis. Previous studies vary considerably in reporting spatial and seasonal trends in sediment metal concentrations. Our results paralleled those found by Zachariadis et al. (2001), demonstrating significant differences in Cd, Pb and Hg for surficial coastal sediments both spatially and seasonally. In addition, there was a lack of significant trends (i.e. higher summer vs. winter) observed for either parameter for the three metals. Man et al. (2004) found that when Cd, Pb, Zn and Cr were examined in two intertidal mudflats there were no significant seasonal or spatial variations in sediment concentrations throughout the ten month study period. When the sites were more diverse in origin significant spatial and seasonal differences were observed, saltmarsh sediments accumulated higher

concentrations of metals versus mudflat sediments with both sites having the highest winter and lower summer levels (Wright and Mason 1999).

Metal levels varied significantly in the collected suspended sediments both within and among the two sites. To put these metal concentrations into context, they were compared to the current Canadian Sediment Quality Guideline levels (SQGL), to evaluate the potential for biological adverse effects in benthic organisms inhabiting the two sites. Copper and cadmium had levels either exceeding the SQG or the probable effect level (PEL) for both sites in all four seasons. Lead exceeded the PEL or SQGL values for both sites in the fall and winter, while spring and summer levels fell below the PEL for both sites. Zinc followed a similar seasonal trend as lead, with Boundary Bay exceeding the PEL value for both fall and winter and Deep Bay for the winter, spring and summer. These levels are extremely difficult to interpret and put into a biological context, as there are no recommended guidelines for organisms either living in contaminated marine sediments or ingesting these sediments.

If the dose makes the poison then it is important to quantify the dose that causes any potential adverse effects in the exposed organism. The SQG levels were derived using a database of studies that examined only aqueous metal uptake from sediments, not dietary uptake. This makes it even more difficult to distinguish whether the current guidelines for metals comprehensively covers both aqueous and dietary uptake in benthic marine species. In addition, if SQG levels are being used to determine potential adverse effects of metal exposure in benthic organisms, it is pertinent to question whether sediment metal levels accurately reflect those found in species ingesting these sediments. Recent studies determined that sediment metal levels were poor indicators of levels found

in both barnacles and mussels (Rainbow et al. 2004: Zachariadis et al. 2001). These species proved to be good indicators of spatial variations in metal levels among coastal areas, but their ability to indicate seasonal changes or correlate with concentrations of metals in sediments was not reliable. Therefore, sediment metal concentrations may not represent good measures of ambient trace metal bioavailabilities to coastal filter feeders.

Overall the intertidal marine sediment trap was extremely successful over the eleven month deployment period at the two sites. Sufficient quantities of sediments were collected from each site in order to run in lab feeding experiments with intertidal bivalves, conduct sediment analysis using TOC and %LOI, and quantify sediment metal concentrations using both sequential extractions and ICP-MS. Up to this point it has been difficult to interpret metal levels in bulk collected sediments as they were too coarse (above 53 μm) and therefore not entirely biologically relevant, or not in sufficient quantities to run the numerous toxicological tests needed to quantify metal concentrations in biota and sediments within a habitat. Our sediment trap collected the most biologically relevant sediment sources ingested by *M. trossulus* in the natural environment, allowing us to examine the feeding behaviour and cadmium assimilation from these sediments under controlled laboratory conditions. In the field, this trap would be extremely successful in coastal monitoring programs to measure fluxes in suspended sediment metal levels over time. Traps could also aid in pinpointing potential "hot spots" of metal contamination, indicating a need to develop biota sampling within that area.

The research presented in this thesis demonstrates that *M. trossulus* is tolerant to a wide range of environmental conditions (salinity and organic carbon), yet variations in these conditions will modify uptake of metals from particulate matter, resulting in

changes in metal tissue levels in this species. For this species to be an effective biomonitor of metal contamination, these variations must be taken into consideration when interpreting metal tissue levels between sites. For ecotoxicologists to build upon the current knowledge base and develop future concepts and methodologies that preserve and sustain our benthic marine habitats, studies such as those contained in this thesis need to be considered and further tested in the field. I conclude by making five suggestions as to how this research can further strengthen our ability to utilize *M. trossulus* as a biomonitor, and detect potentially toxic levels of metals within marine intertidal habitats.

Recommendations

1/ Transplant experiments with caged *M. trossulus* at both Boundary Bay and Deep Bay are needed to investigate the ingestion of metal bound sediments and phytoplankton under field conditions. Particular focus should be on Cu and Cd considering the 2003-2004 sediment levels presented in this study was above Canadian PEL (Cu) and SQGL (Cd) over the four season sampling period. In addition, the use of a second filter-feeding biomonitor species such as a barnacle or oyster would be beneficial to examine possible interspecies differences in metal uptake. Rainbow (2004) demonstrated the importance of using both mussels and barnacles to examine trace metal bioavailability geographically and over time.

2/ Sediments collected demonstrated significant differences between sites and among seasons for %C, %N, and Cu, Cd, and Pb levels. Within site differences need to be examined using the marine intertidal sediment trap by having 3 sampling locations within each water body, each location having a minimum of 5 traps deployed. This will further test the ability of the sediment traps to collect samples within a site and compare

any potential intrasite differences. In addition the sources of Cu and Cd metal input into Boundary Bay and Deep Bay need to be determined and eliminated in order to minimize the potential for adverse biological effects within these ecosystems, as indicated by the Canadian Sediment Quality Guidelines (CCME 2001).

3/ Investigate the subcellular compartmentalization (haemocyte lysosomes, metallothioneins and metal-rich granules) of Cd in *M. trossulus* to determine the level of biologically dexotoxified metal versus total metal tissue concentrations. Non-detoxified metals are considered bioavailable or trophically available metals (cytosol and proteins) that represent the portion of metals in *M. trossulus* tissues that can be passed onto predators up the food chain. The procedures used for determining subcellular partitioning of metal within tissues can also examine possible physiological responses elicited by a reduced salinity in *M. trossulus*, as indicated by the results of Ch. 3. Either laboratory feeding experiments or mussels collected from two sites with varying salinities need to be performed using a protocol developed and tested with clams (Wallace et al. 2003).

4/ Examine the trophic transfer of Cu and Cd using three levels of a trophic food web in Boundary Bay and Deep Bay. Phytoplankton and suspended sediments would represent the primary level of the food web, bivalves the secondary level, and migratory sea ducks the tertiary. Bivalves are able to accumulate high concentrations of Cu and Cd from the large amount of primary level prey that they ingest. They then serve as a dietary source to their predators, who also inherit the large metal burden from their bivalve prey. Whole samples can be collected for the primary and secondary level organisms while blood, feather or fecal samples are sufficient in quantifying effects observed by tertiary

level organisms. Stable isotope analysis coupled with metal tissue analysis (ICP-MS) is currently the most effective analytical tool available to examine metal transfer within a food web. Recent freshwater field studies have demonstrated significant dietary heavy metal exposure via trophic transfer in freshwater (salinity below 5 ppt) ecosystems, but currently none exist for the marine environment (Purkerson et al. 2003, Stewart et al. 2004).

5/ Develop more accurate kinetic models to determine metal assimilation within a species or between the levels of a food web. Recent kinetic models examining Cd and Zn trophic transfer do not factor in any changes in abiotic conditions within these marine ecosystems. In my opinion, this approach cannot be fully utilized until more field studies are conducted (Ke and Wang 2002, Wang 2002). The development of accurate models requires a more biologically relevant approach, accounting for both biotic and abiotic variations that affect metal assimilation in organisms inhabiting distinctly different aquatic habitats.

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Appendices

Appendix 1: Collaborative research published in American Minerologist 89, 1110-1118, 2004, co-authored by J. R. Widmeyer. Title: The structure of the Mn oxide on the sheath of the bacterium *Leptothrix discophora*: An XAFS study. Copyright permission was granted by American Minerologist to include in this thesis.

Appendix 1:

The structure of the manganese oxide on the sheath of the bacterium *Leptothrix discophora*: An XAFS study

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ABSTRACT

In natural waters, manganese oxides (MnO_x) are important in mediating the bioavailability of trace metals such as Ni, Cu, Zn, Cd and Pb, as these metals readily adsorb to the MnO_x surface. Manganese from a variety of anthropogenic sources usually enters the aquatic environment in dissolved form as Mn(II). It is subsequently oxidized under oxic and neutral (pH = 6-7) conditions. Often this oxidation is catalyzed by bacteria, such as *Leptothrix discophora*, as part of their natural metabolic process.

Mn K-edge X-ray Absorption Fine Structure Spectroscopy (XAFS) was used to investigate the local structure of the manganese oxide on the sheath produced by the bacterium *Leptothrix discophora* SP-6. The features observed in the near edge region of the Mn K-edge spectrum indicate the presence of three oxidation states of manganese - Mn^{2+}, Mn^{3+}, and Mn^{4+}. Fitting the experimental XAFS data identifies the bacterial MnO_x as being composed of single-layer- microcrystals with layers similar to those occurring in Na-birnessite. Some MnO_6 octahedra might lie outside the layer plane, sharing corners with those in the layer plane. X-ray diffraction results for the same samples are consistent with the single-layer structure.

