Development and Use of a *mer-lux* Bioreporter for the Measurement and Characterization of Bioavailable Hg(II) in Defined Media and Aquatic Environmental Samples

by Karen Jocelyne Scott

A thesis submitted to the faculty of Graduate Studies
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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FACULTY OF GRADUATE STUDIES

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DEVELOPMENT AND USE OF A *MER-LUX* BIOREPORTER FOR THE MEASUREMENT AND CHARACTERIZATION OF BIOAVAILABLE Hg (II) IN DEFINED MEDIA AND AQUATIC ENVIRONMENTAL SAMPLES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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Dedication

One of the highlights of my doctoral program was participating in the 6th International Conference on Mercury as a Global Pollutant held in Minamata, Japan. I feel privileged not only to have had the opportunity to visit Minamata, but also to have heard the live testimonies of victims of Minamata Disease and to have experienced the generosity and kindness of the residents of Minamata. I would like to dedicate this thesis to the people of Minamata, Japan for their spirit and courage in the face of an environmental, economic, and social tragedy.

Abstract

Mercury (Hg) has gained the title of toxic global pollutant due to its world wide distribution via atmospheric processes and subsequent deposition as inorganic divalent Hg (Hg(II)) to terrestrial and aquatic ecosystems, including in remote areas. Hg(II) is the substrate for two important microbially mediated processes of the Hg cycle, both of which are intracellular, thus requiring Hg(II) to enter the cell or be "bioavailable". They are reduction of Hg(II) to elemental Hg (Hg^0) and the methylation of Hg(II) to the potent neurotoxin methyl Hg (CH_3Hg^+) or dimethyl Hg ($(CH_3)_2Hg$). Identifying the sources of bioavailable Hg and understanding the factors, both chemical and biological, that control the concentration and uptake of Hg(II) is important in understanding and modeling the microbial processes contributing to the biogeochemical cycling of Hg in aquatic ecosystems.

A new analytical method using genetically engineered bacteria that produce light when Hg(II) enters their cells has provided a way to gain insight into the fraction of Hg(II) that is bioavailable and into factors controlling its bioavailability. Part of the research in this thesis contributed to the methods development for the analyses of bioavailable Hg in environmental samples; the remainder used the method to quantify and characterize bioavailable Hg in the environment.

Measurements were made for the first time on a variety of aquatic samples as a preliminary assessment of source strengths of bioavailable Hg entering lakes at the Experimental Lakes Area (ELA) in northwestern Ontario. With the exception of snow, all input sources had detectable but low concentrations of bioavailable Hg representing on average 1 to 2.5% of the total Hg concentration. Bioavailable Hg in snow was

surprisingly high representing ~50% of the total Hg. Conversely, in lakes, bioavailable was never detectable.

The *mer-lux* bioreporter was also used to examine the behaviour of Hg(II) in four distinctly different lakes at the ELA by conducting trace level Hg(II) addition experiments and measuring subsequent changes in its bioavailability. The dissolved phase dominated the complexation of Hg(II) and it seems likely that much of the Hg(II) entering lakes rapidly becomes bound (and unavailable for uptake) by a functional group or ligand class that has a high affinity for Hg(II), such as reduced sulfur.

To further characterize the dissolved phase in relation to its effects on bioavailability, the *mer-lux* bioreporter was used in conjunction with trace level ultrafiltration methods and XAD-8 chromatography for the characterization of dissolved organic carbon (DOC). There was a natural preference for Hg(II) to bind or partition to the higher molecular weight DOC (>10 kDa). Consequently, the bioavailability of Hg(II) increased with decreasing molecular weight of DOC. The low absorptivity and percent aromaticity of the lower molecular weight fraction suggested an autochthonous origin. Weak correlations were observed between bioavailable Hg and parameters such as DOC concentration (R^2= 0.48) and SUVA or molar absorptivity (R^2 = 0.66) suggesting that a much more specific parameter than carbon is needed to characterize the bioavailable reactivity of DOC. In attempts to understand and model the implications of environmental change on the biological fate of Hg(II), the character of the DOC rather than the quantity should be considered.

In addition to DOC, biological and chemical controls on Hg(II) uptake were investigated. The results suggest that the observed relationship of Hg(II) uptake to

aqueous chemical speciation was inconsistent with a mechanism of passive uptake of neutral Hg(II) complexes. Rather, Hg(II) appears to be entering the bacterial cell by an, as yet, undescribed facilitated uptake mechanism. Furthermore, the uptake of Hg(II) does not conform to the free ion activity model frequently applied to metal uptake. This holds even when competition for uptake sites between the free Hg ion (Hg^{2+}) and H^+ is considered. Based on these findings, a greater variety of biologically labile Hg(II) species is likely available to bacteria than previously believed.

Table of Contents

List of Figures

Chapter 1

Chapter 2.

Chapter 3.

Chapter 4.

List of Tables

List of Abbreviations

Da	- Dalton
DOC	- dissolved organic carbon
ELA	- Experimental Lakes Area
FLUDEX	- Flooded UplanD Experiment
FIAM	- Free Ion Activity Model
GMM	- Glucose Minimal Medium
HPOA	- hydrophobic organic acids
HPON	- hydrophobic organic neutrals
Hg	- mercury
Hg^{2+}	- "free ion" of mercury
Hg(II)	- inorganic divalent mercury
Hg^{0}	- elemental mercury
MeHg	- monomethyl mercury
$(CH_3)_2Hg$	- dimethyl mercury
MDE	- Mercury Depletion Event
MW	- molecular weight
Na+/K+-ATPase	- sodium-potassium transporting adenosine triphosphatase
NIST	- National Institute of Standards & Technology
ORNL	- Oak Ridge National Laboratory
NOAA	- National Oceanographic & Atmospheric Administration
SUVA	- specific UV absorbance

Chapter 1

INTRODUCTION

Background of Mercury

Properties & Historical Uses. Mercury (Hg) and its principal ore, cinnabar, were first mined more than 2,300 years ago and have been in use to the present day in an astounding variety of ways. Some uses of Hg, for example, as an elixir believed to prolong life by the early Chinese, were somewhat untenable since Hg is highly toxic. However, many other uses are derived from the distinctive physical properties of this element.

Elemental Hg (Hg^0) is unique because it is the only metal which is a liquid at room temperature. It is a very dense liquid that weighs about 13.6x as much as the same unit volume of water. The fact that Hg remains a liquid over a wide temperature range (freezing point = -38.9° C, boiling point = 356.6° C), together with its almost constant thermal coefficient of expansion (between 0° to ~300° C), accounts for its use in thermometers. Mercury vapour is a poor conductor of heat compared with other common metals, but it is a moderately good conductor of electricity, explaining its wide use in the electrical and control instrument industries. An intriguing property of Hg is its ability to dissolve all the common metals (except iron and platinum) to form alloys or amalgams. Amalgamation also lowers the chemical reactivity of the metal dissolved in Hg. This property was exploited in the chlor-alkali industry, which utilizes the Hg cell method to produce chlorine and caustic soda. Amalgamation to recover gold from its ore has been practiced since the 1st century BC. Some dental fillings are amalgams of Hg (d'Itri 1972).

Organomercurial compounds possess a wide spectrum of fungicidal activity and have been widely used in agriculture. This property together with its low water solubility made Hg attractive as bactericide-fungicide agents to protect water based paints from bacterial fermentation prior to their application and to retard fungus attacks on painted surfaces under damp and humid conditions. The pulp and paper industry once used Hg as a slimicide (d'Itri 1972).

Toxicity. The remarkable properties of Hg have been known since ancient times but so was the toxic nature of this element. The Romans recognized this by sending slaves, who were condemned to death, to work in the Hg mines (d'Itri, 1972). Despite its extensive history, Hg contamination in aquatic ecosystems has only been recognized as a serious environmental problem for the last 40 years. The major impetus for Hg research began in the late 1950s when a mysterious neurological disorder started to afflict the population near Minamata City in Japan. In 1949, the Shin-Nihon Chisso Hiryo Co. fertilizer factory began to produce vinyl chloride and acetaldehyde by means of the catalytic conversion of acetylene by $HgCl_2$ and $HgSO_4$. However, some of the catalyst was unknowingly converted into a methylmercuric compound and was carried via the plant's wastewater effluent into Minamata Bay where it entered the food chain and contaminated the fish and shellfish. By 1956, "Strange Disease", now known as "Minamata Disease", had assumed epidemic proportions (Osame and Takizawa, 2001). No sooner had the complete story of the Minamata disease been pieced together than the same problem arose in the Agano River at Niigata, Japan followed by contamination in the Jintsu and Kumana Rivers of Toyama Prefecture (MCPD, 2000).

Many other incidents of Hg poisoning in the 1950s and 1960s occurred worldwide and were characterized by point source pollution. Subsequent legislative action regulated or halted the major point sources of Hg pollution to aquatic ecosystems in many countries, including Canada, and the use of organomercurial compounds in agriculture was banned worldwide. However, the Hg problem persists today under a different guise; elevated concentrations of Hg in fish in areas far from any point source pollution. This problem is widespread among lakes in eastern Canada, the northeastern and north central United States, and Scandinavia (Wiener *et al.*, 1990). Hg has also been identified by the Northern Contaminants Program/Arctic Environmental Strategy as a priority substance impacting the biota in the Arctic (Downs *et al.,* 1998). The major source of Hg to these remote ecosystems is believed to be long-range atmospheric transport from industrial areas and natural sources (Mierle, 1990). Thus, Hg is now considered a pollutant of global concern. In many ways, the success of Hg as an important industrial commodity virtually guaranteed that it would become a notorious environmental pollutant (Schroeder, 1994).

Biogeochemical Cycling of Hg

The Global Nature of Hg Pollution

The pervasive and global nature of Hg pollution is due primarily to its chemical properties. Hg^0, for example, is characterized by high vapour pressure and low water solubility, and predominates (80-95%) in the atmosphere (Lindquist and Rodhe, 1985). Worldwide, coal combustion and solid waste incineration are two of the more important anthropogenic sources of Hg^0 to the atmosphere (Pirrone *et al.,* 1996; Bullock, 2000);

natural sources include plate tectonic boundaries, volcanoes, and mid-ocean ridges (Nriagu, 1979). Over time, Hg^0 is oxidized to inorganic divalent Hg, Hg(II), through reactions mediated mostly by ozone (Munthe, 1992; Hall, 1995). Solar energy and water vapour may also participate in the oxidation process (Brosset, 1987; Brosset and Lord, 1991). In addition, reactive halogens are believed to participate in a photochemically driven oxidation of Hg^0 after polar sunrise in the Arctic (Lindberg *et al.*, 2002). Generally, the oxidation process is relatively slow, allowing Hg^0 to travel in the atmosphere from days to over a year (Lindquist and Rodhe, 1985) before it becomes oxidized to Hg(II).

The residence time of Hg(II) in the atmosphere is comparatively shorter than Hg^0 due to its lower volatility and higher water solubility. It is, therefore, readily removed or "scavenged" by wet and dry deposition and deposited in terrestrial and aquatic environments, sometimes up to 2,000 km from the original Hg^0 source (Lindqvist, 1994). Hg(II), therefore, is the predominant species that enters remote terrestrial and aquatic systems, including oceanic waters (Mason *et al.,* 1994), via the atmosphere (Lindqvist and Rhode, 1985).

In an aquatic ecosystem, Hg(II) can be reduced to Hg^0 by humic substances (Allard and Arsenie, 1991; Matthiessen, 1998), solar radiation (Xiao *et al.,* 1995; Costa and Liss, 2000) and aquatic microorganisms (Mason *et al.,* 1995). The microbial reduction of Hg(II) is among the best-understood heavy metal resistance mechanisms (Foster, 1987). In the commonly isolated Gram-negative species, Hg resistance is invariably plasmid encoded by inducible *mer* operons. The best understood systems are those borne on the transposons Tn501 and Tn21. Tn21 consists of 6 functional genes,

merTPCAD, and is tightly regulated by Hg(II) (Selifonova *et al.*, 1993). Hg resistance occurs by the reduction of Hg(II) to the volatile, less toxic Hg^0 catalyzed by mercuric reductase (*merA*) (Osborn *et al.,* 1995) an intracellular, cytoplasmic flavoprotein. The product of this process, Hg^0, is volatile and leaves the bacterial cell.

Many surface waters are supersaturated in Hg^0 relative to the atmosphere (Vandal *et al.,* 1991). Due to its relatively high volatility, Hg^0 is readily evaded from water surfaces back to the atmosphere, playing an important part in the global Hg cycle (Mason *et al.,* 1994). Hg^0 can also be oxidized to Hg(II) in lake water (Amyot *et al.,* 1994) and coastal seawater (Amyot *et al.,* 1997). Smith *et al.,* (1998) have demonstrated that the oxidation of Hg^0 can be mediated by bacterial hydroperoxidases. The importance of such reactions in terms of the oxidative phase of the global Hg cycle has not been assessed but certainly warrants further study.

One of the major features of the aquatic-biological Hg cycle is the formation of methyl Hg (MeHg). Although all forms of Hg are toxic, MeHg is of particular concern to humans because it readily biomagnifies in the aquatic food chain resulting in elevated levels in the tissues of higher trophic level organisms such as fish (Boudou and Ribeyre, 1997). Contamination of fish is a serious problem in a large number of remote lakes in the boreal ecozone of Canada, the eastern U.S. and northern Europe and concentrations are increasing in Arctic marine mammals (Braune, 1999). Concentrations of MeHg in fish (tissues) may exceed those measured in the water in which they live by over one million times (Watras and Bloom, 1992) and consumption of tainted fish may lead to serious neurological damage and death in humans and wildlife (Mottet *et al.,* 1997).

The principal source of MeHg today is not industrial pollution but rather naturally produced through the biotic and abiotic methylation of Hg(II). Biological methylation is believed to be carried out primarily by sulfate-reducing bacteria (Compeau and Bartha, 1985) by transfer of a methyl group originating from a methylcorrinoid donor (Choi and Bartha, 1993). The process is enzyme-catalyzed, as opposed to a spontaneous chemical reaction, although the enzyme has not, to date, been identified (Choi *et al.*, 1994). Abiological methylation has received comparatively little attention (Lee *et al.,* 1985; Weber, 1993) because it is generally believed that Hg methylation is predominantly a microbially mediated process carried out primarily in anaerobic environments. Berman and Bartha (1986) did a quantitative comparison of chemical vs. biological methylation using estuarine sediments (10 ppm Hg) and found the environmental significance of abiotic Hg methylation was minor with over 90% of environmental Hg methylation associated with biological activity. Furthermore, *in vivo* enzymatic Hg methylation by a corrinoid protein (methyl donor) was much faster than transmethylation by free methylcobalamin (Choi *et al.*, 1994).

The net amount of MeHg available for trophic transfer is controlled by the rates of its production (methylation) and destruction (demethylation) (Lindqvist, 1994). The only significant abiotic degradation mechanism appears to be photolytic decomposition demonstrated by Sellers *et al.,* (1996) in surface waters of a Boreal lake. The end product of MeHg photodegradation in natural waters is likely Hg^0, however, this has not been experimentally verified. There are two known microbial degradation pathways for MeHg, *mer* mediated and oxidative demethylation. The commonly accepted mechanism of decomposition involves cleavage of the C-Hg bond by the organomercurial lyase

enzyme, encoded by *merB*, yielding methane and Hg^{2+}. Demethylation is then followed by the reduction of Hg^{2+} to Hg^0 by the mercuric reductase enzyme, *mer A* (Griffen *et al.*, 1987). The second mechanism of biotic MeHg demethylation is via an oxidative pathway. Oremland *et al.*, (1995) found that while methane was the sole product of MeHg degradation in aerobic estuarine sediments, aerobic demethylation in freshwater sediments and anaerobic demethylation in freshwater and estuarine sediments produced primarily CO_2, indicating the presence of an oxidative pathway. This process produces Hg^{2+} but it is unclear whether it is subsequently reduced to Hg^0 as it is with *mer*-mediated demethylation.

In aquatic ecosystems, the behaviour of Hg is very complex as it continues to be transformed (cycled) between oxidation states and its major chemical forms by both abiotic and biotic processes including those mediated by the sun, humic substances and microorganisms. Bacteria play a pivotal role in aquatic Hg cycling with Hg(II) being the substrate for many of these microbial reactions. To become microbially transformed, Hg(II) must first enter the bacterial cell. In other words, it must be "bioavailable". Not all species of Hg(II) are bioavailable since there are many chemical and biological factors that can affect the complexation and speciation of Hg and in turn its subsequent bioavailability. For example, both Hg(II) and MeHg have a high affinity and tendency to form complexes with organic and inorganic ligands, particularly sulfur. The result is a dynamic variety of chemical species complexed to varying degrees by ligands, or adsorbed on or within particles (Sunda and Huntsman, 1998). Thus, an aquatic bacterium is never exposed to Hg as a single entity *per se*, but rather as a variety of chemical species each differing in its bioavailability (Luoma, 1983). Understanding the factors

which control the concentration of bioavailable Hg is important to understanding microbially mediated transformations of Hg(II) in aquatic ecosystems and their contribution to the global cycle of Hg.

Biosensors

The analysis of Hg in unperturbed ecosystems has historically been hindered by technical difficulties, particularly contamination of samples owing to the very low concentrations being measured. During the past twenty years, there have been many advances in the measurement and detection of trace levels of a variety of Hg species (Bloom and Crecelius, 1983; Bloom, 1989; Gill and Bruland, 1990; Morrison and Watras, 1999). Though often costly and complex, these methods are highly accurate and sensitive, and are important for establishing certain environmental standards (Kohler *et al.,* 2000). Nonetheless, the measurement of Hg concentrations by these chemical methods does not reflect how much Hg is available in a form that can be transported into cells (Steinberg *et al.*, 1995). Consequently, these methods fail to provide any information on the bioavailability of Hg, or on its effects on biological systems (Kohler *et al.*, 2000). This holds for other heavy metals and organic contaminants as well. There is, therefore, a need for analytical methods that report not only on the presence of a chemical but also on its bioavailability. Recognizing this need, there has been a rapid development of "biosensors" or "bioreporters" based on genetically engineered bacteria. Apart from being readily amenable to genetic manipulation, bacteria are attractive candidates because of their large population sizes, rapid growth rates, low costs and relatively easy maintenance (Kohler *et al.*, 2000).

In order for a bacterial cell to function as a microbial biosensor, it has to contain two linked genetic elements: a "sensor" (promoter-operator) and a "reporter". The sensing element detects the target compound and turns on the reporter which emits a detectable signal (Kohler *et al.*, 2000). The beauty of this type of manipulation is that a promoter sequence from one bacterial species can be genetically fused to a reporter gene from a second microorganism and introduced into the cells of a third, thereby capitalizing on the properties of a number of organisms. In practice, the regulatory mechanism of the promoter is required in order for it to sense its target compound (Kohler *et al.*, 2000). The resultant construct is normally introduced into the host cell as a plasmid where it is replicated along with the cell's normal DNA.

The Reporter

The ideal reporter gene(s) should provide a sensitive, real-time measurement of gene expression and encode a product that is easily detectable. The assay for the ideal reporter gene product should be simple, inexpensive to perform, and should not require cell disruption (Selifonova *et al.*, 1993). Bioluminescence satisfies these criteria. The measurement of light is among the most sensitive techniques for measuring concentrations of many compounds. It can be easily, inexpensively, and non-invasively measured in real-time with photographic film, a scintillation counter (with coincidence turned off), or a luminometer, among other methods of detection (Korpela *et al.*, 1989).

Bacterial Bioluminescence as a Reporter Signal. In luminescent bacteria, 21 different genes from at least three genera (*Photobacterium*, *Vibrio*, *Photorhabdus*) have been implicated in the luminescent system and are designated as *lux* genes. Although all

luminous bacteria encode biochemically similar luminescence systems, only five of these *lux* genes (*luxCDABE*) have been found in all luminescent bacteria (Meighen, 1994).

From a biochemical viewpoint, bacterial luminescence requires oxygen, a source of energy (a reduced flavin mononucleotide, $FMNH_2$), a luciferase enzyme and a long-chain fatty aldehyde (Stewart and Williams, 1992); relatively simple compounds that are closely related to the basic metabolites of the cell (Meighen, 1994).

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + \text{light (490 nm)}$$

Luciferase is a heterodimeric enzyme encoded by the *luxA* and *luxB* genes (Stewart and Williams, 1992). All bacterial luciferases that have been purified consist of an α subunit (~42,000 mol wt) and a β subunit (~37,000 mol wt). The synthesis of the long-chain aldehyde is catalyzed in an ATP- and NADPH-dependent manner by a multi-enzyme fatty acid reductase complex comprising a reductase, a transferase, and a synthetase encoded by *luxC*, *luxD*, and *luxE* genes, respectively (Heitzer *et al.*, 1992).

Most microorganisms lack the genetic blueprint for luciferase and fatty acid reductase but they can supply $FMNH_2$, generated by an NAD or an NADP-dependent oxidoreductase (Belas *et al.*, 1982). For a dark bacterium to become bioluminescent, therefore, all that is required is the genetic transfer of the genes for luciferase and fatty acid reductase (*luxCDABE*). In practice, the aldehyde can be provided exogenously to bacterial cultures expressing only the luciferase component (*luxAB*) of the *lux* operon (Stewart and Williams, 1992). However, the addition of aldehyde is generally not required for light emission if the *luxCDE* are also transferred. Given that the *lux* operons from *V. fischeri* and *V. harveyi* were the first to be cloned, the majority of vector

constructs use *lux* DNA from these sources (Stewart and Williams, 1992), conveniently transferred as one unit (Meighen, 1991). Most Hg biosensor constructs use bacterial *lux* genes as the reporter although the firefly luciferase *luc* system (Virta *et al.*, 1995) and fusions to β-galactosidase are also in use (Barkay *et al.*, 1998).

The Sensing Element

In contrast to the reporter, the sensing element is different in each bacterial sensor. In most cases, the sensing element is a protein which controls a promoter that a bacterium would normally turn on in response to specific or general environmental changes. In the recombinant strain, however, the selected promoter drives the synthesis of the reporter protein(s) instead (Kohler *et al.*, 2000).

Many of the environmental biosensors have been specifically designed for the detection of heavy metals since pollution by metals is considered to be among the most serious of environmental problems (Birch *et al.,* 1996). In many of these constructs, inducible promoters of genes involved in metal-resistance mechanisms are used as the sensing elements and luminescence as the reporter. Examples of metal-*lux* constructs include aluminum, arsenic, cadmium, chromate, copper, iron, and zinc (Kohler *et al.*, 2000) and, of course, Hg.

MerR as a Sensing Element. The sensing element in the Hg biosensors described to date is based on the regulatory region of the Hg resistance operon (Figure 1). Hg resistance (*mer*) is widespread in both Gram-negative and Gram-positive bacteria (Foster, 1987). The *mer* operon of Gram-negative bacteria consists of *merR*, the gene encoding the regulatory protein MerR, and structural genes encoding Hg(II) transport

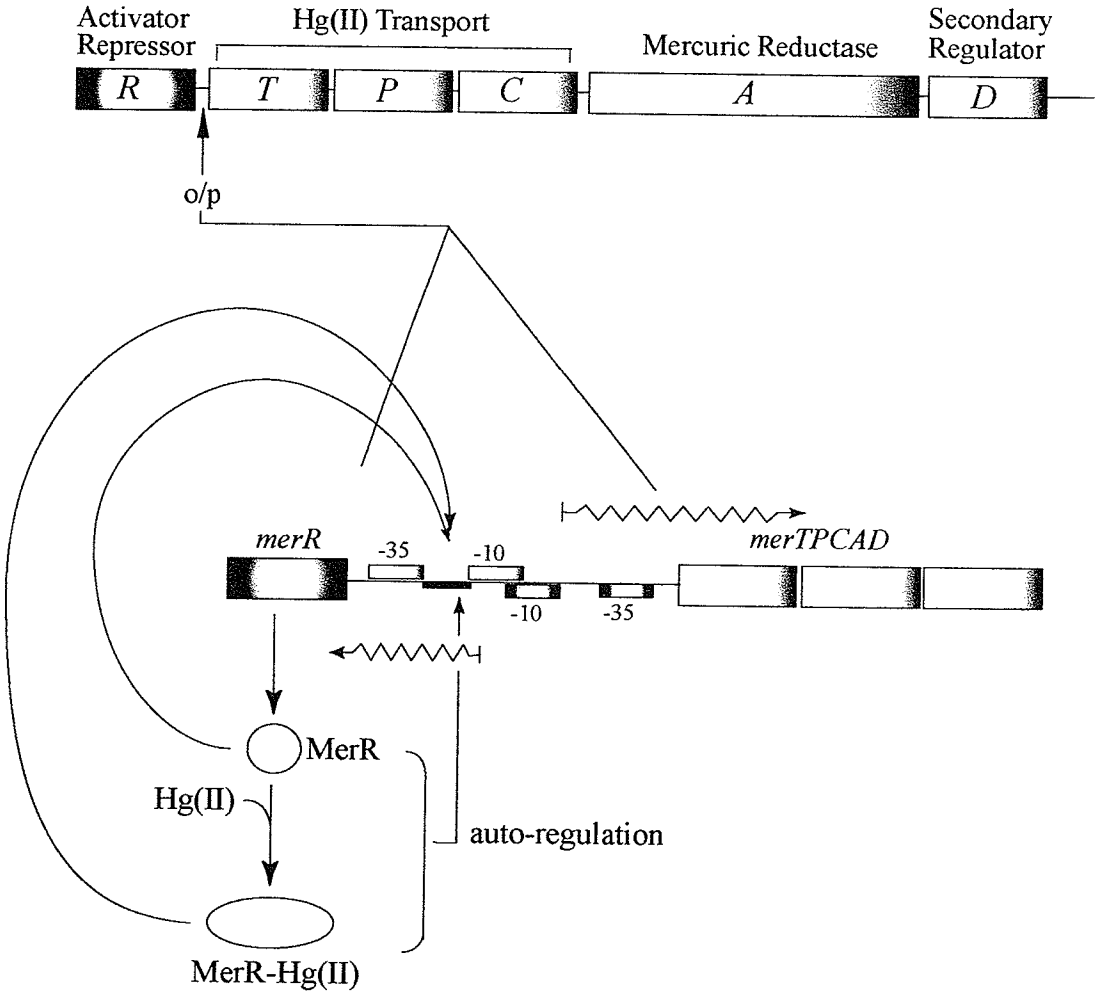

Figure 1.1 General structure and regulation of the *mer* operon. *merR* is the gene encoding for the regulatory protein MerR, which controls its own transcription and that of the divergently transcribed structural genes, *TPCAD; merTPC* encode proteins involved in the uptake of Hg(II), *mer A* encodes mercuric reductase which reduces Hg(II) to Hg^0, *merD* is a proposed transcriptional down-regulator. The MerR binding site between the -10 and -35 regions of P_{TPCAD} is indicated with a black bar, the o/p is the operator and promoter region. Modified from Heltzel (1990), Condee and Summers (1992) and Summers (1992).

(*merTPC*), which brings Hg(II) through the cytoplasmic membrane, and Hg(II) reduction (*merA*). The *merA* gene encodes mercuric reductase, a cytosolic, NADPH-dependent, flavin adenine dinucleotide-containing disulfide oxidoreductase which reduces Hg(II) to volatile Hg^0, which then diffuses away from the cell (Summers, 1992). The most promoter-distal gene, *merD*, encodes a small, low-abundance protein proposed to turn off structural gene transcription once Hg(II) has been reduced by mercuric reductase (Condee and Summers, 1992) by binding competitively to the MerR binding site in the *mer* operator/promoter (o/p) region (Osborn *et al.,* 1995).