INTRODUCTION

Manganese-oxidizing bacteria of the strain *Leptothrix discophora* are of importance in the environmental chemistry of manganese (Adams and Ghiorse 1987, 1988; Boogerd and De Vrind 1987; Emerson and Ghiorse 1992; Nelson et al. 1999a). These microbes adsorb aqueous Mn^{2+}, which is then oxidized by a protein-catalyzed process (Adams and Ghiorse 1987). The MnO_x oxidation product is deposited on the sheath, a structure composed of polysaccharides and proteins that forms the exterior of the bacterium's cell wall. Heavy metals, in particular Pb^{2+}, can be incorporated into the sheath through chemisorption (Nelson et al. 1998, 1999a, 1999b). As the bacteria are eaten by mussels and other organisms, accumulation of these metals on the MnO_x may represent an important route for their introduction into the food chain. The actual adsorption process by Pb^{2+} and other heavy metal ions onto the MnO_x is not well understood at present. One reason for this is that the crystal structure of the MnO_x is unknown.

The average manganese oxidation state of the bacterial MnO_x, as determined by iodometric titration, is of the order of 3.3 to 3.7 (Adams and Ghiorse 1988), indicating that at least two different oxidation states of manganese are present. Additional measurements of the oxidation reaction rates suggest that a mixed Mn(III/IV) oxide is produced (Adams and Ghiorse 1988). In nature, manganese (III/IV) oxides and oxyhydroxides occur in a variety of different, often poorly, crystalline forms (Post 1999). Nelson et al. (1999a, 1999b) proposed that the bacterial MnO_x is amorphous. It has been suggested that Mn-oxidizing bacteria contribute to the formation of rock varnishes (McKeown 2001). A mineralogical characterization of these materials with Mn K-edge absorption spectroscopy (McKeown 2001) indicated that they contain several different crystal phases of MnO_x, that range from structures with large tunnels, such as todorokite, to layered structures, such as birnessite.

We have used x-ray absorption fine structure (XAFS) spectroscopy (Konigsberger and Prins 1988) to study the MnO_x produced by the bacteria strain *Leptothrix discophora* SP-6. The Mn K-edge was investigated to determine the short-range order and local crystal structure as well as the oxidation state of manganese. We have also applied x-ray diffraction to the bacteria samples to determine if long-range order is present in the MnO_x sheath.

MATERIALS AND METHODS

Preparation of samples

Samples of the bacteria strain *Leptothrix discophora* SP-6 (American Type Culture Collection (ATCC) #51168) were grown under controlled laboratory conditions at room

temperature. The glassware used was acid washed prior to the growth process in order to remove all trace metals from the system. Modified versions of the growth media ATCC #1503 (with Mn^{2+}) and ATCC #1987 (without Mn^{2+}) were used. The amounts of peptone and glucose were doubled and $FeSO_4$ was added. The composition of the growth medium used is given in Table 1. Except for the $MnSO_4$, the control sample was grown in the same medium. The samples were harvested after 48 hours of growth at 20°C with shaking. The bacteria and fluid were separated by centrifugation (5 minutes, 1000 rpm), and the supernatant was removed. The culture was washed once by re-suspension in 25 mL of distilled deionized water, followed by centrifugation (5 minutes, 1000 rpm) and removal of the supernatant. The remaining bacterial pellet was lyophilized/freeze dried for 24 hours. Five different samples prepared by this method were used in the XAFS and X-ray diffraction experiments.

Measurements were also made on nine manganese oxide mineral standards, two of which we synthesized. Na-birnessite was prepared using the method described by Ressler et al. (1999). Vernadite (δ-MnO_2) was prepared according to the method given by Manceau et al. (1992a).

X-ray diffraction

The powder x-ray diffraction patterns of the freeze-dried bacteria and the manganese oxide mineral standards were collected with a Siemens D5000 diffractometer. The Cu-K_α x-ray source (1.5406 Å) was operated at 45 kV with a current of 35 mA. The divergence slit was set at 0.3 mm and the antiscatter slit at 0.5 mm. The samples were ground to a fine powder and tightly packed into a square cavity (~1 cm x 1 cm, with a depth ~0.1cm) centered in a glass sample holder such that the sample surface was flush

with the surface of the holder. The sample holder was then secured and aligned in the diffractometer. The data were collected in the coupled ($\theta - 2\theta$ geometry) mode over a 2θ range from 3.0° to 75.0° with a step size of 0.1° and a dwell time of 0.1 seconds per point. Because of a low count rate, over 300 scans were taken per data set and summed. In order to identify the diffraction peaks resulting from the MnO_x sheath, two bacteria samples were investigated. The first sample was exposed to $MnSO_4$ during growth, whereas the second sample was not, and thus did not have the MnO_x deposited on the sheath.

X-ray absorption fine structure (XAFS) spectroscopy

Historically, XAFS has been divided into the spectroscopy of the near edge structure (XANES) referring to the structure in the x-ray absorption coefficient within ~50 eV of the absorption edge and the extended x-ray absorption fine structure (EXAFS) referring to the structure at higher energies and extending to as much as 2 keV above the edge. With improved understanding of the origin of the structure it has become conventional to use XAFS to encompass both regions. In this paper we use XAFS when both regions are being considered and EXAFS or XANES when just one of the regions is being considered.

The Mn K-edge absorption spectra were collected using the PNC-CAT undulator beamline (Heald et al. 1999), Sector 20 at the Advanced Photon Source, Argonne National Laboratories, Argonne, IL. The linearly polarized X-ray beam from a Si-(111) double crystal monochromator was detuned to 65% at 6800 eV to eliminate the higher-order harmonics. For additional harmonic rejection, a rhodium-coated glass mirror was placed into the path of the beam preceding the He-filled parallel-plate ionization chamber

used to measure the incident photon flux (I_0). Mn K-edge fluorescence spectra were collected using a 5-electrode (Ni-mesh) ion chamber (Jiang and Crozier 1998) containing argon. Distortion of the spectra due to self-absorption effects was not an issue. The freeze-dried bacteria samples were spread onto adhesive KAPTON® tape to form a thin layer of sample sandwiched between two layers of tape. Measurement with an optical microscope indicated the thickness of the manganese-containing layer was less than 10 μm. Given that the concentration of Mn was 0.68 wt% per dry weight of bacteria, the effective thickness of Mn was less than 1% of the Mn K-edge attenuation length. To reduce radiation damage, the sample was mounted along the circumference of a Plexiglas disk with diameter of 3 in. and rotated at 1200 rpm as the XAFS spectra were collected. No radiation damage was observed in the Mn K-shell spectra after 10 hours of exposure to the x-ray beam when this method of sample mounting was employed, whereas, with a stationary sample, radiation damage was observed after 20 minutes of exposure.

The Mn K-edge fluorescence spectra of several manganese oxide minerals – hausmannite (Mn_3O_4), manganite (γ-MnOOH), bixbyite (Mn_2O_3), todorokite (MnO_2), synthetic birnessite (MnO_2), and synthetic vernadite (δ-MnO_2) – and commercial MnO (manganosite), $MnSO_4 \cdot H_2O$, and β-MnO_2 (pyrolusite) powders (Aldrich) were measured under the same experimental conditions to aid in the identification of the local structure of the bacterial MnO_x. Three different oxidation states of manganese (Mn^{2+}, Mn^{3+}, and Mn^{4+}) were represented by these minerals. The photon energy scale in the Mn K-edge region was calibrated through measurement of the Mn K-edge spectrum of manganese metal powder in epoxy. The maximum of the first derivative was defined as the edge energy and set at 6537.67 eV (Kraft et al. 1996).

ANALYSIS AND DISCUSSION

X-ray diffraction

The x-ray diffraction pattern of the freeze-dried bacterium (Fig. 1) is dominated by a single broad peak at $2\theta = 19.55° \pm 0.13°$, possibly associated with the sheath surrounding the bacterium that is composed of polysaccharides and proteins (Emerson and Ghiorse 1993a, 1993b). In addition to the above peak, the diffraction pattern of the bacterium incubated with the MnO_x (Fig. 1) has two asymmetric peaks at $2\theta = 36.73° \pm 0.03°$ (d = 2.445Å ±0.002Å) and $65.59° \pm 0.04°$ (d = 1.422Å ± 0.001Å). These two peaks were isolated from the rest of the pattern by normalization (the height of the peak at 19.55° was set to 1.0 and the pattern of the bacterium was subtracted from the pattern of the bacterium with MnO_x) (Fig. 1, upper panel). The pronounced asymmetry of the first MnO_x peak is characteristic of x-ray diffraction from single layers (the Warren effect) (Warren and Bodenstein 1965; Yang and Frindt 1993) that produce only (hk0) lines and no (hkl) lines.

Manganese dioxide has a variety of different stable crystal structures (Li et al. 1988; Manceau and Combes 1988) in which edge-sharing MnO_6 octahedra form layers or chains. The latter are connected by corner-sharing octahedra giving rise to the tunnel structure of manganese dioxide. The layers, on the other hand, are unconnected. In a crystal they are stacked on top of each other with a spacing of 7 to 9 Å. Water molecules and metal ions (eg. Na^+, Mg^{2+}, Ca^{2+}) are typically found in the interlayer regions and inside the larger tunnels.

The diffraction pattern observed for δ-MnO_2, a synthetic vernadite, is similar to that of the bacterial MnO_x (Fig. 1, upper panel). However, vernadite's two peaks occur at slightly larger angles: $2\theta = 37.36° \pm 0.05°$ (d = 2.405Å ± 0.003Å) and $66.56° \pm 0.03°$ (d = 1.404Å ± 0.001Å). There are also two features peaking at $11.59° \pm 0.05°$ (d = 7.63Å ± 0.34Å) and $22.73° \pm 0.24°$ (d = 3.91Å ± 0.07Å) that are not observed in the pattern of the biogenic MnO_x (Fig. 1, upper panel). If they had been buried underneath the polysaccharide peak, then they should have appeared when the latter was removed by the background subtraction. These two peaks in the XRD pattern of our vernadite sample probably correspond to the (001) and (002) lines, respectively. This peak assignment contradicts the results of Manceau et al. (1992b) that synthetic vernadite should not have any basal reflections. It is possible that our sample has a structure closer to 2-D' phyllomanganate (a layered structure) than do those studied by Manceau et al. (1992b), and thus we observe the basal reflections.