The expression of the Hg resistance *mer* operon is controlled and regulated by MerR, a Hg(II)-responsive regulatory protein. In Gram negative *mer* operons, *merR* is divergently transcribed from the structural genes (Figure 1). The MerR protein binds as a dimer to a region of dyad symmetry located between the –35 and –10 RNA polymerase recognition hexamers of P_{TPCAD} in the *mer* o/p region (Lund and Brown, 1989) (Figures 1 & 2). MerR binds as a transcriptional repressor in the absence of Hg(II) and as an activator in the presence of Hg(II). With or without Hg(II) induction, MerR binding fosters the occupancy of the uninduced P_{TPCAD} by RNA polymerase (Heltzel *et al.*, 1990) (Figure 2). In the absence of Hg(II) ions, MerR represses expression of the structural genes by preventing formation of an open complex by RNA polymerase. In the presence of Hg(II), the MerR-*mer* o/p complex undergoes a conformational change causing structural distortions in the DNA on the 5' side of the –10 hexamer within the region of dyad symmetry, thus allowing active transcription of the structural genes by RNA polymerase (Heltzel *et al.*, 1990, Ansari *et al.*, 1991; Summers, 1992). MerR also

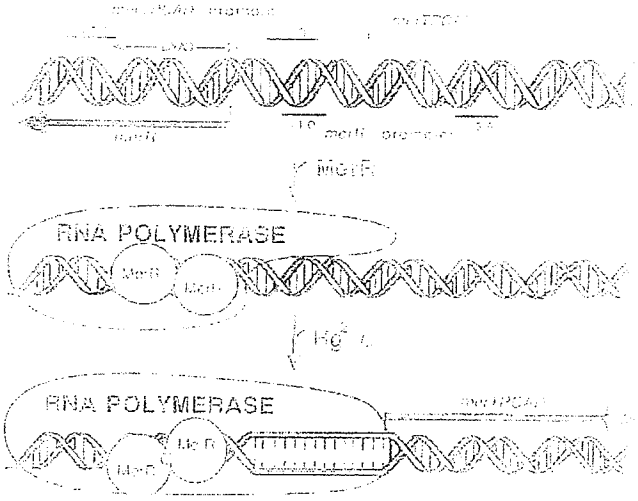

Figure 2.1 Model for the regulation of the *mer* operon. The derepressed *mer* operator/promoter (top). In the absence of Hg(II), MerR represses initiation of the *merTPCAD* transcript (middle). In the presence of Hg(II), MerR initiates activation of the *merTPCAD* transcript (bottom). From Condee and Summers (1992).

negatively regulates its own expression by repressing its own transcription, whether Hg(II) is present or not (Zeng *et al.,* 1998).

As a receptor, MerR has a high affinity for and is extremely sensitive to Hg(II) (Ralston and O'Halloran, 1990). This can be explained by the metal receptor site of MerR (Tn501) where Hg(II) is believed to bind in a rare three-coordinate Hg(II) thiolate complex ($Hg(S\text{-}Cys)_3$). Other group IIb metals prefer tetrahedral metal-thiolate coordination, so even though Cd(II), Zn(II) (and Au(I), Ag(I), and Au(III)) have been found to partially stimulate transcription in the presence of MerR, concentrations at least two to three orders of magnitude greater than for Hg(II) are required (Ralston and O'Halloran, 1990). Thus, MerR can discriminate between Hg(II) and Zn(II) or Cd(II) (Ralson and O'Halloran, 1992) while maintaining sensitivity to Hg(II) (Wright *et al.,* 1990); important attributes to impart to a biosensor.

The remaining discussion will focus on the development of "*mer-lux*" biosensors; recombinant bacteria containing fusions of Hg resistance genes with promoterless *lux* genes under the regulation of MerR.

mer-lux Biosensors

The earliest *mer-lux* biosensors (using the entire Tn21 *mer* operon in an *E. coli* host) were constructed to evaluate *in vivo* gene expression rates of the *mer* structural gene promoter, P_{TPCAD} of Tn21 (Condee and Summers, 1992). *In vivo* gene expression kinetics corresponded well with those previously determined *in vitro* (Ralston and O'Halloran, 1992); apparent $K_{0.5}$ for Hg(II) induction by MerR *in vivo* was 9.3×10^{-8} M vs. 1×10^{-8} to 5×10^{-8} M *in vitro*. A large increase in luciferase activity over a narrow range of Hg(II)

inducer concentrations producing a sigmoidal curve was also observed. This response is known as the "ultrasensitive threshold effect", and again showed good agreement with the *in vitro* observations of Ralston and O'Halloran (1992). The authors concluded that the luciferase assay (with an intact Hg(II) transport system) is capable of faithfully portraying gene expression kinetics *in vivo*.

To address the effect of the *mer* structural genes on the performance of *mer-lux* biosensors, Selifonova *et al.,* (1993) constructed three *mer-lux* reporter plasmids, pRB28, pOS14, and pOS15 (Figure 3) which were transformed into *E.coli* HMS174 cells and used in assays of Hg(II)-dependent *lux* induction. In plasmid pRB28, the luciferase genes *luxCDABE* are located downstream from a 0.7-kb fragment carrying the regulatory gene *merR*, the *mer* o/p, and only a portion (87 bp) of *merT* encoding 29 amino acids. Plasmid pRB28 does not specify a Hg(II) uptake system and induction of light production by Hg(II) was believed to be solely by a passively transported inducer. Indeed, until recently it was generally believed that with the exception of microbial Hg resistance determinants that encode their own Hg(II) transport systems, the sole mechanism of Hg(II) uptake was passive diffusion of neutral lipophilic complexes (Gutknecht, 1981; Bienvenue, 1984; Mason *et al.,* 1996). This will be discussed further in Chapter 6. Plasmid pOS14 carries a 2.9-kb *mer* fragment that includes *merR*o/p*TPC* i.e. a transport system but no mercuric reductase. Construct pOS15 contains a 5.3-kb *mer* insert that spans an intact *mer* operon. Thus, pOS15 specifies Hg(II) transport and reduction (Selifonova *et al.*, 1993).

The sensitivity of the *mer-lux* biosensors to Hg(II) was increased by the introduction of transport functions, *merTPC,* (in pOS14), and decreased by including an

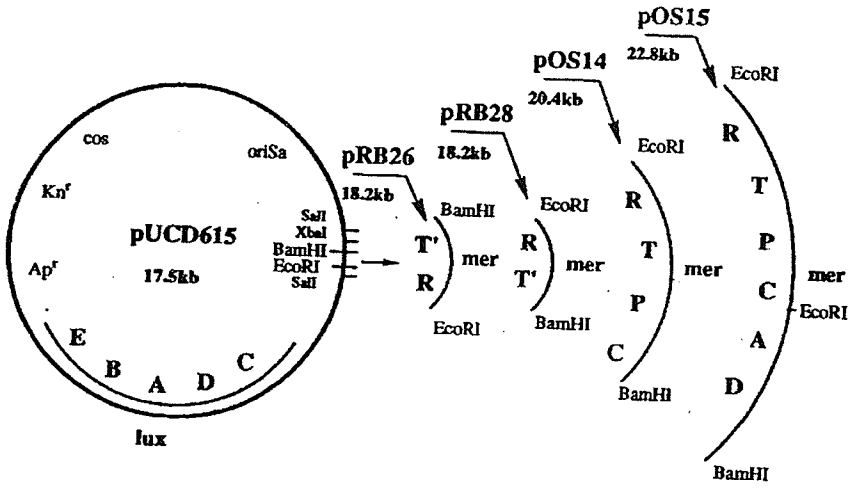

Figure 3.1 Construction of the *mer-lux* fusion plasmids pRB28, pOS14, and pOS15. From Selifonova *et al.*, 1993.

active mercuric reductase, *merA*, (in pOS15). However, bioluminescence by pRB28 (no Hg(II) transport proteins or mercuric reductase) was induced at a Hg(II) concentration that was almost as low as that needed for induction by pOS14. The luminescence response increased with Hg(II) concentration observed over the range of 0.1 nM to 1000 nM. Thus, these experiments established the quantitative response of the bioreporters within a specific range of Hg(II) concentrations under defined model conditions (Selifonova *et al.,* 1993).

These biosensors were also used to detect the presence of bioavailable Hg in natural water samples. The detection of Hg(II) was at the 0.5 and 1,000 nM concentration range, which covers levels commonly encountered in contaminated environments. The sensitivity was inadequate for detection of trace Hg(II) concentrations (pM range) that exist in some "pristine", remote lakes with high levels of MeHg in fish. Since no bioassay is environmentally relevant unless it is functional in the correct concentration range (Kohler *et al.,* 2000), it appeared that the *mer-lux* biosensor would likely be of little use in these types of lakes because Hg(II) concentrations are below those that induce the *mer* operon.

Tescione and Belfort (1993) also evaluated an *E. coli mer-lux* biosensor consisting of the *mer* operon from *Serratia marescens* (*merR*, *merT*, and an inactive *merA*) and the *lux* genes were from *V. fischeri*. Responses were generally parabolic with regions in the mid-range exhibiting linearity. The range of sensitivity for Hg(II) was 10 nM to 4 μM, again inadequate for the detection of trace concentrations of Hg(II).

The belief that *mer* specified reactions were induced only at high concentrations of Hg(II) was disproved by Rasmussen *et al.,* (1997) who reported an increase in the

sensitivity of a *mer-lux* assay using *E. coli* HMS174 pRB28 from nM to pM concentrations. This was achieved by simply reducing the biomass in the assay from 10^7 to 10^5 cells mL^{-1}. The increase in sensitivity was purported to be due to a reduction in the number of cellular binding sites that may compete with the regulatory protein MerR, for binding of the inducer, Hg(II). The sensitivity of the *mer-lux* assay was sufficiently improved for the detection of Hg(II) in most contaminated natural waters and some pristine waters depending on what percent of the total Hg pool is bioavailable.

Herein, the *mer-lux* biosensor of choice was *E. coli* HMS174 pRB28, specifying neither an uptake system nor an active mercuric reductase. The typical sigmoidal response observed *in vitro* (Ralston and O'Halloran, 1992) and *in vivo* with an intact *mer* operon (Condee and Summers, 1992) was also observed with pRB28. This indicates that under the employed assay conditions light production was determined by the Hg(II)-dependent activation of the *mer* promoter rather than by factors relating to the synthesis and activity of the luminescence system (Rasmussen *et al.*, 1997).

Despite the limitation of insufficient detection for low level Hg environments, the *mer-lux* biosensor (*E. coli* pRB28) was still used to study factors affecting the bioavailability of Hg(II) such as pH, chloride and dissolved organic carbon concentrations. In conjunction with thermodynamic speciation modeling to predict the predominant Hg(II) species in solution, Barkay *et al.*, (1997) found that the negatively charged species of Hg(II) induced less light production than the electrochemically neutral forms such as $Hg(OH)_2$ and $HgCl_2$. Furthermore, despite the difference in the octanol water partition coefficients between $Hg(OH)_2$ and $HgCl_2$, these neutral species of Hg(II)

appeared to be equally bioavailable. They also found that the bioluminescent reaction was severely affected by pH but provided no explanation as to why.

An important addition to the *mer-lux* bioassay method came with the introduction of *E. coli* pRB27, the *lux* constitutive mutant of pRB28 (Barkay *et al.,* 1997). Restriction enzyme analysis of pRB27 showed a small deletion upstream of the *merR*o/p*merT'* insertion that created pRB28 which might be responsible for the constitutive phenotype. Changes in light production by pRB27 reflect conditions affecting the physiology of the *mer-lux* organism but not the Hg(II)-dependent induction of the *mer-lux* gene fusion (Barkay *et al.,* 1997). Although the quantitative usefulness of pRB27 is somewhat limited owing to the different energy requirements between constitutive and Hg(II)-induced light production, the advantages that a constitutive organism could provide in defining factors affecting the *lux* reaction have not been fully exploited.

Of value to the recent development of the method was the description on data analyses in Barkay *et al.*, (1998), especially the determination of a standard curve using the logarithmic increase in the rate of light production. Further improvements to the detection limit were made at the Freshwater Institute (Department of Fisheries and Oceans, Canada) in the laboratory of J.W.M. Rudd. These improvements included increasing the volume of the bioassay from 2 to 20 ml and the use of a laminar flow unit in a HEPA-filtered "Hg clean" lab to minimize Hg contamination, standard protocol for the chemical analyses of trace concentrations of Hg. No "trace metal clean" techniques to minimize contamination by other metals were, however, introduced to the bioassay method. A new *mer-lux* organism *Vibrio anguillarum* was also transformed at the University of Manitoba; *V. anguillarum* pRB28 by Fiona Punter and *V. anguillarum*

pRB27 by George Golding. This organism is a salt-water fish pathogen, considered more environmentally relevant than *E. coli*, which is enteric.

Research Objectives

The primary goal of this research was to develop and utilize the *mer-lux* bioreporter, *Vibrio anguillarum* pRB28, to explore the nature of the chemical and biological factors controlling Hg(II) bioavailability in remote lakes of the Boreal region, typical of those impacted by non-point source Hg pollution. To accomplish this goal, the following objectives were defined.

- Develop the methodology for the analyses of bioavailable Hg(II) in environmental samples
- Identify and quantify sources of bioavailable Hg(II) to lakes
- Explore the reactivity of dissolved organic carbon and Hg(II) in terms of bioavailability
- Examine the role of chemical speciation on the bioavailability and uptake of Hg(II)

Chapter 2

GENERAL METHODS

Sampling Sites

Experimental Lakes Area. The Experimental Lakes Area (ELA) is located in northwestern Ontario, Canada (93°30'-94°00' W and 49°30'-49°45' N) at an altitude of 360-380 m above sea level (Figure 1). The area is characterized by Precambrian acid granites overlain in some areas by thin glacial drift composed largely of sand and gravel. All of the lakes are oligotrophic and, based on conductivity measurements, are the most dilute lakes known in the Canadian Shield with the exception of some large lakes in the N.W.T (Armstrong and Schindler, 1971). Their watersheds drain into the Winnipeg River, in the Hudson Bay drainage system (Brunskill and Schindler, 1971).

A survey of 20 lakes and four experimental reservoirs was conducted in 1999 for bioavailable Hg (Figure 1). Some general characteristics of these lakes are in Table 1. Four of theses lakes were studied in detail in 1998 and 1999, chosen primarily to provide a range of dissolved organic carbon (DOC) concentrations. Lake 224 is a very clear lake with low DOC concentrations, while lake 661 is a brown water pond and contains high concentrations of DOC. Lakes 240 and 239 are intermediate in DOC concentrations. Roddy lake and three experimental reservoirs created as part of the FLooded UplanD EXperiment (FLUDEX) were also studied in some detail. FLUDEX is a whole ecosystem experiment whereby three reservoirs were created in areas of differing soils and vegetation (Table 2) to simulate hydroelectric development and its impact on the

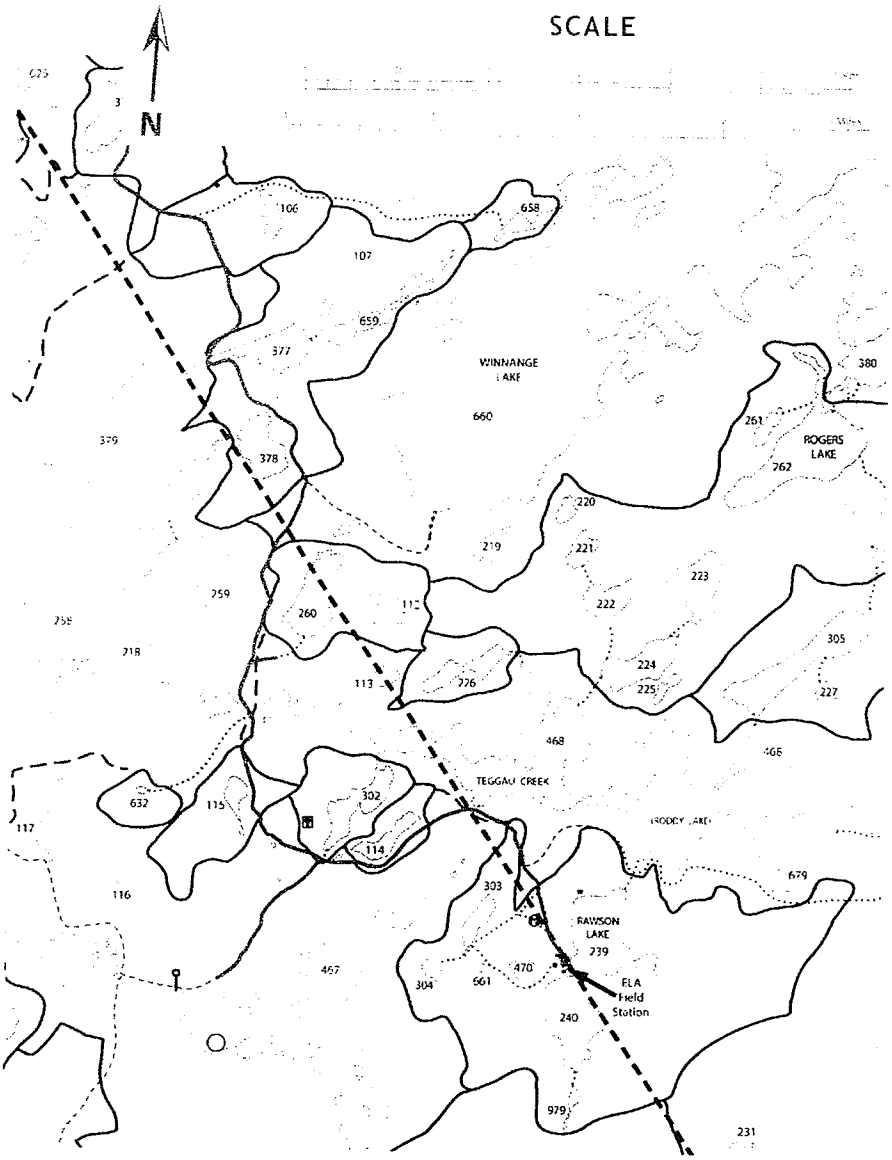

Figure 1.2 Map of part of the Experimental Lakes Area, northwest Ontario. Lakes are indicated by number, the experimental lakes by a darker shade of gray. The Meteorological Site is indicated by an Ⓜ northwest of the ELA Field Station.

Table 1.2 Lakes and reservoirs at the Experimental Lakes Area surveyed in 1999 for bioavailable Hg. Lake characteristics, where available, include surface area (A*o*), maximum depth (z_m), Secchi depth visibility (SDV), total dissolved solids (TDS), and conductivity at 25° C. (C) Cleugh and Hauser (1971), mean depth* (*z*), (Brunskill and Schindler, 1971), dissolved organic carbon (DOC) (ELA survey, 1986).

Lake	***Ao (ha)***	z_m ***(m)***	***Z (m)***	***SDV (m)***	***TDS (mg L^{-1})***	***DOC (μM)***	***C (μmoh cm^{-1})***
114	12.1	5.0	1.7	2.5	9	650	17
115	6.5	1.5		1.5	18	1060	19
222	17.5	5.4		2.2	30	900	32
223	30.2	11.2		5.0	40		23
224	25.4	16.7		7.0	40	290	18
225	5.6	1.8		1.7	50	750	17
227			4.4				
239	56.1	30.4	10.5	4.8	20	550	25
240	44.1	13.1	6.1	4.2	20	580	22
259	96.9	20.3		6.3	30	580	18
302S	10.9	10.6	5.1				
302N	12.8	13.8	5.7	2.8	50		19
303	9.9	2.5	1.5	2.5	50		17
304	3.6	6.7	3.2	1.9	40	700	16
468							
470						980	
632							
658							
660							
661						1600	
Reservoirs							
979						710	
FLUDEX 1							
FLUDEX 2							
FLUDEX 3							

Table 2.2 Characteristics of the three FLUDEX reservoirs.
Data marked 'ξ' are from Heubert (1999); 'Φ' are from Boudreau (2000).

	Reservoir 1	***Reservoir 2***	***Reservoir 3***
Dominant vegetation$^{\xi}$ (percent coverage)	*Pinus/Ledum/Sphagnum* (53%) *Pinus/Polytrichum* (47%)	*Pinus/Betula* (100%)	*Pinus/Vaccinium* (73%) *Polytrichum/Cladina* (27%)
Total soil C incl litter$^{\Phi}$ (kg C ha^{-1})	38,400 (58.2%)	25,000 (47.4%)	21,300 (51.9%)
Total C above-ground vegetation$^{\xi}$ (kg C ha^{-1})	27,590 (41.8%)	27,732 (52.6%)	19,762 (48.1%)
Surface area (m^2)	7,358	4,966	6,271
Drainage area (m^2)	47,842	7,334	929
Reservoir watershed area (m^2)	55,200	12,300	7,200
Volume above flooded soils (m^3)	6,870	4,266	7,117
Volume in flooded soils (m^3)	1,807	1,813	795
Water renewal (d)	11	10	7

production of methyl Hg (MeHg) and greenhouse gases. Roddy lake is the water source used to fill the reservoirs.

Precipitation and through-fall were sampled at the ELA meteorological station (Figure 1). Terrestrial run-off samples were collected from three distinct areas in 1999 and 2000; the lake 114 inflow catchment (Figure 1), a number of catchments in the 302 uplands (Figure 2), and the wetland and upland streams at lake 658. The lake 114 inflow catchment is 5.73 hectares and dominated by dense stands of young jack pine and paper birch. The forest is young due to logging in 1976. Ground cover was characterized by thin glacial till covered with moss and lichen with some exposed areas of granodiorite bedrock (St. Louis *et al.*, 1994). Conversely, the 302 uplands are generally characterized by patches of treed soil deposits interspersed among large areas of lichen-covered bedrock (Allan *et al.*, 1993). The specific catchments sampled in the 302 uplands included U8, U2, U4, and the sub-catchment U1f, all located on a granitic ridge top ~40 m above the small headwater lake 302 (Figure 2). The physiographic features of these catchments are described in Table 3. Sub-catchment U1f was also the pilot study area for the Mercury Experiment to Assess Atmospheric Loadings in Canada and the U.S. (METAALICUS) in 1999 and 2000. METAALICUS is an ambitious, multi-disciplinary whole ecosystem experiment designed to elucidate some of the pathways of Hg entering aquatic food chains through the use of stable isotopes of Hg. The full-scale METAALICUS project, which started in 2001, used lake 658 and its watershed. I analyzed the upland and wetland streams entering lake 658 in 2000 before the full-scale experiment began.

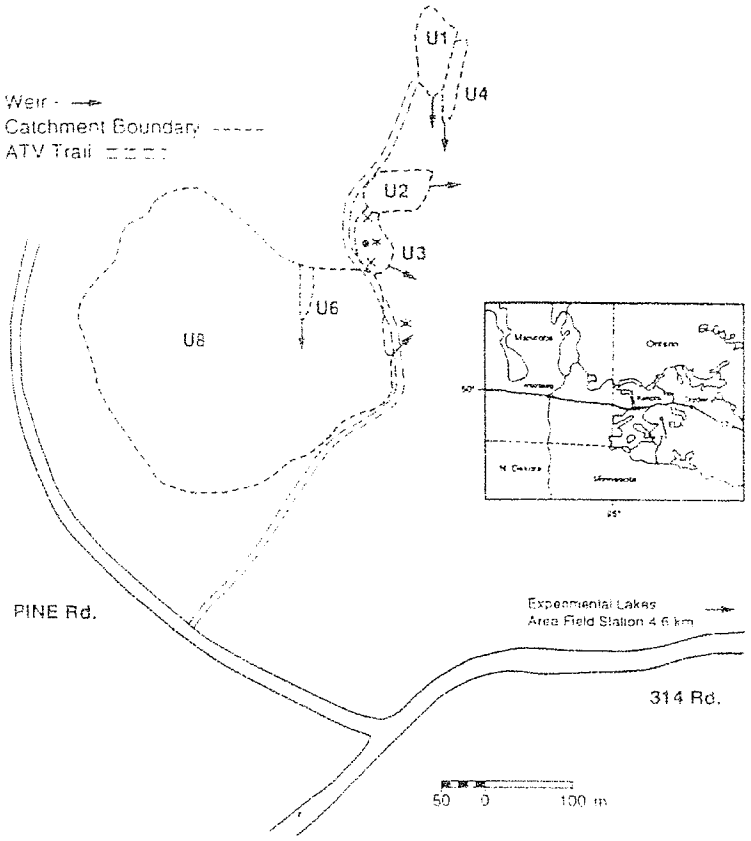

Figure 2.2 Map of the 302 Upland catchments at the Experimental Lakes Area, northwest Ontario. (from Allan *et al.*, 1993)

Table 3.2 Physiographic features of the 302 upland catchments. (Modified from Allan *et al.*, 1993).

Site	***Area (m^2) Ha 114***	***Bedrock (%)***	***Forest (%)***	***Mean Soil Depth (cm)***
114IF	5.73	0	100	--
U1f	1102	62	38	11
U2	4694	65	35	11
U4	1730	60	40	10
U8	71,970	--	--	--

-- = not measured

Barrow, Alaska. Snow samples were collected at the Climate Monitoring and Diagnostics Laboratory (CMDL) clean air sector of the U.S. National Atmospheric and Oceanographic Administration (NOAA) in Barrow, Alaska (71°19'N, 156°37'W). The CMDL is located approximately two km from the Arctic Ocean near the peninsula at Point Barrow, where the two distinct water masses of the Chukchi and Beaufort seas converge. Barrow is approximately 1,600 km south of Alert, Nunavut, the site where "mercury depletion events" were first discovered (Schroeder *et al.*, 1998).

Sample Collection, Handling & Analyses

Field Sample Collection. All Teflon samplers (bottles and wide-mouth jars) for Hg sampling were cleaned and prepared by Flett Research Inc. (Winnipeg, Manitoba). Cleaning and preparation involved washing the bottles or jars in hot HNO_3 and filling them with a 1% HCl solution. Bottles were always stored in two Ziploc bags and transported to the field in clean coolers that were placed in two plastic bags tied with twist-ties. All Hg sampling was carried out using established Hg "clean hands, dirty hands" protocol as described in St. Louis *et al.,* (1994). Briefly, this protocol requires that two people sample together at all times. The "dirty hands" person handles the outer Ziploc bag while the "clean hands" person handles the inner Ziploc bag and the Teflon sampler only. Both people must wear gloves at all times during sampling and must not touch anything other than the Ziploc bags and/or Teflon samplers.

Lake water and terrestrial run-off samples for all Hg analyses (total, methyl and bioavailable Hg) were collected in either 125 ml or 250 ml Teflon bottles. Most of the lakes were sampled from an aluminum motor boat; however, the motor was never used.

Lakes 239, 240, 979, and the three FLUDEX reservoirs were sampled from a fiberglass or Kevlar canoe when possible. Lake 661 was sampled from land at the V-notch of the outflow weir. For precipitation sampling, 250 ml wide-mouth Teflon jars were placed on acid-washed plastic holders that were screwed into a wooden platform approximately 4.5 feet above the ground. Through-fall sampling was similar except it was directed via a trough that was attached to a tree by a wooden holder into 500 ml wide-mouth Teflon jars. Samplers for both precipitation and through-fall were set out at the beginning of a rain event. As soon as the rain event ended, the water collected in the wide-mouth jars was promptly poured into Teflon bottles. Snow was collected in 2 litre wide-mouth Teflon bottles by using the lid to gently remove and scoop the surface layer of snow into the bottle. All samples for Hg analyses (total, methyl, and bioavailable) were placed in double plastic bagged coolers as part of the "clean" sampling protocol and to protect them from sunlight. Environmental samples for water chemistry were collected in polypropylene bottles at the time of Hg sampling.

Sample Handling. If samples required filtering, it was done immediately upon returning from the field. Sterile cellulose acetate milli-pore (0.45 μm) filters were pre-rinsed with 100 ml of 5% HCl followed by ~650 ml milli-Q water prior to filtering actual samples. This volume of water was required to ensure that the filter was free of residual dissolved carbon that could potentially affect the bioavailability of Hg(II).

Samples for total Hg and MeHg analyses were then immediately preserved with 0.25 ml concentrated hydrochloric acid per 125 ml sample (total Hg) or by freezing (MeHg) upon returning from the field. Samples for bioavailable Hg analyses were always analyzed within a few hours of being sampled because they could not be

preserved (see Chapter 3 for details). Furthermore, between sampling and analyses, it was essential to keep water samples for bioavailable Hg analyses in the dark at all times, both in the field and in the lab (see Chapter 3 for details).