We calculated the XRD pattern for two other crystal phases of manganese dioxide: Na-birnessite and todorokite. Synthetic Na-birnessite has a layer structure with the Na^+ ions located in the interlayer region. In contrast, todorokite has a tunnel structure. For todorokite the unit cell parameters given in the literature (ICSD 2000a) were used for the calculations. For Na-birnessite we derived a primitive triclinic unit cell (Fig. 2a) from the parameters of the monoclinic unit cell of synthetic Na-birnessite (Post and Veblen 1990; ICSD 2000b): a = 2.9535 Å, b = 2.8500 Å, c = 7.3360 Å, $\alpha = 90°$, $\beta = 101.52°$, $\gamma = 61.15°$; space group = P-1; Mn at 0, 0, ½; O at 0.248, 0.376, 0.367. Shifting the atom positions by ½ c in the cell ensures that all atoms of a single layer are contained in the cell, instead of the top half of one layer and the bottom half of another. Cations and water

molecules located in the interlayer region of Na-birnessite and inside the tunnels of todorokite were ignored in the calculations. XRD patterns were calculated for crystals containing between 4 and 2500 unit cells.

In the simulation of the XRD pattern of 3D Na-birnessite several diffraction lines occur between 35° and 38°, with the most intense peak at 36.7°. Similarly, there are several lines between 63° and 69 °. The calculated diffraction pattern of a 12a × 12b 2D (single layer) crystal has two asymmetric peaks at 36.6° and 64.0° (Fig. 2c). When the area is extended to 20a × 20b, the feature at the higher angle has a double peak. Upon further extension to 50a × 50b cells, both the low angle and high angle features have two partially resolved peaks. This is not observed in the experimental data. Similarly, the simulated XRD pattern of a crystal with two layers and an area of 12a × 12b unit cells already show substantial differences from the biogenic MnO_x XRD pattern, in particular a very strong peak at 10.7° with a amplitude ~5 times greater than the "single layer" peak at 36.6°. Based on these calculations the bacterial MnO_x is possibly composed of small single layers, containing about 144 manganese atoms (Fig. 2b and Fig.1, upper panel). A todorokite-like structure is unlikely, as even a crystal with only four unit cells is predicted to have diffraction peaks not observed in the experimental pattern.

X-ray absorption near-edge structure (XANES)

The Mn K-edge x-ray absorption spectrum of the freeze-dried bacterium is shown in Fig. 3. The low intensity pre-edge features observed in the energy region between 6539 and 6542 eV (Fig. 3, inset) result from the dipole-forbidden, but quadrupole-allowed, excitation of Mn(1s) electrons into unoccupied Mn(3d) orbitals. The energy of the main

white line peak (6560.5 ± 0.1 eV) of the bacterial sample is close to the energies of the white line peaks in the MnO_2 compounds: birnessite, todorokite, vernadite and pyrolusite (Table 2). The spectral features in the XANES region of the bacterium sample are very similar to those of Na-birnessite and δ-MnO_2 (Fig. 4a). There is, however, a feature observed in neither reference compound: a low energy shoulder that precedes the white line peak and peaks at about 6552 eV, close to the white line peak of $MnSO_4 \cdot H_2O$ (Table 2). These observations suggest that three different oxidation states of manganese (Mn^{2+}, Mn^{3+}, and Mn^{4+}) are present in the sample containing the bacteria.

To estimate the amount of Mn^{2+} present, linear combinations of the normalized XANES spectra of $MnSO_4 \cdot H_2O$ and Na-birnessite, todorokite or vernadite were fitted to the normalized XANES spectra of the bacterial MnO_x using the least squares method. The best result was obtained for a linear combination of the XANES spectra of $MnSO_4 \cdot H_2O$ and Na-birnessite (Fig. 4b). The five bacteria samples studied all had different amounts of Mn^{2+}, ranging from 17% ± 3% to 43% ± 5% with an average value of 29% ± 10% of the total Mn atoms present in the sample. The high concentration of Mn^{2+} and its variation arise from different amounts of $MnSO_4$ remaining after the washing process used in the preparation of the bacterial samples. Our wash process, designed to minimize cell destruction, may not have been entirely effective in removing unreacted Mn^{2+}. There is also another source of Mn^{2+}: it might be bound to the biogenic MnO_x. Prior to their iodometric titration, Adams and Ghiorse (1988) washed their samples in a 10 mM $CuSO_4$ solution to remove any bound Mn^{2+}. Our wash process did not involve this step, and thus our samples are expected to still contain Mn^{2+} from this source.

The other component in the best linear fit to the bacterial MnO_x, Na-birnessite, is a manganese oxide mineral containing two oxidation states of manganese: Mn^{3+} and Mn^{4+}. The idealized formula of Na- birnessite, $Na_4Mn_{14}O_{27}$, gives an average oxidation state of 3.57 for Mn. Synthetic Na-birnessite has an average oxidation state of 3.66 (Table 2), with Mn^{3+} and Mn^{4+} in the layers and a small amount of Mn^{2+} in the interlayer regions (Drits et al. 1997; Silvester et al. 1997). Other forms of birnessite have different oxidation states. In hexagonal birnessite, for example, the average oxidation state is 3.88 (Drits et al. 1997; Silvester et al. 1997). Our finding, the presence of the mixed $Mn(III)Mn(IV)O_x$ mineral birnessite with possibly some Mn(II) bound to it, agrees with Adams and Ghiorse (1988) and Nelson et al. (1999a) that the bacterial MnO_x contains multiple oxidation states of manganese. The average oxidation state of Mn in the bacterial MnO_x determined by iodometric titration, 3.62 (Adams and Ghiorse 1988), is in good agreement with the values expected for Na-birnessite.

XRD probes the long-range order and crystal structure of the sample, while XANES provides information about the oxidation state and the local chemical environment of the Mn atom. Our XANES results indicate that the local short-range structure of the bacterial MnO_x is akin to that of Na-birnessite and our XRD data suggests that the material is composed of small 2-D crystals, possibly with single layers similar to those occurring in Na-birnessite. To obtain more quantitative information, EXAFS was used to determine the atomic positions in the first and second coordination shells.

Extended x-ray absorption fine structure (EXAFS)

The EXAFS interference function, $\chi(k)$ (Fig. 5a), was extracted from the Mn K-edge fluorescence spectrum using standard procedures (Crozier and Seary 1981). This involved polynomial background subtraction and normalization to the edge jump to put the data on a per atom basis. A correction for the atomic absorption of Mn was applied using the McMaster coefficients (McMaster et al. 1969) for the Mn K-edge. The absorption edge energy E_o was set equal to the first inflection point in the spectrum. Fig. 5b shows the $k^3\chi(k)$ that was used in calculating the Fourier transform.

The magnitudes of the Fourier transform of $k^3\chi(k)$ are shown in Fig. 6 for the freeze-dried bacterial sample and several Mn compounds. The transforms were calculated over the range 2.03 to 11.85 $Å^{-1}$ using a 30% Gaussian window. The transform of the bacterial MnO_x has two dominant peaks, as emphasized by the vertical lines, which coincide with those in Na-birnessite. Their positions are determined by the first neighbor Mn-O and second neighbor Mn-Mn (edge-sharing octahedra) interatomic distances, respectively. In vernadite the Mn-O peak occurs at a smaller value than in the bacteria sample, suggesting a shorter average Mn-O bond distance in vernadite. Recalling that the XRD of the vernadite is also different from that of the biogenic MnO_x, vernadite was thus eliminated as a model for the bacteria. The shoulder peak at 3.1 Å in the transform of the bacterial MnO_x is not observed for Na-birnessite (Fig. 7), but it was observed by Silvester et al. (1997) for hexagonal birnessite. The position of the shoulder corresponds to the Mn-Mn distance for corner sharing octahedra, indicating that the biogenic MnO_x has a defect layer structure with out-of-plane corner-sharing octahedra, as shown in Fig. 8.

To determine the actual structural parameters of the bacterial MnO_x, theoretical EXAFS spectra were obtained using the computer program FEFF 7.0 (Ankudinov 1996; Zabinsky et al. 1995) to generate electron scattering amplitudes $F_j(k)$ and phase shifts $\delta_j(k)$ for each scattering path. The experimental data were fit in r-space using the program WINXAS (Ressler 1997).

To evaluate the fitting procedure and establish a value for S_0^2 and the mean square relative displacements, σ^2, for the MnO_2 (Na-birnessite) and $MnSO_4$ systems, fits were performed on EXAFS spectra of commercial $MnSO_4 \cdot H_2O$ powder and the synthetic Na-birnessite mineral. Simplified crystal structures were used in the initial calculation of the theoretical $\chi(k)$ to minimize the number of fit parameters. This simplification involved adjustments to the unit cell parameters and to the atom positions within the unit cell, to make all first coordination shell Mn-O bond lengths the same value in a given crystal structure and to limit the second coordination shell Mn-Mn distance in Na-birnessite to one value, 2.919 Å for edge-sharing MnO_6 octahedra. This simplification also recognizes that the spatial resolution of EXAFS is limited by the finite range of the data: with k_{min} = 2 $Å^{-1}$ and k_{max} = 12 $Å^{-1}$, the minimum resolvable separation of two bond lengths is ≈0.16 Å. The coordination numbers and interatomic distances for single scattering paths are listed in Table 3 for both the real and the simplified crystal structures.