Chemical Analyses. Total Hg concentrations were determined by cold vapour atomic fluorescence spectrophotometry (Brooks Rand Ltd. Model 2) at Flett Research Ltd., Winnipeg, Manitoba. MeHg concentrations were determined at Flett Res. Ltd. by distillation extraction, aqueous phase ethylation and atomic fluorescence detection (Bloom, 1989). Water chemistry included DOC, chloride, sulfate, suspended carbon, alkalinity, pH and conductivity as described in Stainton *et al.*, (1974) and was carried out at the ELA and the Freshwater Institute chemistry labs.

The general approach taken for the analysis of bioavailable Hg was based on that described in the early literature using E. coli biosensors (Selifonova *et al.*, 1993; Barkay *et al.*, 1997). Some unpublished details relevant to the general method are described in this chapter. However, because many modifications and additions were contributed to the method during the course of this research, the methodology for the analysis of bioavailable Hg is described in a Methods Development chapter, in addition to the experimental evidence to support how the changes were derived.

Bioavailable Mercury

Equipment. In the laboratory, culture flasks, test tubes, Teflon centrifuge tubes, reagent storage bottles, and spectrophotometer tubes were acid washed in 30% H_2SO_4 and thoroughly rinsed in milli-Q water prior to use. With the exception of the spectrophotometer tubes and glass scintillation vials used for the assays, equipment used

for the bioassay was sterile. All procedures including culture transfers, media and reagent preparation, and assays were carried out in a Class 100 laminar flow hood in a HEPA-filtered air clean lab at either the Freshwater Institute (Winnipeg, MB) or at the Experimental Lakes Area research station (NW Ontario).

Reagents. Reagents used for the *mer-lux* bioassay are listed in Table 4. The total Hg (1 to13 ng L^{-1} or 5 to 65 pM) measured in the reagents was assumed to be unavailable for uptake by the *mer-lux* organisms because blanks did not induce on a regular basis. This includes the milli-Q water, which contained 0.3-0.5 ng Hg L^{-1} (1.5 to 2.5 pM). The NaCl reagent was heated (muffled) at 800 °C for four hours to reduce the amount of organic materials that could bind Hg. The melting points of the other reagents were too low for muffling.

Hg(II) Standard Preparation

Hg Stock. The Hg stock was a 1 μg ml^{-1} $HgNO_3$ solution prepared and provided by Flett Research Ltd., using National Institute of Standards and Technology standard reference material #3133 (10 g Hg L^{-1} in 10% HNO_3). For some experiments, the standard may have contained 1% (by volume) BrCl, (27 g KBr in 2.5 L of 12N HCl and 38 g $KBrO_4$) to prevent reduction and volatilization of Hg(II), and to oxidize organic contaminants in the standard solution.

Working Hg Standards. The primary standard was diluted twice in milli-Q water in Teflon vials for a final concentration of 0.0625 ng ml^{-1} (0.313 nM). Both dilutions of the primary standard were prepared and used immediately to avoid degradation or possible changes in the bioavailability that might occur over time.

Table 4.2 Growth and assay media reagents used for the *mer-lux* bioassay.

Reagent	***Brand***	***Grade***	***Concentration***
Growth Medium			
Na_2HPO_4	BDH	Analar (99%)	0.5 M
KH_2PO_4	Fisher	ACS certified (99.7%)	1 M
NH_4Cl	Aristar		2 M
Trace elements*			
$MgSO_4$-$7H_2O$	Fisher	ACS certified (100%)	1 M
Vitamin B_1			1 mg/ml
Kanamycin			10 mg/ml
Assay Medium			
D-glucose	Fisher	ACS certified	30 %
$(NH_4)_2SO_4$	Fisher	ACS certified (99.8%)	12 %
K_2HPO_4	BDH	analytical	0.95 M
NaH_2PO_4-H_2O	BDH	analytical	
Treatments			
Na_4EDTA	Sigma	Min 99%	1 M
NaCl	Fisher	ACS certified	1 M
NaOH	Fisher	ACS certified (98.1%)	1 M

*trace elements = $FeSO_4$, H_3BO_3, $CoSO_4$, $CuSO_4$, $MnCl_2$, Na_2MoO_4, $ZnSO_4$

Standards were generally run in duplicate and always included blanks. The detection limit for the *mer-lux* bioreporter was ~0.25 ng L^{-1} with the luminometer and ~0.05 ng L^{-1} with a scintillation counter.

Thermodynamic Speciation Modeling

The aqueous chemical speciation of some of the treatment solutions was modeled using the thermodynamic speciation model KINETEQL, created by Dr. Robert Hudson, Department of Natural Resources and Environmental Sciences, University of Illinois. Modeling efforts included determining the effect of other metals present in the reagents on the binding capacity of EDTA and potential changes in the speciation of Hg(II). Stability constants (Table 5) from Morel (1983) and the National Institute of Standards and Technology (NIST) standard reference database (version 6.0) of critically selected stability constants (Martell and Smith, 2001) of metal complexes were used.

Activity coefficients were calculated using the Davies equation (1) from Morel (1983) to account for the interactions between electrolytes in the assay solutions.

$$\ln\gamma_i = -AZ_i^2 \left[I^{1/2} / (1 + I^{1/2}) - bI\right] \qquad (1)$$

where γ is the activity coefficient, Z_i is the charge, and I is the ionic strength. A and b are constants with values of 1.17 and 0.3 respectively. Ionic strength (I) was calculated using equation (2) where m is molar concentration of ionic species in solution, and Z is the electrochemical charge of species.

$$I = \tfrac{1}{2} (m_1Z_1^2 + m_2Z_2^2 + m_3Z_3^2 + \ldots) \qquad (2)$$

Table 5.2 Stability constants used in thermodynamic speciation modeling.

Species	*Log K*	*Species*	*Log K*
Hg^{2+}	0	$CuHEDTA^{-}$	23.9
$HgCl^{+}$	7.2	$CuEDTAOH^{3-}$	8.6
$HgCl_2$	14	Zn^{2+}	0
$HgCl_3^{-}$	15.1	$Zn(NH_3)_2^{2+}$	4.5
$HgCl_4^{2-}$	15.4	$Zn(NH_3)_3^{2+}$	6.9
$HgOHCl$	4.1	$Zn(NH_3)_4^{2+}$	8.9
$HgOH^{+}$	-3.4	$ZnHPO_4$	15.7
$Hg(OH)_2$	-6.2	$ZnH_2PO_4^{+}$	21.2
$Hg(OH)_3^{-}$	-21.1	$Zn_3(PO_4)_2$	35.3
$HgNH_3^{2+}$	8.8	$ZnEDTA^{2-}$	18.3
$Hg(NH_3)_2^{2+}$	17.4	$ZnHEDTA^{-}$	21.7
$Hg(NH_3)_3^{2+}$	18.4	$ZnEDTAOH^{3-}$	9.4
$Hg(NH_3)_4^{2+}$	19.1	Al^{3+}	0
$HgPO_4^{-}$	9.5	$AlEDTA^{-}$	18.9
$HgHPO_4$	20.3	$AlEDTAH$	21.6
$HgEDTA^{2-}$	23.5	$AlEDTAOH^{2-}$	12.6
$HgHEDTA^{-}$	27	Ca^{2+}	0
$HgEDTAOH^{3-}$	13.6	$CaHPO_4$	15.2
Cu^{2+}	0	$CaH_2PO_4^{+}$	21
$Cu(NH_3)_2^{2+}$	7.5	$CaEDTA^{2-}$	12.4
$Cu(NH_3)_3^{2+}$	10.3	$CaHEDTA^{-}$	16
$Cu(NH_3)_4^{2+}$	11.8	Mg^{2+}	0
$CuHPO_4$	16.5	$MgH_2PO_4^{+}$	20.8
$CuH_2PO_4^{+}$	21.3	$MgEDTA^{2-}$	10.6
$CuEDTA^{2-}$	20.5	$MgHEDTA^{-}$	15.1

Chapter 3

ANALYSIS OF BIOAVAILABLE MERCURY IN AQUATIC SAMPLES METHODS DEVELOPMENT

Introduction

When I began this research, the *mer-lux* bioreporter was a relatively new method in development for the determination of bioavailable Hg. The host organism was *E. coli*, an enteric bacterium, and its use had been primarily in the lab under defined conditions. Further, the quantification of bioavailable Hg in environmental samples was limited to contaminated environments. The bioreporter had, therefore, never been used in "pristine" environments, nor had it been used to characterize or describe the behaviour of Hg(II) in such environments. As with any new method in development, the existing protocol was modified or changed and new additions made. This chapter describes some of the changes and additions made to the already published method of the *mer-lux* bioreporter. It also includes a description of the new host organism, *Vibrio anguillarum*, and its growth requirements. *V. anguillarum* is believed to be a better representative than *E. coli* for the analyses of bioavailable Hg in aquatic environments because it is not an enteric organism.

The mer-lux Biosensor

***V. anguillarum* pRB28.** The biosensor used for this research was *Vibrio anguillarum* pRB28. It is similar to the original *E. coli* HMS174 pRB28 biosensor (Selifonova *et al.*, 1993), however, as described below, it can be used in a wider range of natural waters, from freshwater to sea water. This is because *V. anguillarum* is a pathogen of marine and

estuarine fish and has also been readily isolated from other aquatic environments (Guerin-Faublee *et al.*, 1995). This also makes it a more environmentally relevant organism than *E. coli* (enteric) for studying aquatic ecosystems. Briefly, pRB28 is a plasmid containing a gene fusion comprising the regulatory region of the narrow spectrum Tn21 *mer* operon and *lux* genes coding for luciferase (*luxAB*) and fatty acid reductase (*luxCDE*). The construct does not specify a Hg(II) uptake system (*merTPC*) or an active mercuric reductase (*merA*). Hg(II) entering cells containing this plasmid will not be reduced, but will cause transcription of the *luxCDABE* genes. Light production under defined conditions is proportional to the amount of Hg(II) entering the cells.

***V. anguillarum* pRB27, the constitutive control.** *V. anguillarum* pRB27 contains a mutant plasmid derived from plasmid RB28, with constitutive expression of *luxCDABE* resulting in continuous light production. Restriction enzyme analysis of pRB27 showed a small deletion upstream of the merR operator/promoter in pRB28 that might have caused the constitutive phenotype (Barkay *et al.*, 1997). *V. anguillarum* pRB27 was used as a control to identify changes in the light-emitting reaction caused by the chemical environments of treatments or environmental samples as opposed to Hg(II)-induced changes in light production. Bioassays using *V. anguillarum* pRB27 were always run in conjunction with assays using *V. anguillarum* pRB28.

Cell Preparation for the mer-lux Bioassay

General Protocol. There are two stages for preparing the *V. anguillarum* pRB27 and pRB28 cells for a *mer-lux* bioassay; growth and harvest. This general cell preparation protocol was modified from Selifonova *et al.*, (1993) for a number of reasons. The first

was to accommodate the different growth and media requirements of *V. anguillarum* (as opposed to *E. coli*). The second reason was to reduce the time required to prepare the cells from three days to 24 hours. A shorter preparation time allows greater flexibility in the field. This is particularly important if there is no control over when samples can be taken (i.e. rain) since samples for bioavailable Hg analyses cannot be preserved or stored for any length of time (discussed below). Finally, to more accurately maintain the sample chemistry during the analyses, the pH of the buffers used to wash and re-suspend the cells was often manipulated to correspond to the pH of the environmental sample being analyzed.

1) Growth. All growth media, including LB broth, LB agar (Difco) and glucose minimal medium (GMM) (Table 4, Chapter 2), contained 50 μg ml^{-1} kanamycin for selection and maintenance of plasmids. One colony of *V. anguillarum*, growing on LB agar plates, was inoculated into two ml LB broth and grown for two hours with shaking (100 rpm) at 26.5° C. 50 μl (*V. anguillarum* pRB28) or 100 μl (*V. anguillarum* pRB27) of the LB culture was then transferred into five ml GMM and grown for 18 hours (*V. anguillarum* pRB28) or 21 hours (*V. anguillarum* pRB27). 20 ml of GMM was then added to the culture flask and cells were grown to mid-log phase and harvested. *V. anguillarum* pRB28 required approximately 2.5 hours to reach mid-log and *V. anguillarum* pRB27 approximately six to eight hours. *V. anguillarum* pRB27 likely requires a longer growth period to attain mid-log due to the energy requirements associated with constitutive light production.

The time at which the biosensor cells were harvested was found to have an important effect on the stability of the light produced. Figure 1 shows two assays

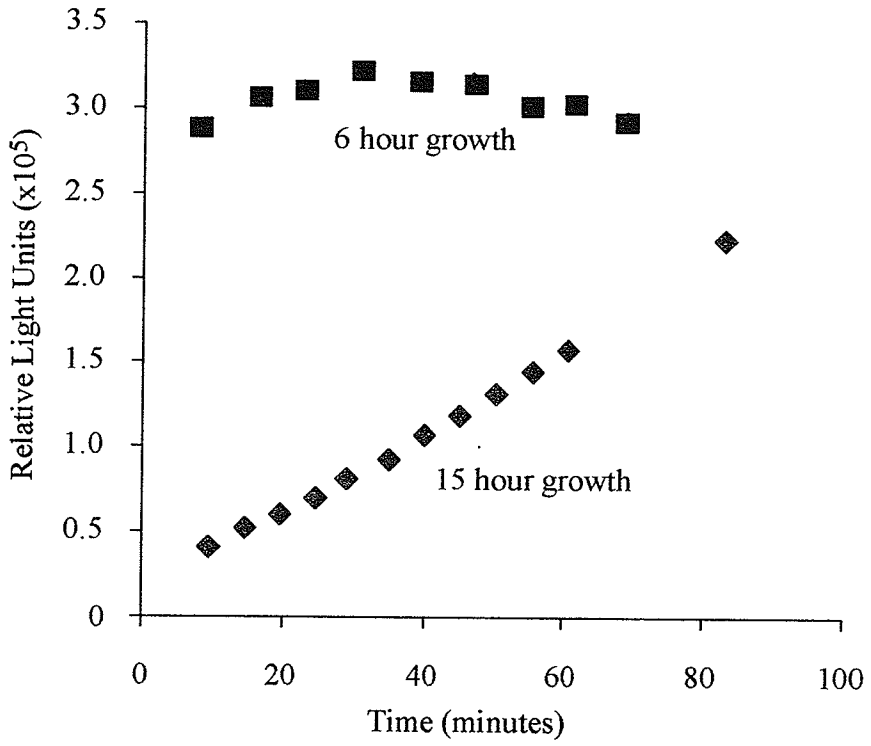

Figure 1.3 The effect of growth time on the stability of light production by *V. anguillarum* pRB27 during a *mer-lux* bioassay. After the second transfer into glucose minimal medium, cells were grown for an additional 6 or 15 hours before being harvested for the bioassay.

conducted with *V. anguillarum* pRB27 at 23 g L^{-1} NaCl. Cells were harvested six hours following the second transfer of GMM (~mid-log), and after 15 hours (closer to stationary phase). Luminescence produced by the log phase cells was more stable than that of stationary phase cells. The observed response with growth phase could reflect metabolic factors affecting the availability of substrate(s) required for the *lux* reaction, such as reducing power for $FMNH_2$ or aldehyde synthesis. With the addition of the assay medium components (nitrogen, carbon and phosphorous), light production increases. This, of course, is undesirable because the relief of no longer being deprived could mask the effects of the treatment being investigated. This would apply to *V. anguillarum* pRB28 as well especially if it is not functioning at full capacity during the induction period. It is, therefore, important to harvest *V. anguillarum* cells near mid-log. However, the effect of the harvest time on luminescence should be verified for any new biosensor.

2) Harvest. Cells were harvested, centrifuged at 10,000 rpm (4° C.) for 10 minutes, and washed once in 67 mM Na-K phosphate buffer. The final pellet was resuspended in three ml buffer and diluted to an OD_{600} of 0.4. This suspension was then diluted at 1:10 in buffer. Depending on the purpose of the experiment being conducted, the pH of the buffer used to wash and re-suspend the cells was often manipulated to maintain the pH of the environmental sample being analyzed. This was accomplished by changing the ratio of $H_2PO_4^-$ and HPO_4^{2-} of the buffer.

Dealing with the question of pH from an analytical perspective is a challenge. In addition to Hg having a pH dependent speciation, the response of the *mer-lux* bioreporter is also strongly pH dependent. Figure 2 shows the bioavailability of Hg(II) decreasing with increasing pH even though it appears that higher pH is more favourable for light

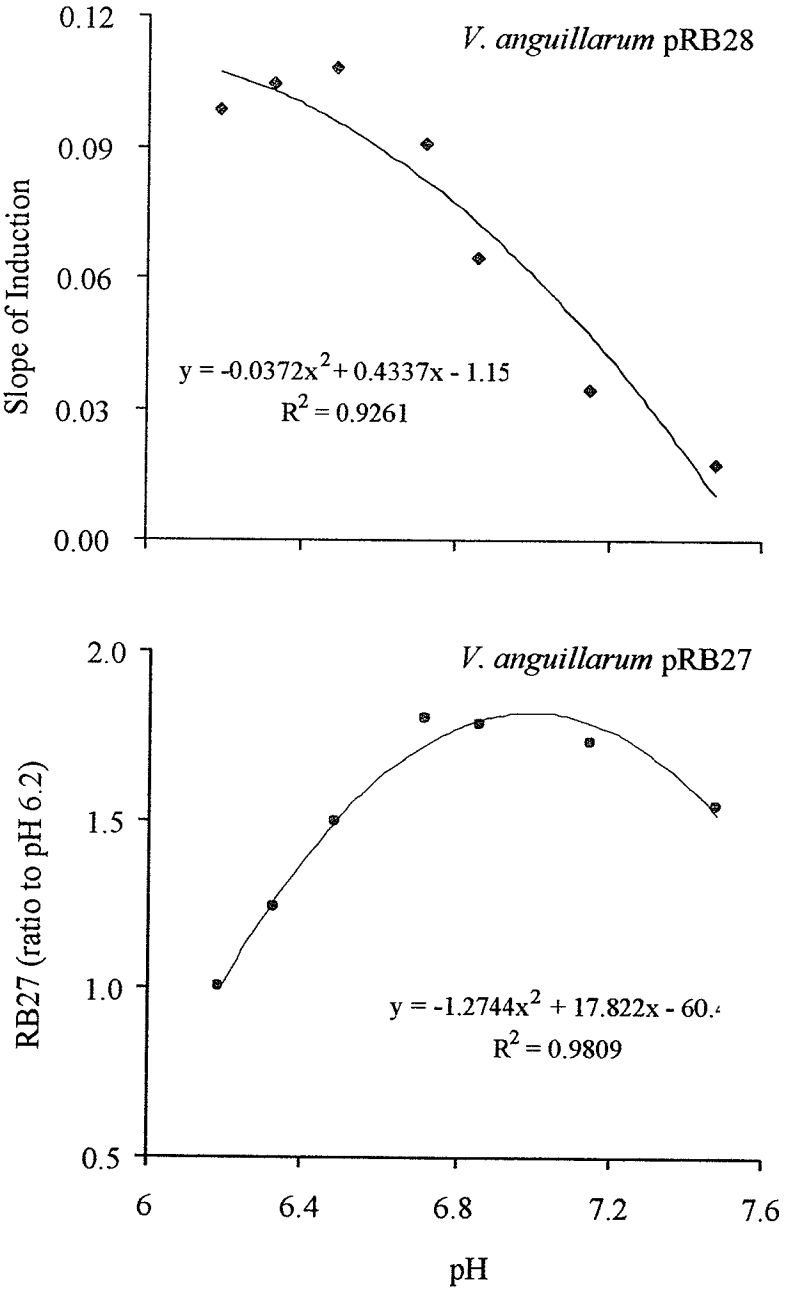

Figure 2.3 Effect of pH on the bioavailability of Hg(II) as measured with the *mer-lux* bioreporter *V. anguillarum* pRB28 and on light production by the constitutive control organism *V. anguillarum* pRB27.

production by *V. anguillarum* pRB27. To confound matters, pH governs the protonation of functional groups on dissolved organic carbon (DOC) in natural waters (Thurmann, 1985). It has also recently been demonstrated that DOC interacts with cell surfaces more readily at low pH thus potentially influencing chemical and physical processes at the cell-surface interface (Campbell *et al.,* 1997), such as enhancing the permeability of the membrane to lipophilic compounds (Vigneault *et al.*, 2000). All of these factors could, in turn, affect the bioavailability of Hg(II). So the general approach taken in this research was to manipulate the pH of the buffers to maintain the pH, hence chemical matrix, of the environmental sample. An exception was in 1999 when rain, run-off and through-fall samples were analyzed at pH 7 to enable a direct comparison with lake waters, which, at the Experimental Lakes Area are mostly circumneutral.

General Protocol Modified for Sea Water. To examine the effects of chloride (Cl^-) on the bioavailability of Hg(II), it was necessary to subject the biosensor cells to high concentrations of NaCl (up to 23 g L^{-1}). To avoid osmotic shock going from a low Cl^- growth medium to a high Cl^- treatment, cells were grown at the same concentration of Cl^- at which the bioassay was to be carried out. In addition, the phosphate buffer used to wash and resuspend the cells contained the same concentration of Cl^- as the medium in which the cells were grown (and bioassayed) thereby maintaining a constant exposure to the same concentration of Cl^- during all stages of the cell preparation. This approach can be extended to, and is recommended for, the analyses of estuarine and seawater samples.

Glycine Betaine. Although *V. anguillarum* is slightly halophilic, it was not able to grow in minimal medium at NaCl concentrations exceeding 10 g L^{-1}. Consequently, to

work at salinities common to estuarine and sea water environments, glycine betaine, an osmoprotectant, was added to the growth medium. Between 0.50 and 0.86 mM glycine betaine was adequate to allow growth of *V. anguillarum* in NaCl concentrations exceeding 10 g L^{-1}. Glycine betaine is a trimethylated derivative of the amino acid glycine, with the three methyl groups attached to the nitrogen ($(CH_3)_3N^+CH_2COO^-$) (Le Rudulier *et al.,* 1984) and was one of the first organic molecules known to be involved in osmoregulatory functions in the plant and animal kingdoms (Galinski, 1995). It is also one of the preferred external osmolytes readily taken up by non-halophilic prokaryotes in order to extend their range of salt tolerance (Galinski, 1995). Glycine betaine also accumulates intracellularly as a function of the external osmotic strength (Le Rudulier *et al.*, 1984). Thus, the addition of glycine betaine to the assay medium (as opposed to the growth medium) was not required to sustain the cells during the assay itself. This was desirable since potential changes in the speciation of Hg(II) or in the rate of uptake of Hg(II) by the cells due to glycine betaine was avoided. Table 1 shows the cell counts for the final cell suspension of *V. anguillarum* cells grown under a variety of NaCl concentrations.

The mer-lux Bioassay

Samples. A "sample" refers to a standard sample, a treatment sample, or an environmental sample such as rainwater. For the standards and treatments, sterile, low Hg milli-Q water was used. All samples were weighed directly into pre-rinsed sample vials for better precision between replicates. Sample vials for the luminometer were disposable polypropylene luminometer tubes (two ml) and for the scintillation counter

Table 1.3 Cell counts for *mer-lux* organisms grown at differing NaCl concentrations.

	RB27			RB28		
NaCl ($g\ L^{-1}$)	Cell count (10^8 cells ml^{-1})	CV%[a]	n[b]	Cell count (10^8 cells ml^{-1})	CV%[a]	n[b]
0	1.87	24	6	2.54	18	7
0.1	1.84	13	5	2.3	31	10
0.5	1.92	16	2	1.73		1
1	1.88	9.1	5	2.47	27	8
2.5	1.62	5.8	2	2.19		2
5	1.48	15	7	2.09	21	3
10	1.56	20	4	2.15		2
15	1.45	21	5	2.23		2
23	1.67	14	1	2.04	11	8

a = coefficient of variability between counts repeated n times

b = number of times that plate counts were determined using quadruplicate plates

were borosilicate glass scintillation vials (20 ml).

The Assay Medium. To support metabolic activity and protein synthesis by the *mer-lux* organisms during the assay, the addition of nutrients (carbon, nitrogen, phosphorus) to all samples, including environmental samples, is essential. However, Hg(II) forms coordinate and covalent bonds with ammonia and phospho groups resulting in speciation changes of Hg(II). Therefore, the criteria used to determine the most favourable combination of nitrogen, carbon, and phosphorus included (1) achieving an optimal detection limit, (2) minimizing stress to the organisms, and (3) adding as little as possible to the samples.

Unlike its predecessor *E. coli* HMS174 pRB28, *V. anguillarum* pRB28 does not require the addition of 67 mM phosphate to the assay medium. In fact, *V. anguillarum* pRB28 performs equally well when no phosphate, other than the 3 mM carried over in the cell suspension, is added to the samples (provided 9.1 mM $(NH_4)_2SO_4$ is also added). This is advantageous when analyzing seawater since phosphate forms a precipitate with carbonates. Figure 3 shows the relative effects of varying concentrations of $(NH_4)_2SO_4$ and phosphate as determined by the "Method of Ratios" described below. Overall, the highest signal and therefore best detection was achieved using 3 mM phosphate and 9.1 mM $(NH_4)_2SO_4$, or else 67 mM phosphate at either 9.1 mM or 0.091 mM $(NH_4)_2SO_4$. The combination of low phosphate (3 mM) and low $(NH_4)_2SO_4$ (0.091 mM) was an undesirable option because it resulted in a reduced signal, greater variability and more stress imposed on the organisms as shown by the constitutive control *V. anguillarum* pRB27 (Figure 3). Ultimately, 9.1 mM $(NH_4)_2SO_4$, 3 mM phosphate and 5 mM glucose were added as nutrients to all samples described in this thesis, unless otherwise stated.

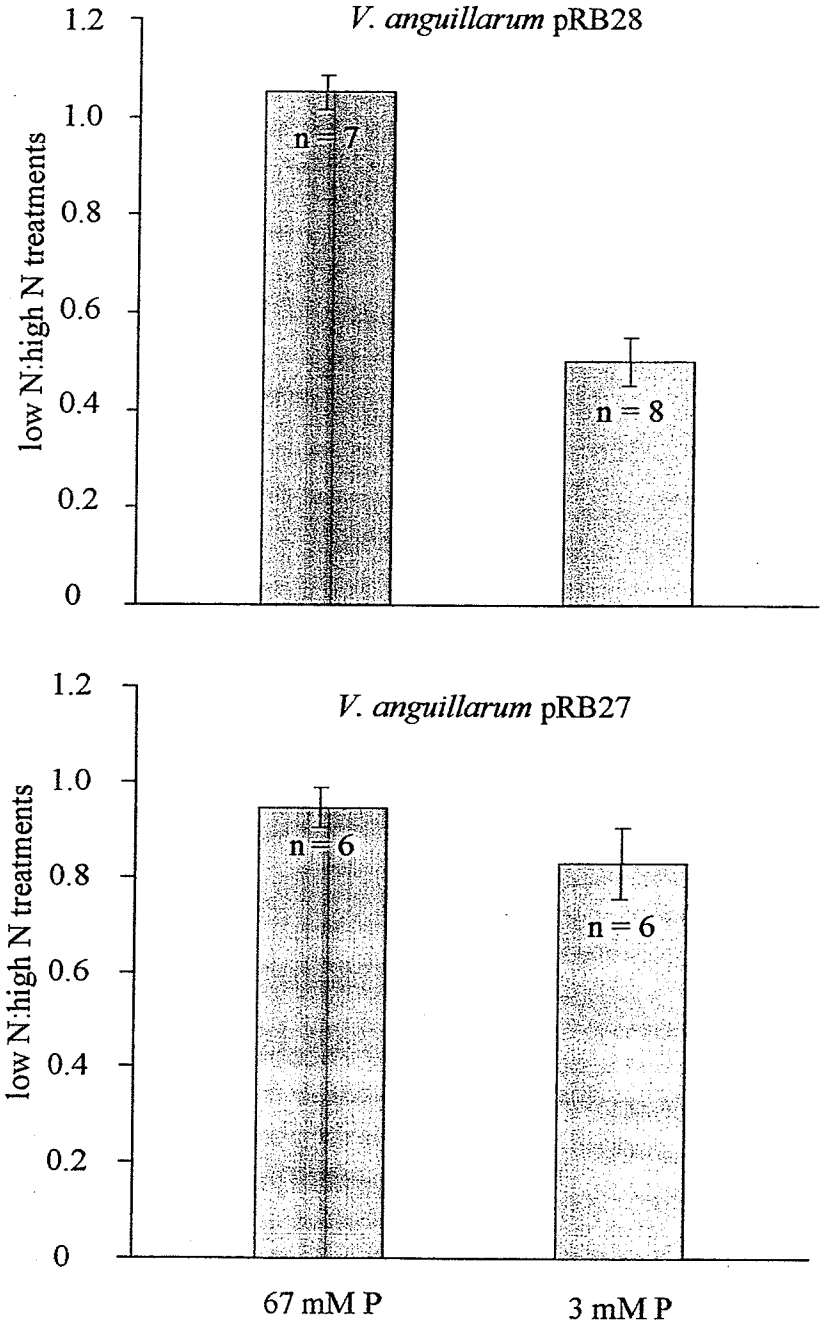

Figure 3.3 The relative effects of "low" (0.09 mM) and "high" (9 mM) nitrogen (N) as $(NH_4)_2SO_4$ in the presence of 67 mM or 3 mM phosphate buffer (P) on the Hg(II)-induced response (*V. anguillarum* pRB28) and on luminescence (*V. anguillarum* pRB27). Error bars are standard error. 'n' is the number of replicate assays, with all treatments run in duplicate.