For the Na-birnessite, the r-space fit spanned the region from 0.86 to 4.14 Å. Four single (SS) and three triangular multiple (MS) scattering paths were used. The shift in the edge energy (ΔE_0) was limited to one value, and the coordination numbers (N_j) for all paths were fixed. The path distance (R_j) and the σ_j^2 of each MS path were correlated to those of the corresponding SS paths. The remaining parameters (S_0^2, ΔE_0 and R_j and σ_j^2

for the SS paths) were allowed to vary freely. The fit results for Na-birnessite (Fig. 7a) are indistinguishable from the data over the fit range. The theoretical $\chi(k)$ calculated by FEFF based on the simplified structures, and the fit procedure used, provide a good description of the local environment of the manganese atoms. The SS bond distances determined by the FEFF fit for Na-birnessite are listed in Table 3. The bond distances for the first two coordination shells are slightly less, but within experimental error of the expected values. The distances for the third and fourth shell are overestimated. The EXAFS of $MnSO_4 \cdot H_2O$ was used to determine a single Mn^{2+}-O bond length that could be used as a starting point to the fitting of the sample containing the bacteria. One SS path was sufficient to fit the first peak in the Fourier transform of $k^3\chi(k)$ (Fig.7b).

The starting point for the FEFF fit of the bacterial MnO_x data was the simplified Na-birnessite structure with a few additional paths resulting from the presence of Mn^{2+} and the possible presence of out-of-plane MnO_6 octahedra. To generate these paths, the FEFF calculation was performed twice: the first time with all atoms in the positions for the simplified birnessite structure, and the second time with the six oxygen atoms in the first shell shifted so that the Mn-O distance for that shell was 2.196 Å, which is the Mn-O distance of the simplified $MnSO_4$ structure, instead of 1.935 Å, which is the Mn-O distance of the simplified birnessite. To obtain the scattering paths resulting from an out-of-plane MnO_6 unit, the FEFF calculation was also done with one of the six second-shell Mn atoms moved out of the plane so that the Mn-Mn distance equalled 3.438 Å, which is the Mn-Mn distance for corner-shared MnO_6 octahedra (Table 4).

The FEFF fit was limited to the two main peaks, the shoulder at 3.1 Å, and the minimum at 4.12 Å in the Fourier transform of the bacterial MnO_x $k^3\chi(k)$ (Fig. 7c),

covering a range from 0.57 to 4.12 Å. The value of S_0^2 was fixed at the value determined for the synthetic Na-birnessite. Similarly, the values for σ_j^2 of each SS path were fixed: for the first shell oxygen atoms at 2.196 Å the value determined for $MnSO_4 \cdot H_2O$ was used and for all other SS paths the values determined for the synthetic Na-birnessite were used. The shift in the edge energy (ΔE_0) was limited to one value. The coordination chemistry of manganese requires that all manganese atoms are bonded to six oxygen atoms. Thus, the overall coordination number for the first shell (first neighbor Mn-O) was fixed at six. The XANES results implied that on average 29% of the manganese atoms present are in the form of Mn^{2+}, whereas the rest are in the form of Mn^{3+} or Mn^{4+}.This means that ~29% of first-shell scattering distances have R_1^{2+} = 2.196 Å (Mn^{2+}-O), while the rest have R_1^{4+} = 1.935 Å (Mn^{4+}-O). EXAFS spectroscopy probes the local chemical environment of all manganese atoms, and the resulting overall interference function $\chi(k)$ is the sum of the individual $\chi_i(k)$'s from each manganese atom. The coordination number N_j of each shell j obtained from $\chi(k)$ represents an average value of the corresponding coordination numbers for each manganese atom. Hence, there are, on average, N_1^{2+} = 0.29 × 6 = 1.74 oxygen atoms at R_1^{2+} and N_1^{4+} = 0.71 × 6 = 4.26 oxygen atoms at R_1^{4+} (Table 4). These two values of N_1 were correlated so that their sum always equalled 6.

SS paths with larger distances were included. The presence of corner-shared MnO_6 octahedra was considered, so the starting values for the coordination numbers of Mn-Mn scattering paths were set at 5 and 1 for Mn-Mn(edge) and Mn-Mn(corner), respectively, instead of the theoretical values of 6 and 0 for Na-birnessite. In the Na-birnessite structure there is a Mn-O distance (3.502 Å) with coordination N_3 involving edge - sharing octahedra that is close to the Mn-Mn (corner) distance (3.438 Å). It was included

in the calculation. To extend the r-space fit range, we also included a second Mn-O edge distance at 4.559 Å with coordination N_4. The values of N_j for the third and fourth shell Mn-O single scattering paths of Na-birnessite were fixed at the theoretical values of six and twelve for the Na-birnessite structure, respectively. The presence of a vacancy (out-of-plane octahedron) will not alter N_3 and N_4 since it is created by the removal of an in-plane Mn and not oxygen atoms.

MS paths were included in the fitting. N_j, R_j and σ_j^2 of the MS paths were calculated from the values of the corresponding SS paths at each iteration. The remaining parameters - R_j for all SS paths, ΔE_0 and N_2 - were unrestricted The paths used for the FEFF fit and the initial values for N_j, R_j are given in Table 4. The fit results (shown in Fig. 7c for a typical bacteria sample) are in good agreement with experiment. The final values for the SS paths are listed in Table 4 as an average of the five bacteria samples.

These calculations have been done with and without the inclusion of the MS paths. Their inclusion in the FEFF fit has minimal effect on the coordination numbers and radial distances of the first two shells (first shell: Mn^{4+}-O and Mn^{2+}-O; second shell: Mn-Mn(edge) and Mn-Mn(corner)). However, the inclusion of the MS paths does increase the Mn-O distance obtained for the third shell by 0.12 Å from 3.59 Å to 3.71 Å. This is a significant change. Of the three triangular paths listed in Table 4, the path with the largest effective radial length (3.869 Å Mn^{4+}-O-O) makes the largest contribution to the Fourier transform of the bacterial $\chi(k)$. The atoms involved in the MS are in the same octahedron and lie on the straight line joining corner oxygens to the center Mn. The photoelectron travels from the Mn absorber to a corner O, is backscattered through 180 degrees, travels past the Mn without scattering to the O at the other end of the line where it is

backscattered through 180 degrees and returns to the center Mn. The phase shift is large, about -0.7 Å, so the magnitude of the Fourier transform of the MS triangular path peaks at 3.13 Å. It interferes with the SS contribution of the Mn-O(edge) who's transform magnitude peaks in the same region and actually has a smaller amplitude than does the MS peak. MS cannot be neglected, particularly since the atoms involved in the MS are in the same octahedron. The three Mn^{4+}-O-O triangular paths involve atoms in the same octahedron and the fit distances are stable, not changing significantly under different fit criteria. The fit results for the MS paths are not included in Table 4, as they do not contribute significant structural information.

Some differences were observed among the transforms of the five bacteria samples regarding the height, width, and shape of the first peak (Fig. 7c, only one of the samples is shown). These can be attributed to the different concentration of Mn^{2+} in each of the five samples, as the Mn-O bond length is ≈ 0.3 Å longer for Mn^{2+} than for Mn^{4+} (Table 4). The percent of Mn^{2+} in each sample can be estimated from the FEFF fit results by dividing N_I of the second SS path by 6, the coordination number of the first shell. The compositions thus obtained agreed within 6 % with those obtained by the analysis of the XANES spectra.

The bond lengths obtained for the first and second coordination shells are in good agreement for the five samples, giving average values of 1.90 Å ± 0.02Å, 2.21Å ± 0.02 Å, 2.88 Å ± 0.025 Å and 3.50 ± 0.025 Å for the Mn^{4+}-O, Mn^{2+}-O, Mn-Mn (edge-sharing octahedra), and Mn-Mn (corner-sharing octahedra) interatomic distances, respectively. The coordination numbers of 4.3 ± 0.5 and 1.3 ± 0.4 obtained for edge-sharing and corner-sharing MnO_6 octahedra, respectively, are significantly different from the

theoretical values of six and zero for the Na-birnessite structure. Our values are comparable to those obtained by Silvester et al. (1997) for hexagonal birnessite, which has MnO_6 octahedra in corner-sharing positions above layer vacancies. The Mn-Mn (corner-sharing octahedra) interatomic distance of 3.50 Å ± 0.025 Å also suggests that the Mn atoms above the layer plane are probably a mixture of Mn^{2+} and Mn^{3+} (Sylvester et al. 1997).

An estimation of the number of layer vacancies can be made from the Mn-Mn coordination numbers for edge-sharing and corner-sharing octahedra. The coordination numbers obtained from $\chi(k)$ represent an average value of the corresponding coordination numbers for each manganese atom. However, for very small crystals or crystals with many layer vacancies, the atoms with less than six edge-sharing neighbors (those near the crystal edge or next to a vacancy) represent a significant portion of the Mn atoms present, so the lower coordination numbers decrease the value of N_2 (edge) significantly. Similarly, the number and position of vacancy sites affect the value of N_2 (corner). To estimate the number of vacancies three assumptions were made: the vacancies are restricted to the Mn^{3+} rich rows, all vacancy sites are occupied by corner-sharing MnO_6 octahedra, and the crystal size is 12 × 12 two-dimensional unit cells (144 Mn atoms). The crystal size effectively reduces the coordination number for edge-sharing octahedra to a maximum of 5.4, if no vacancies were present. Thirteen vacancies would reduce the coordination number for edge-sharing octahedra to 4.3, and give a coordination number of 1.1 for the corner-sharing octahedra. A coordination number of 1.3 for the corner-sharing octahedra is obtained when there are between 16 and 19 vacancy sites. The number of vacancies required is dependent upon their location within

the layer. Taking the experimental error of the coordination numbers into account, the number of vacancy sites could range between 10 and 25. On average a layer is expected to have between 15 and 20 vacancies. The structure shown in Fig. 8 has 19 layer vacancy sites, resulting in coordination numbers of 4.1 and 1.3 for the edge-sharing and corner-sharing octahedra, respectively.