These concentrations were insufficient to support growth and replication thus permitting a constant cell density throughout the bioassay.

Final Steps. Appropriate volumes of Hg(II) were added to the standards and treatments (see *Hg(II) Standard Preparation* in Chapter 2). As the final step, the biosensor cells were added to every sample (standards, treatments and/or environmental sample) at either 30 or 45-second intervals. When light measurements were made with a field luminometer (MGM Instruments, Model BG-P), 100 µl of the prepared cell suspension was added; with a scintillation counter (Beckman LS 6500, single photon counting), 1 ml of cell suspension was added. The final concentration of cells per sample was 1.1×10^5 cells ml^{-1} and 8.5×10^4 for RB28 and RB27, respectively. Light measurements were started immediately after the addition of cells and continued at 10-minute intervals for up to two hours. Because samples were counted every 10 minutes, the maximum number of sample vials was restricted to between 12 and 16.

Standardization of the mer-lux Bioassay

Linear Regression Analyses. Figure 4 (top panel) shows a typical induction curve for a *mer-lux* bioassay conducted at pH 7. There is an initial lag period, followed by an increase in light production (induction slope) and a plateau. The lag period likely reflects the time required for Hg(II) to enter the cell, protein synthesis to occur and light production to commence. The plateau likely occurs when the substrates for the light reaction are no longer readily available to the cell. So far, the parameter that best relates the biological response of the *mer-lux* organisms to the Hg(II) concentration in the standards is the initial log linear induction slope at its maximum rate of increase (Barkay

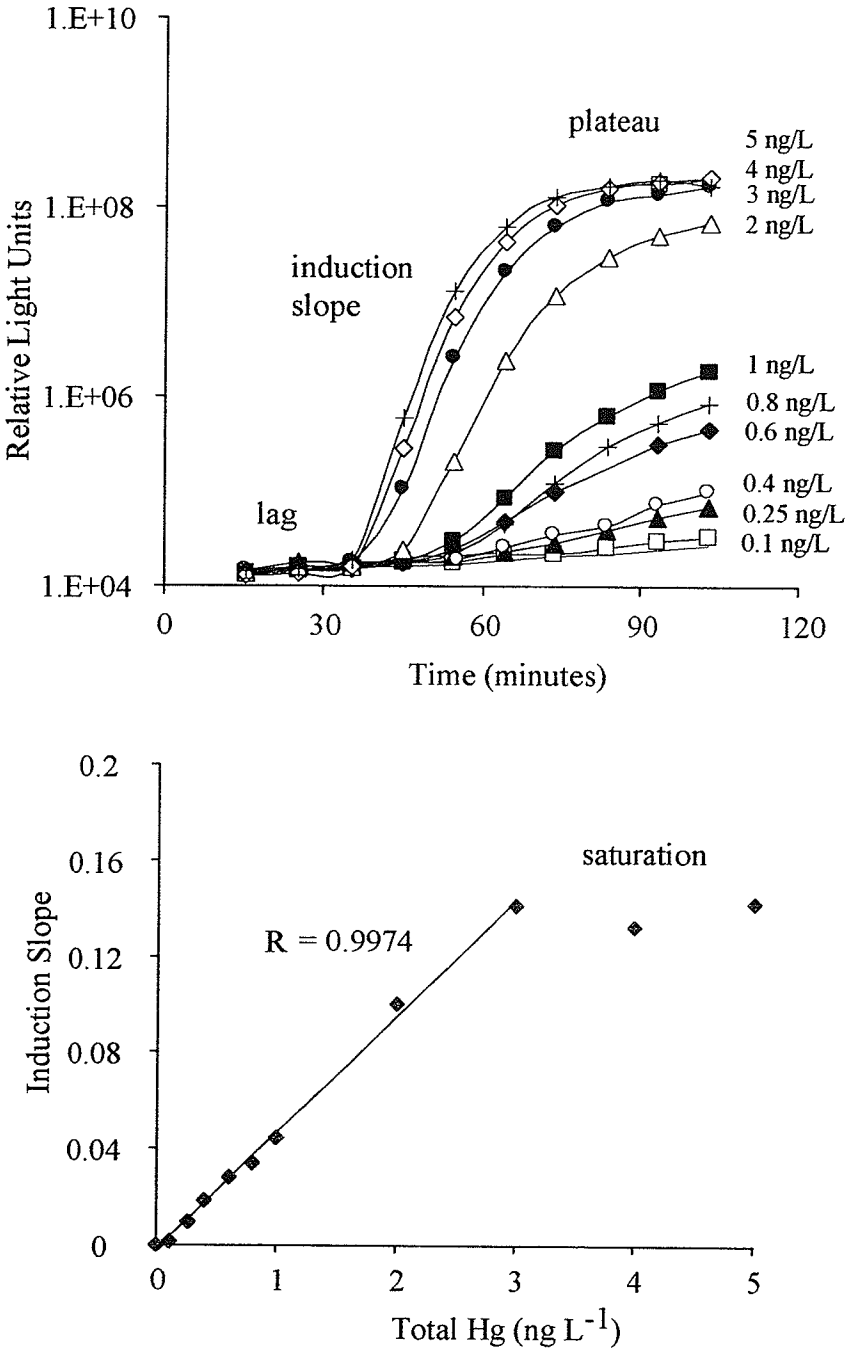

Figure 4.3 Raw induction curves (top panel) for a *mer-lux* bioassay conducted at pH 7 in 5 mM glucose, 9.1 mM $(NH_4)_2SO_4$, 3 mM phosphate and varying concentrations of Hg. Standard curve (bottom panel) determined by linear regression analyses using the log of the slope of induction as indicated in the top panel.

et al., 1998).

The relationship between induction slope and Hg(II) concentrations in the standards is generally highly linear (Barkay *et al.,* 1998) and is, therefore, used to determine the standard curve regression (Figure 4, bottom panel). At pH 7, saturation of the *mer-lux* system occurs at ~3 ng L^{-1} Hg (15 pM). At pH 6, however, saturation occurs at a lower concentration of Hg(II) (~0.75 ng L^{-1} or 3.75 pM) due to the greater sensitivity of the assay at this pH. It is, therefore, very important to work within the linear range at the respective pH. For the quantification of bioavailable Hg in environmental samples, a standard curve was run for each assay and was generally comprised of duplicates of three concentrations of Hg, in addition to duplicate blanks.

The "Method of Ratios". Due to the limited number of samples that could be analyzed at one time, it was necessary (sometimes preferable) to maximize the number of treatments at the expense of the standard samples. I refer to this approach as the "Method of Ratios", normally used when relative differences between given treatments were sought and a standard curve was not essential. This approach has limitations, of course, because replicate bioassays can not be averaged and statistically analyzed without standardizing the assays first. To overcome this drawback, I calculated ratios between the treatments within a given assay and then averaged replicate assays using those ratios. In essence, this approach is a relative standardization as opposed to a quantitative standardization that a standard curve derived from a linear regression would provide.

The "Method of Ratios" and *V. anguillarum* pRB27. This 'relative' approach was also implemented for the assays using the constitutive organism *V. anguillarum* pRB27, which does not respond to Hg(II) unless it causes a toxic or stimulatory response.

In this case, duplicate distilled water blanks containing the required bioassay nutrients (described above) were always run in conjunction with the environmental samples being analyzed. The ratio of the light produced by the environmental sample relative to the light produced by the distilled water blanks was determined at each interval that the samples were counted (usually every 10 minutes) and then averaged. A ratio exceeding one indicated that the constituents in the environmental sample stimulated light production by the constitutive organism; a ratio less than one inhibited light production.

Initially, this ratio was used in a quantitative fashion by applying it as a correction factor to the Hg(II)-induced response generated by *V. anguillarum* pRB28. Upon further reflection, however, I questioned the validity of this approach since it uses a ratio, derived from absolute values of light produced, to correct an exponential change over time. Furthermore, physiological effects resulting in a decrease in light production does not necessarily equate to decreased uptake of Hg(II). Consequently, instead of applying a correction, data from both organisms (*V. anguillarum* pRB28 and pRB27) are presented separately.

Analytical Considerations for the Analysis of Bioavailable Hg in Aqueous Samples

Sample Storage. Traditional Hg sample preservation methods could not be employed for the preservation of water for bioavailable Hg analyses. For example, freezing could change the structure of the DOC molecules which would in turn affect the bioavailability of Hg, and acidification would be toxic to the *mer-lux* organisms. Moreover, bioavailable Hg is not stable. Figure 5A shows the rapid change in the ambient (natural)

concentration of bioavailable Hg measured with *V. anguillarum* pRB28 in unfiltered stream run-off after one day and 24 days of storage. The instability of bioavailable Hg will differ depending on the source of the water; atmospheric samples, such as rain and snow, appear to be the most labile. Interestingly, storage of water samples can also stimulate light production as shown by *V. anguillarum* pRB27 (Fig 5B). This response may be due to the change in water chemistry over time resulting in a greater availability of substrates, which stimulate light production. These types of observations led to the conclusion that environmental samples for bioavailable Hg analyses cannot be stored and they were, therefore, always analyzed within hours of being sampled. It was, however, impossible to analyze snow samples on site and they were maintained frozen until analyzed. The stability of bioavailable Hg in stored snow samples remains to be tested.

Sample Handling in the Field. Between sampling and analyses, it is essential to keep water samples for bioavailable Hg analyses in the dark at all times, including while still in the field. This was particularly important for water containing high concentrations of DOC. Figure 6 shows the effect of exposing a highly coloured water sample (from the northeast inflow stream entering lake 239 at the ELA) to direct sunlight on background luminescence, measured with a scintillation counter. The high background noise, presumably chemiluminescence caused by the absorption and release of radiant energy by DOC molecules, could easily mask the Hg(II)-induced response during the analysis of bioavailable Hg. Guarding against UV exposure also prevents photolytic speciation changes such as the reduction of Hg(II) to elemental Hg (Hg^0). In addition to water samples, snow samples were kept in the dark at all times including during the bioassay.

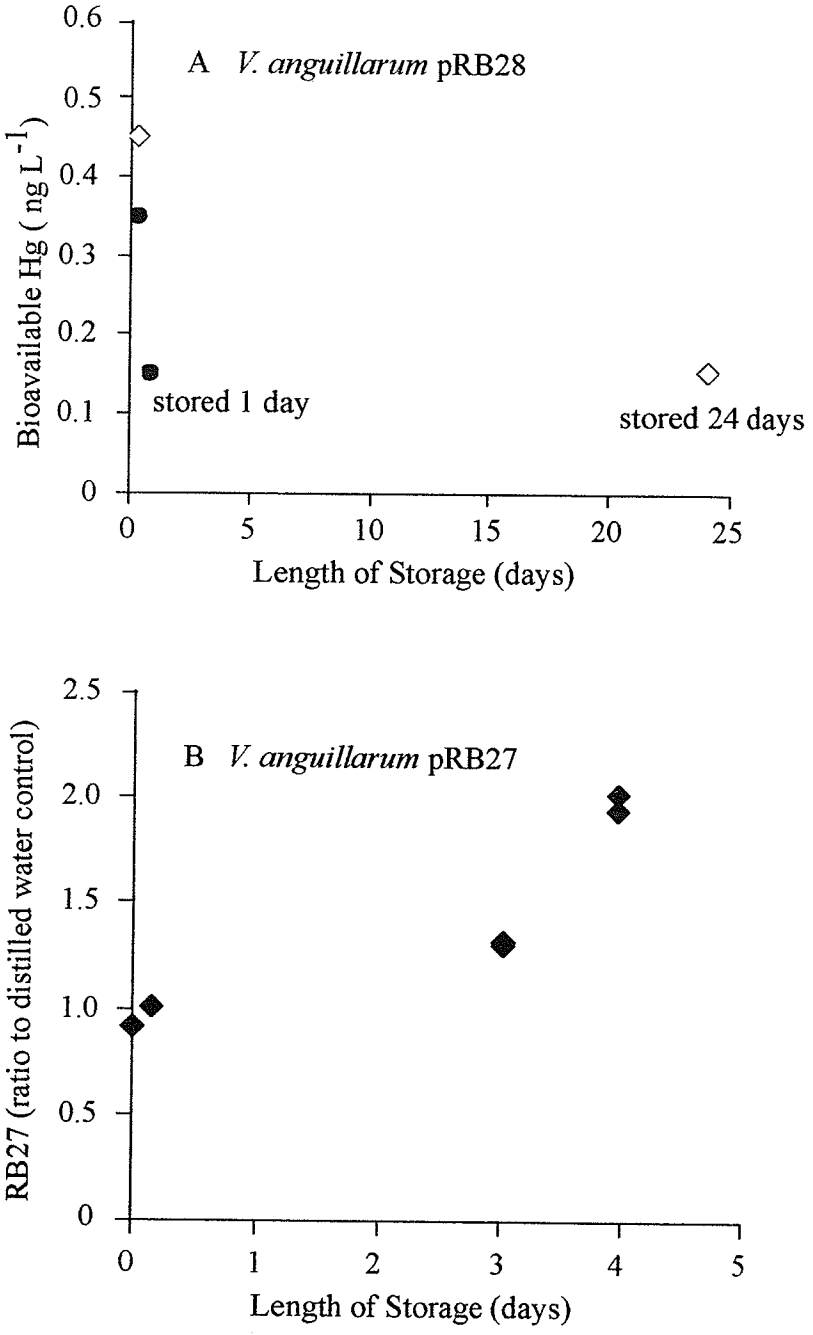

Figure 5.3 The effect of sample storage on the response of *V. anguillarum* pRB28 (top) and pRB27 (bottom). All samples were from terrestrial run-off streams at the Experimental Lakes Area; U1f sub-catchment in the 302 uplands (top) and the upland stream at lake 658 (bottom).