It should be noted that all measurements were done on freeze-dried samples. The drying process could have caused changes to the oxidation state of Mn in the sample, and it might have changed the crystal structure of the bacterial MnO_x. For example, drying of the layered MnO_x mineral buserite results in the formation of birnessite, as evidenced by a reduction in the interlayer spacing from 10 Å to 7 Å (Silvester et al., 1997). As this shows, the possible effects of the drying process on the bacterial MnO_x need to be investigated.

An EXAFS study of two types of synthetic birnessite (Silvester et al. 1997) showed that the structure of this mineral is dependent upon the pH of its environment. Na-rich birnessite is prepared in a basic solution (pH $\geq$ 9). It has primarily a layered structure with few vacancies and little evidence for corner-sharing MnO_6 octahedra. Upon suspension in an acidic solution (pH $\leq$ 5), however, layer vacancy sites are formed and MnO_6 octahedra adsorb at these sites in corner-sharing positions.

Based upon the various studies of the Mn^{2+} oxidation by *L. discophora* (Adams and Ghiorse 1987, 1988; Boogerd and De Vrind 1987; Emerson and Ghiorse 1992; Nelson et al. 1999a), Silvester et al.'s (1997) observations about the structure of birnessite and our experimental results, we propose the following mechanism as a possible way for the formation of the bacterial MnO_x: Aqueous Mn^{2+} is oxidized by the bacteria, and the

reaction products Mn^{3+} and Mn^{4+} form a birnessite-like single layer on the polysaccharide sheath. Layer vacancies are created through the disproportionation reaction (Sylvester et al. 1997) $Mn^{3+}_{layer} + Mn^{3+}_{layer} \rightarrow Mn^{4+}_{layer} + Mn^{2+}_{aq}$, which involves adjacent Mn atoms in the same layer, the migration of Mn^{3+}_{layer} from the layer into corner-shared $Mn^{3+}O_6$ positions above vacancy sites, and disrupted crystal growth. The bacterial MnO_x is produced in a slightly acidic environment (pH = 6 - 7), so Mn^{2+} can adsorb above layer vacancy sites to form corner-sharing octahedra. The resulting crystals are small and without periodicity in the c direction. The XRD pattern suggests that the average crystallite size has an area the order of 36 Å × 36 Å (12 × 12 two-dimensional unit cells). A sketch of this structural model of the bacterial MnO_x is shown in Fig. 8. Further studies are required to clarify the reaction mechanism.

In this model, Pb^{2+}_{aq} and other aqueous trace metal (TM) ions would be expected to adsorb above layer vacancy sites to form $(TM)O_6$ octahedra that share corners with the MnO_6 octahedra in the layer, competing with Mn^{2+}_{aq} and possibly replacing adsorbed MnO_6 octahedra through ion exchange or redox reactions. An EXAFS study on Pb-exchanged Na-birnessite (Manceau et al., 2002) has shown that Pb^{2+} ions adsorb in the interlayer region in corner-shared positions.

ACKNOWLEDGEMENTS

This project received financial support from operating grants by Health Canada and Environment Canada as part of the Toxic Substances Research Initiative (TSRI, Project #4), and the Natural Sciences and Engineering Research Council Canada (NSERC). A major facilities access grant was also provided by NSERC. The United States Department of Energy, Basic Energy Sciences, Office of Science supported experiments performed at the PNC-CAT beamlines, APS, ANL under Contract numbers W-31-109-Eng-38 (APS) and DE-FG03-97ER45628 (PNC-CAT).

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Table 1. Composition of the growth medium for *Leptothrix discophora* SP-6

Substance	Amount per L
Peptone	1.0 g
Yeast	0.5 g
Glucose	1.0 g
$MgSO_4$	0.6 g
$CaCl_2$	0.07 g
Hepes Buffer (pH: 7)	2.38 g
$FeSO_4$	10 μmol
$MnSO_4$	1 mmol

Table 2. The Experimental Mn K-edge Absorption Energies

Name	Oxidation state of Mn	Edge Energy (eV)*	White Line Peak Energy (eV)*
Mn metal foil	0	6537. 7†	
$MnSO_4 \cdot H_2O$	2	6545.9	6551.6
MnO (manganosite)	2	6543.8	6553.8
γ-MnO(OH) (manganite)	3	6546.0	6557.6
Mn_3O_4 (hausmannite)	2.67	6545.9	6558.0
Mn_2O_3 (bixbyite)	3	6546.9	6558.9
MnO_2 (birnessite)	3.66‡	6548.0	6559.7
MnO_2 (todorokite)	4	6549.5	6560.9
δ-MnO_2 (vernadite)	4	6549.9	6561.1
MnO_2 (pyrolusite)	4	6551.4	6559.0
Leptothrix discophora SP-6 Average of 5 samples		6547.9±0.6	6560.5±0.1

* uncertainty: ±0.5 eV.

† calibration standard (Kraft et al. 1996)

‡ average value based on the idealized chemical formula $Na^{+}_{0.33}Mn^{2+}_{0.055}(Mn^{4+}_{0.722}Mn^{3+}_{0.222}Ø_{0.055}O_{\sim 2})$ where Ø is a vacancy (Drits et al. 1997; Silvester et al. 1997).

Table 3. The first four coordination shells about Mn in Na-birnessite

	Real Structure*	Simplified Structure†	FEFF Fit‡
Mn-O	N = 4, R = 1.9183 Å N = 2, R = 1.9675 Å	N = 6, R = 1.935 Å	N = 6, R = 1.90 ± 0.02 Å
Mn-Mn	N = 2, R = 2.8500 Å N = 4, R = 2.9535 Å	N = 6, R = 2.919 Å	N = 6, R = 2.90 ± 0.025 Å
Mn-O	N = 4, R = 3.4632 Å N = 2, R = 3.5794 Å	N = 6, R = 3.502 Å	N = 6, R = 3.60 ± 0.20 Å
Mn-O	N = 4, R = 4.4637 Å N = 4, R = 4.5754 Å N = 4, R = 4.6378 Å	N = 12, R = 4.559 Å	N = 6, R = 4.78 ± 0.20 Å

* These distances are based upon the unit cell and atom positions determined by Post and Veblen (1990) for Na-birnessite.

† The distances for birnessite are based upon a primitive unit cell with slightly modified parameters from those of Post and Veblen (1990): a = b = 2.9190 Å, c = 7.3360 Å, $\alpha = 90.0°$, $\beta = 101.39°$, $\gamma = 60.0°$; space group: P-1; atom positions: Mn at 0.000, 0.000, 0.500 and O at 0.75470, -0.37735, 0.63303.

‡ These are the results of fitting our experimental EXAFS data with FEFF calculations based upon the simplified structures.

Table 4. The initial parameters and final results for the FEFF fit of the bacterial MnO_x.

Paths and initial parameters used in the FEFF fit of the bacterial MnO_x.

	N_j	R_j (Å)		N_j	R_j (Å)
$S_0^2 = 0.66$					
Mn^{4+}-O	4.26*	1.935	Mn-Mn (corner)	1	3.438
Mn^{2+}-O	1.74*	2.196	Mn-O (edge)†	6	3.502
Mn-Mn (edge)	5	2.919	Mn^{4+}-O-O	6	3.869
Mn^{4+}-O-O	12	3.205	Mn-O (edge)†	12	4.559
Mn^{4+}-O-O	12	3.394			

Fit results for the SS paths, average values for five bacteria samples

	N_j	R_j (Å)
Mn^{4+}-O	$N_1 = 4.1 \pm 0.6$‡	$R_1 = 1.90 \pm 0.02$
Mn^{2+}-O	$N_1 = 1.9 \pm 0.6$‡	$R_1 = 2.21 \pm 0.02$
Mn-Mn (edge)	$N_2 = 4.3 \pm 0.5$	$R_2 = 2.88 \pm 0.025$
Mn-Mn (corner)	$N_2 = 1.3 \pm 0.4$	$R_2 = 3.50 \pm 0.025$
Mn-O (edge)§	$N_3 = 6$ (fixed)	$R_3 = 3.71 \pm 0.20$
Mn-O (edge)§	$N_4 = 12$ (fixed)	$R_4 = 4.77 \pm 0.20$

* From the XANES results, about 71% of the Mn atoms are Mn^{4+} and 29% are Mn^{2+}. Since all Mn atoms have six first-shell neighbors, there are, on average, $0.71 \times 6 = 4.26$ atoms at 1.935 Å and $0.29 \times 6 = 1.74$ atoms at 2.196 Å.

† (edge) refers to the fact that this oxygen is part of a neighboring edge-sharing MnO_6 octahedron within the layer plane.

‡ This is the average coordination number of this path, obtained from the combined signal of all Mn atoms. Using the fact that the first shell coordination number is six for both Mn^{4+} and Mn^{2+}, these average coordination numbers correspond to 68% ± 10% Mn^{4+} and 32% ± 10% Mn^{2+} in the sample.

§ (edge) refers to the fact that this oxygen is part of a neighboring edge-sharing Mn octahedron within the layer plane.

FIGURE CAPTIONS

Figure 1. The intensity of the X-ray diffraction pattern of *Leptothrix discophora* SP-6, lower panel: (a) with and (b) without the MnO_x sheath. The XRD pattern of the bacteria with Mn has been offset by 12 counts/sec for clarity. Upper panel: The diffraction peaks of the MnO_x sheath (top) with the background removed as described in the text. At the bottom is the experimental diffraction pattern for δ-MnO_2. In the middle is the calculated diffraction pattern for one of the individual layers of birnessite. The single layer contains 144 manganese and 288 oxygen atoms [12a×12b two dimensional primitive unit cells].

Figure 2. (a) The crystal structure of Na-birnessite. The layers are composed of edge-sharing $Mn(III/IV)O_6$ octahedra, and Na^+ ions are located in the interlayer region (only seven atoms are shown to indicate the position of Na^+ in the crystal). (b) A single layer of the Na-birnessite crystal with 144 manganese atoms (12a × 12b two dimensional unit cells) used to calculate the XRD pattern in Fig. 1, upper panel. The triclinic unit cell used for the XRD calculations is shown. (c) Simulated XRD patterns produced by single layers having differing numbers of two-dimensional unit cells and by a crystal of Na-birnessite having two layers

Figure 3. The Mn K-edge fluorescence spectrum of *Leptothrix discophora* SP-6. The pre-edge peaks are shown in the inset.

Figure 4. (a) The Mn K-edge XANES spectra of the bacterium (solid), δ-MnO_2 (dash-dotted), the synthetic Na-birnessite (dashed) and $MnSO_4 \cdot H_2O$ (dotted). (b) The Mn K-edge XANES spectrum of the bacterium (solid) and the best least-squares fit (crosses) using a linear combination of the spectra of Na-birnessite (dashed) and $MnSO_4 \cdot H_2O$ (dotted).

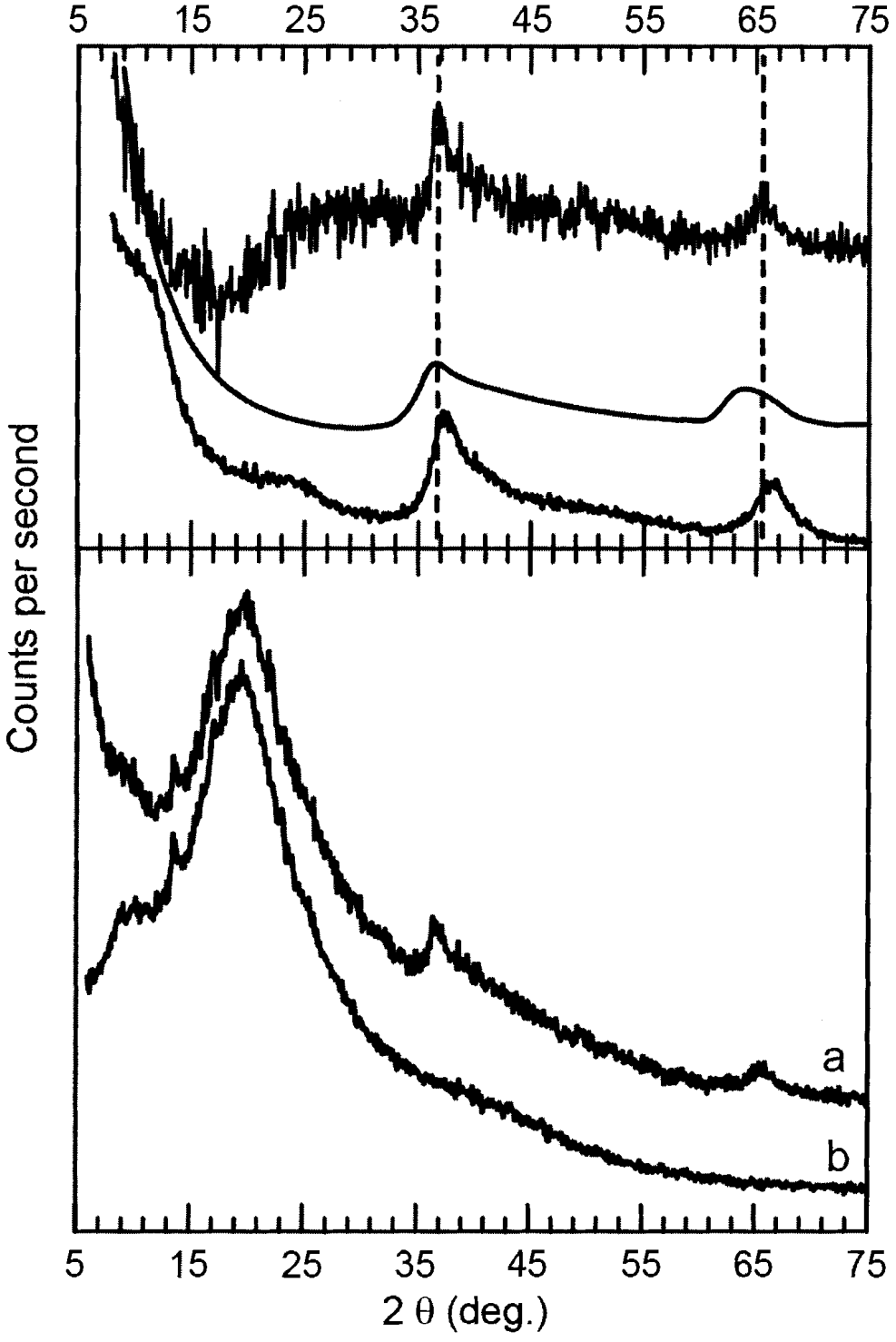
Figure 5. For *Leptothrix discophora* SP-6, (a) The Mn K-edge EXAFS interference function $\chi(k)$ and (b) $k^3\chi(k)$.

Figure 6. An overlay of the magnitudes of the Fourier transform of the Mn K-edge $k^3\chi(k)$ of bacteria sample (solid), synthetic Na-birnessite (dashed), δ-MnO_2 (dash-dotted), and $MnSO_4 \cdot H_2O$ (dotted). The data were transformed over the range 2.03 to 11.85 $Å^{-1}$ with a 30% Gaussian window function. The electron scattering process introduces a phase shift that causes the peaks in the transform to appear at radial distances that are shorter than the actual bond lengths.

Figure 7. The magnitude and imaginary component of the Fourier transform of the Mn K-edge $k^3\chi(k)$ of (a) Na-birnessite, (b) $MnSO_4 \cdot H_2O$ and (c) the biogenic MnO_x . The dotted line represents the FEFF generated function fitted to the experimental data (solid line). The transform range and window function are the same as in Fig. 6.

Figure 8. A sketch of the proposed structure of the biogenic MnO_x. The combination of EXAFS and XRD indicates a single layer with a local structure similar to the layers of birnessite and containing vacancies. But the structure is not definitive: Variation both in the lateral dimensions of the layer and in the number of vacancies is consistent with the data analysis.

Figure 1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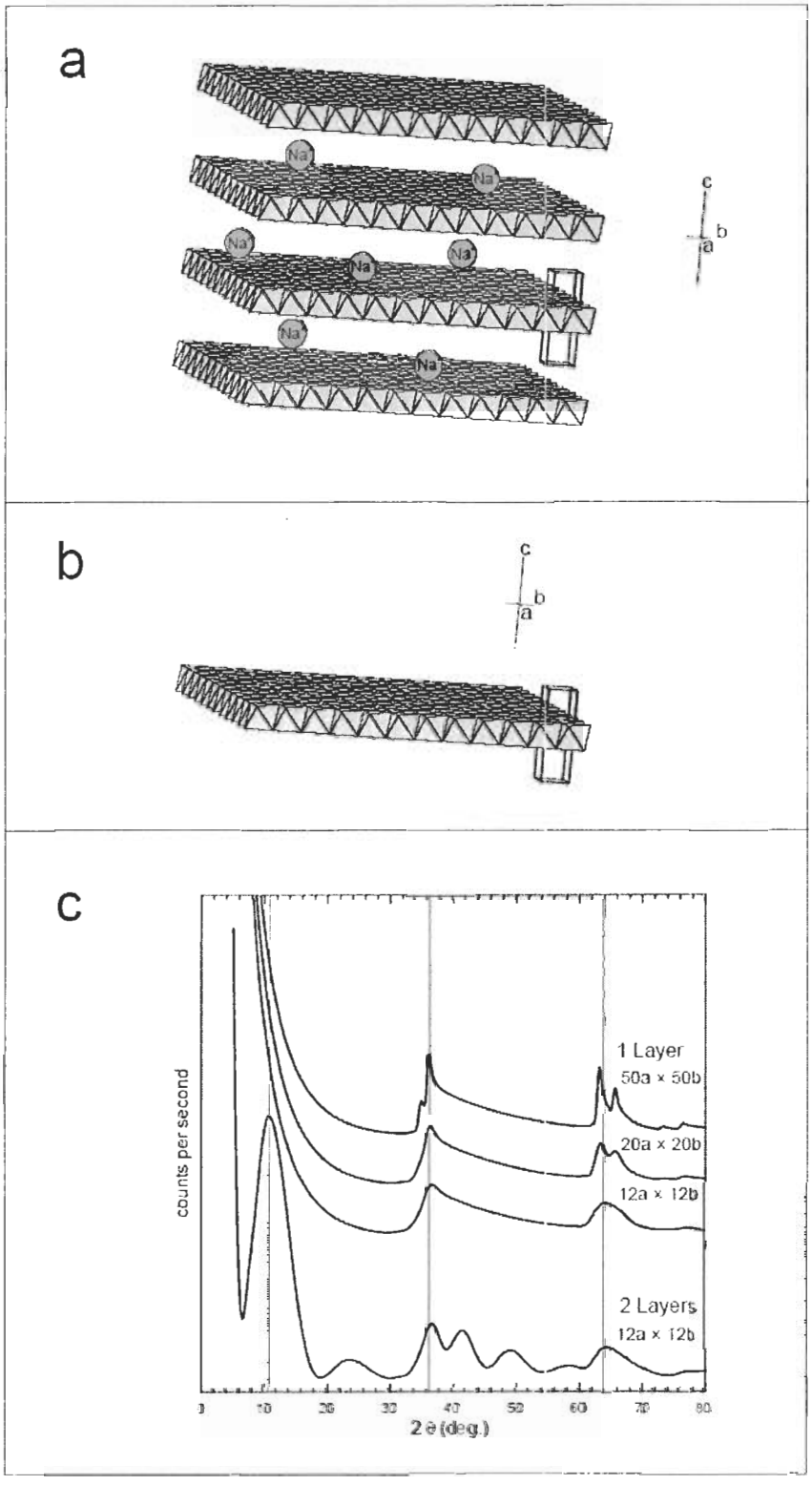
2 θ (deg.)

Figure 2


a
Na
c
b
a
b
c
b
a
c
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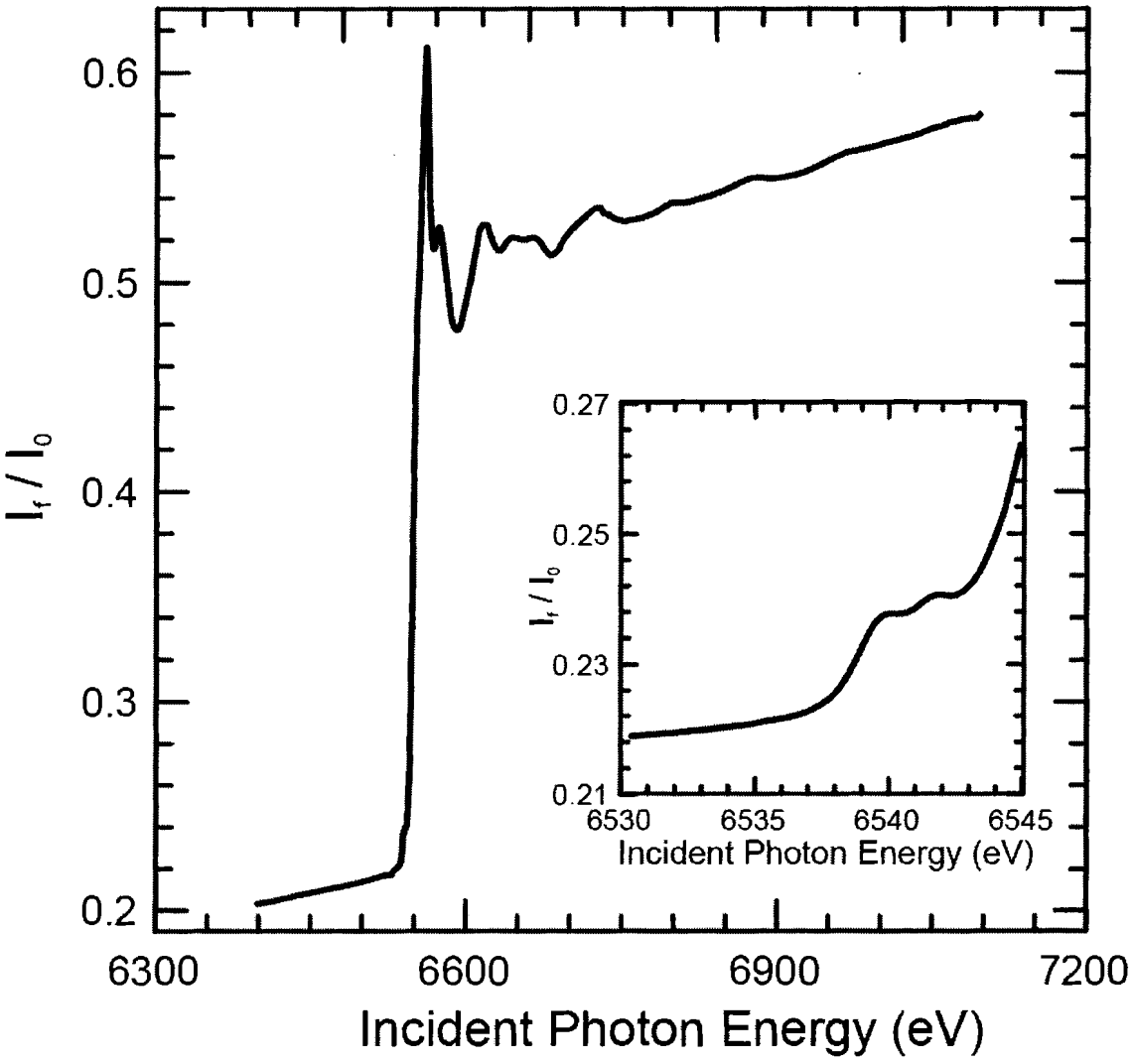
2 Layers
12a × 12b
2 è (deg.)

Figure 3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0.21
I_f / I_0
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6535
6540
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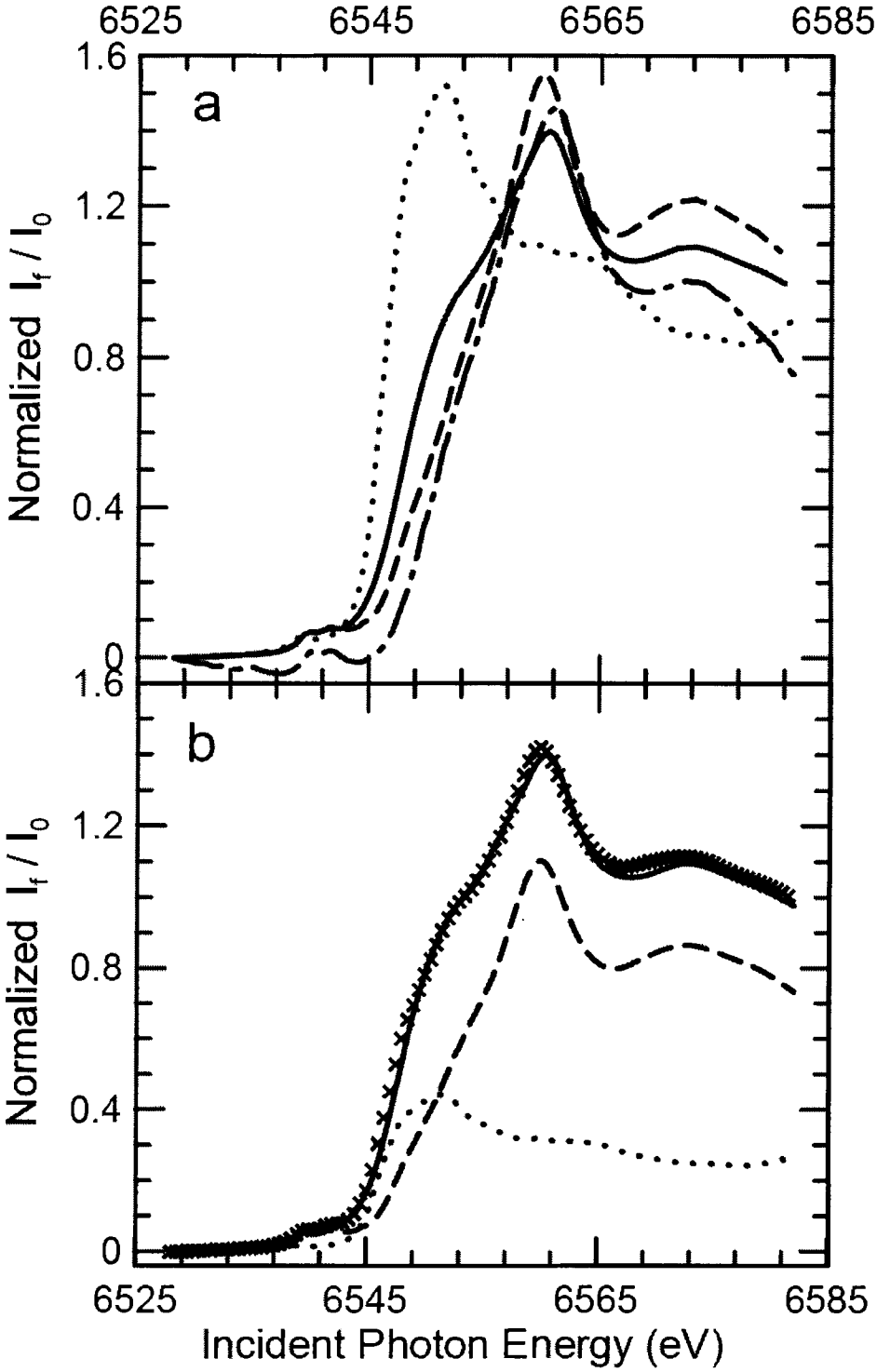
Incident Photon Energy (eV)

Figure 4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b
Normalized I_f / I_0
Incident Photon Energy (eV)

Figure 5

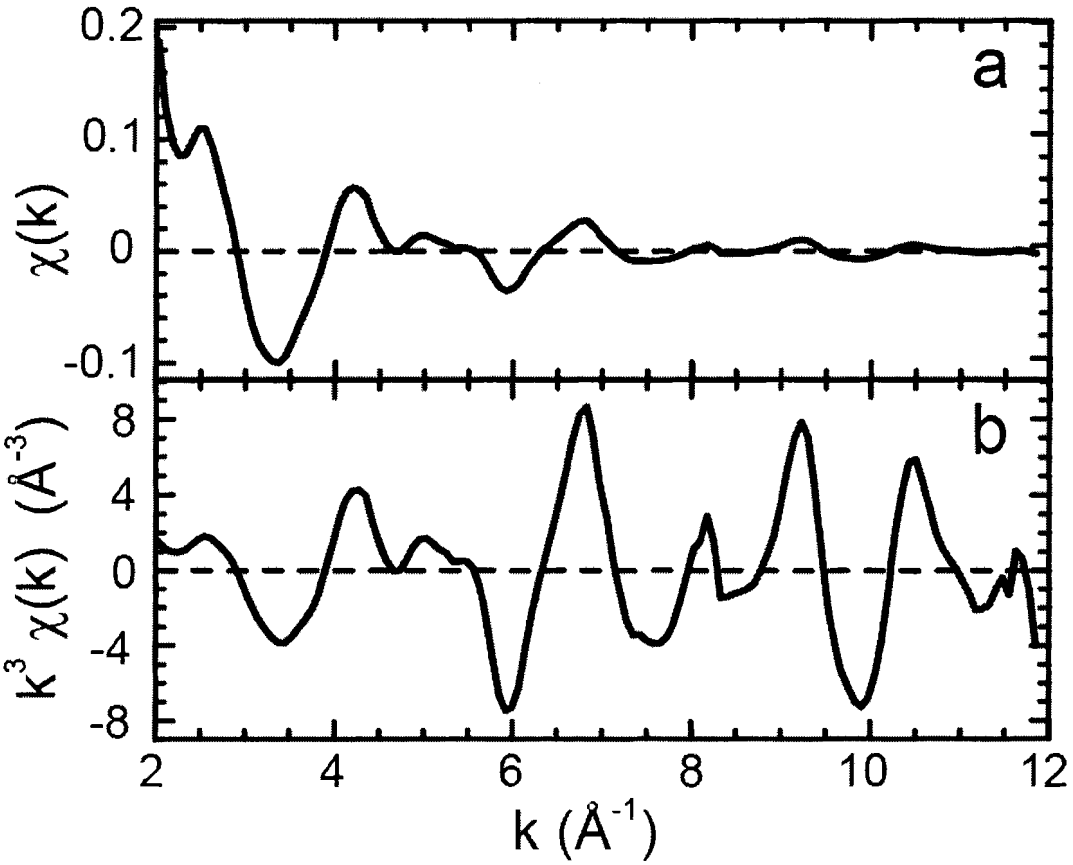
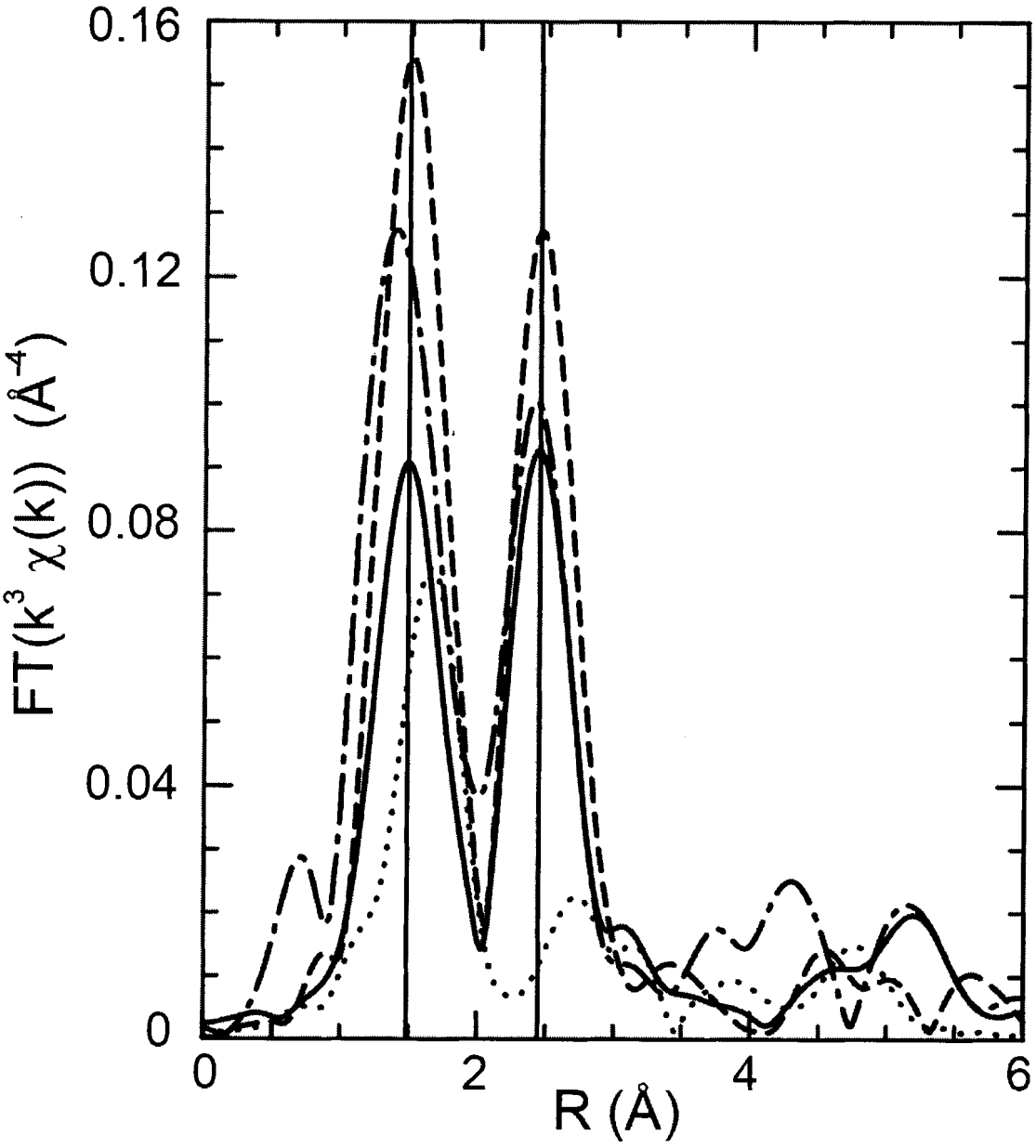


Figure 6



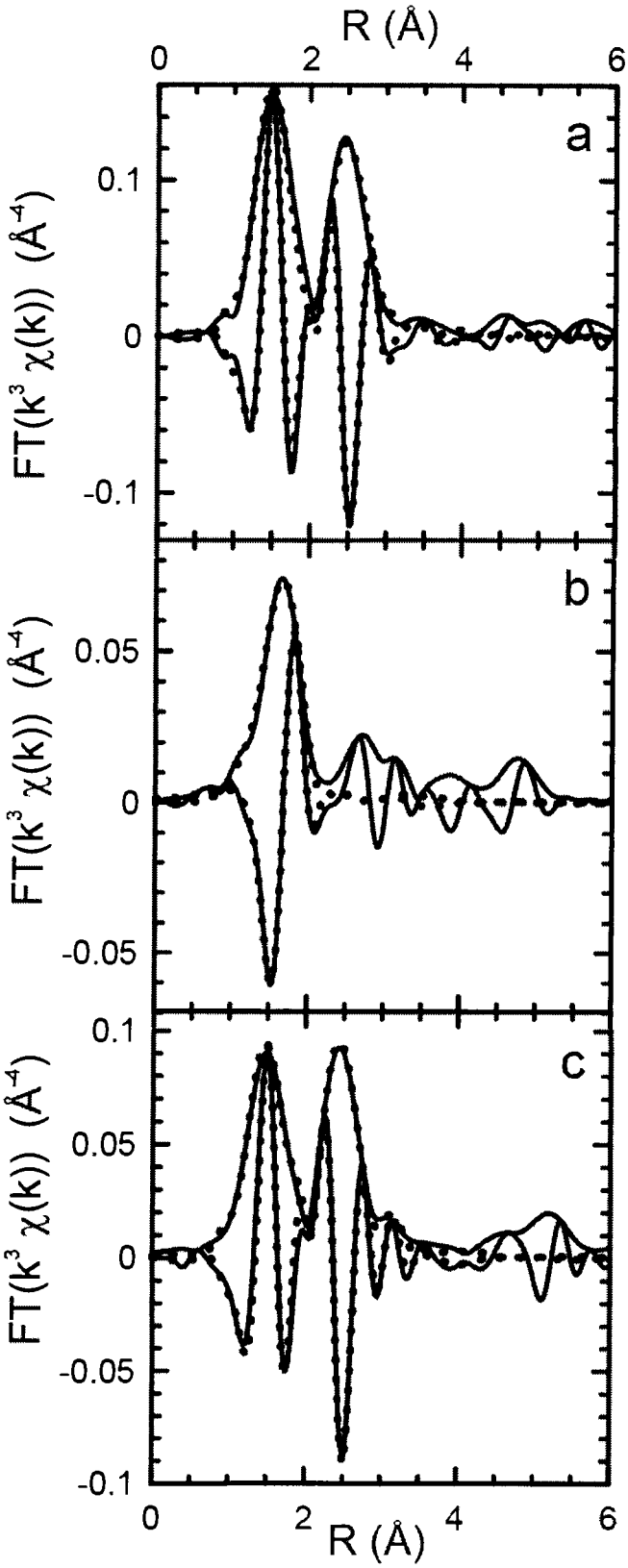


Figure 7

Figure 8