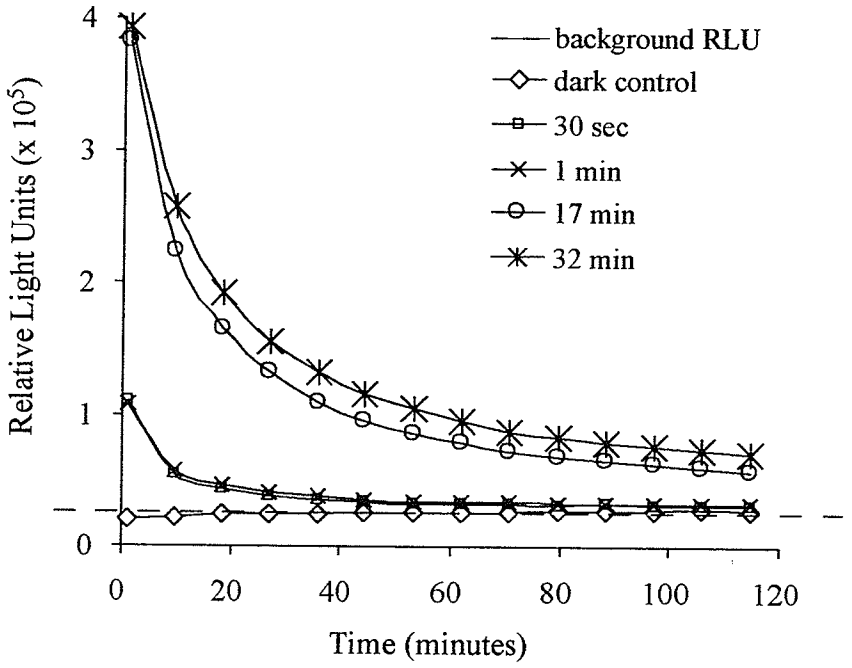

Figure 6.3 Chemiluminescence emitted by a high DOC stream water sample (lake 239 east in-flow) exposed to sunlight for varying lengths of time up to 32 minutes. Light measurements were made with a scintillation counter. Samples did not contain *mer-lux* biosensor cells.

Sample Handling in the Lab. Sample handling in the lab is equally important as in the field. Figure 7 shows the effects of temperature and light on *V. anguillarum* pRB27. Duplicate stream water samples from the lake 658 watershed were either covered at 20° C., left uncovered (but not in direct sunlight) at 20° C, or kept at 10° C. Temperature would logically be an important factor affecting bacteria and a low sample temperature clearly has a profound effect on the biosensor response (Figure 7). Although the effect of *elevated* temperatures on luminescence was not tested, it would also likely affect the response of the biosensors since the *in vitro* half-life of the luciferase (*V. fischeri*) enzyme at 38° C. is only ~1 minute. So even a transient increase of the biosensor temperature for a few minutes could accelerate the decay of bioluminescence signal (Heitzer *et al.*, 1994). Regulating temperature is not always easy in a field lab, especially during the spring and fall. Nonetheless, unlike other Hg analyses, extra care must be taken to ensure that water samples, reagents used for the bioassay, and the Hg clean lab itself are maintained under appropriate and consistent conditions for the analyses of bioavailable Hg. Inconsistency in sample and reagent handling will lead to increased variability in the results.

Hg Addition Approach to Studying Bioavailable Hg. As will be discussed in the following chapters, the concentration of bioavailable Hg in most aquatic samples was very low and in lakes, not detectable. This finding was not unexpected since bioavailable Hg would only be a fraction of the total Hg concentrations, which are also generally low. To study the behaviour of bioavailable Hg in lake waters and reservoirs, Hg additions were required. There are inherent limitations with this approach within which one must work and design experiments accordingly.

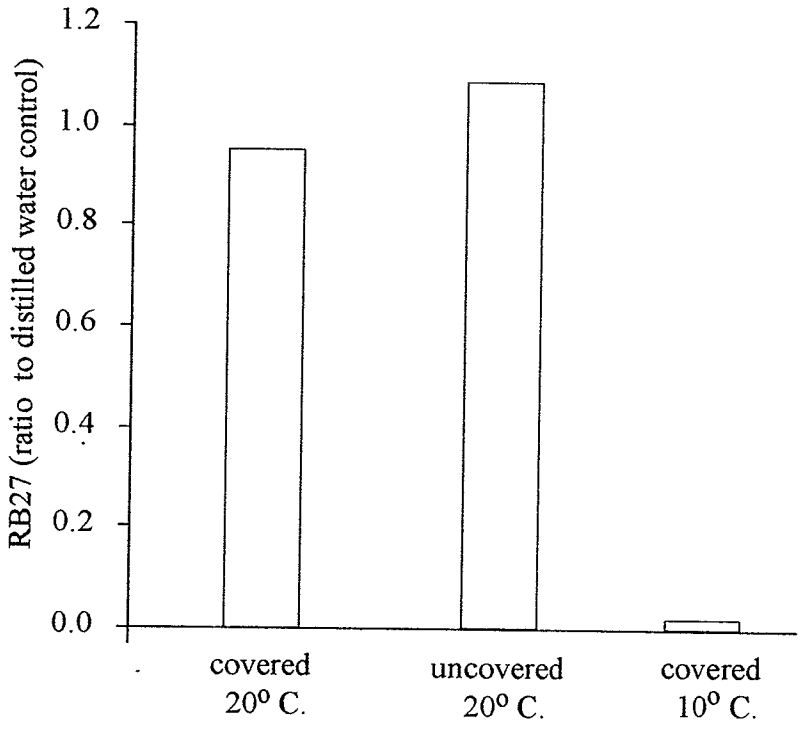

Figure 7.3 In lab variables affecting the light production by *V. anguillarum* pRB27 in lake 658 upland stream water samples. Uncovered samples were not exposed to direct sunlight.

When Hg is added to lake water for example, the concentration of bioavailable Hg changes over time –so kinetically, the system does not reach equilibrium. Moreover, the concentration of bioavailable Hg will increase with increasing concentrations of total Hg added, thereby making it difficult to quantitatively compare the bioavailability between samples. This would be expected to a certain degree; add more total Hg and bioavailable Hg increases as well. However, the extent to which bioavailable Hg increases is not consistently proportional over a range of Hg concentrations added. This is illustrated in Figure 8 where the Hg addition approach was used on fractionated water samples from the FLUDEX reservoirs (see Chapter 5 for details). In this particular example, the permeate sample shows saturation at low Hg concentrations whereas the retentate fraction does not. It is, therefore, important to characterize the sample to define the range of Hg additions in which to work. For environmental work, it is also essential to work at low concentrations of Hg. Not only can the linear range of the *mer-lux* system be exceeded (Figure 4), but the capacity of specific functional groups on DOC to bind Hg could be surpassed (see Chapter 4). Moreover, if Hg is indeed being brought into the cell by a facilitated uptake mechanism (see Chapter 6), high concentrations of Hg could saturate the transporter.

Future Considerations for the Development of the mer-lux Bioreporter

Contaminant Trace Metals. The biological and chemical implications of contaminant metals in the reagents and growth medium could be an important variable affecting the uptake of Hg(II) and/or binding capacity of synthetic and organic ligands in natural waters. The concentrations of metals present in the respective analytical reagents used in

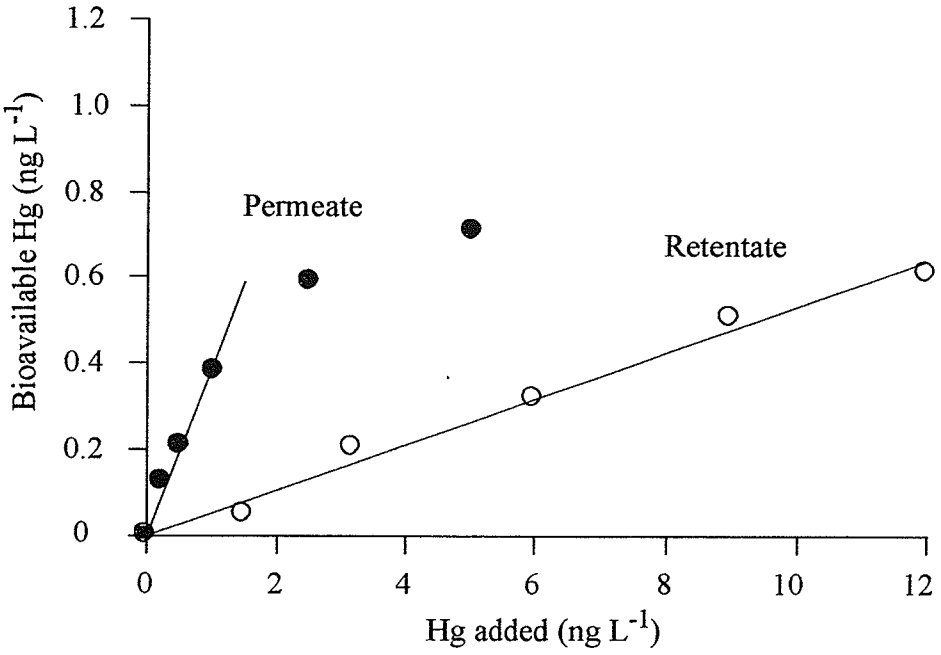

Figure 8.3 Comparative difference in the bioavailability of Hg(II) added to fractionated water from the FLUDEX reservoirs. The permeate fraction required lower concentrations of Hg to saturate the *mer-lux* system than did the retentate fraction.

the *mer-lux* bioassay are listed in Table 2 and were included in thermodynamic speciation modeling efforts involving the chelator EDTA. An important point regarding contaminant metals is that a variety of metals present at low concentrations can theoretically compete with Hg for EDTA despite the large stability constants of Hg-EDTA complexes (Table 5, Chapter 2). This is illustrated in Figure 9 where increased concentrations of EDTA are required to overcome the contaminant metals. The reason for this is largely due to the fact that we are working at extremely low levels of Hg, exceeded by other metals even when their concentrations are low. With the achievement of improved levels of detection comes the responsibility of ensuring that there are no artifacts associated with it. At present, we do not know what the effects of contaminant metals are on the *mer-lux* bioassay apart from what can be predicted using thermodynamic speciation modeling. Therefore, a critical next step in the future development of the bioreporter is to address this issue experimentally by using some of the already existing trace metal clean techniques typically followed by oceanographers and trace metal chemists. These include following an extremely strict protocol in the lab to minimize random contamination from equipment (R. Hudson, pers. comm.), using chelex resins to remove metals from reagents (B. Landing, pers. comm.), and carefully controlling the organisms' exposure to trace metals during growth with metal ion-buffered growth media (Sunda & Huntsman, 1992).

Table 2.3 Concentrations of trace elements in reagents used in the *mer-lux* bioassay. Samples were acidified and analyzed using ICP mass spectrophotometry by Enviro-Test Laboratories Manitoba Technology Centre. A/C = autoclaved

Element	Detection Limit mol L^{-1}	glucose (1.67 M) mol L^{-1}	$(NH_4)_2SO_4$ (0.91 M) mol L^{-1}	NaH_2PO_4 (1 M) mol L^{-1}	K_2HPO_4 (1 M) mol L^{-1}	NaCl (1 M) mol L^{-1}	Hg stock (1 ug ml^{-1}) mol L^{-1}	MQ A/C mol L^{-1}
Al	3.7E-07	3.7E-07	3.7E-06	4.1E-06	5.8E-05	3.3E-06	3.7E-07	
Sb	8.2E-09		2.3E-07	1.6E-07	1.5E-07	8.7E-08		
As	6.7E-09				2.4E-06			
Ba	2.2E-09	7.3E-09	6.6E-09	5.8E-08	2.7E-07	8.7E-09		
Be	1.1E-07				4.4E-07			
Bi	4.8E-10	3.8E-08	2.1E-07	1.7E-06	1.1E-05	4.6E-07		1.1E-07
B	2.8E-07				4.6E-08			
Cd	1.8E-09		2.7E-09	5.3E-09				
Ca	1.2E-06	9.7E-05	1.1E-05	8.2E-06	3.3E-05	1.1E-05		
Cs	7.5E-10		8.3E-08		2.3E-07	2.1E-07		
Cr	1.9E-08	3.8E-08	9.6E-09	1.5E-08	2.1E-08	1.2E-08		
Co	3.4E-09	1.2E-08	1.7E-08	2.1E-07	2.1E-07			
Cu	7.5E-09				1.1E-08			
Fe	1.8E-07		1.4E-05		9.5E-06	5.9E-06	3.6E-07	
Pb	4.8E-10	1.9E-09	3.4E-09	1.7E-08	1.2E-08	7.2E-09	1.4E-09	
Li	1.4E-07							
Mg	4.1E-07	8.2E-07	8.2E-07	2.9E-06		1.2E-06		
Mn	3.6E-09	3.6E-08	2.4E-08	3.8E-08	1.2E-06	6.4E-07	2.2E-08	
Mo	1.0E-09		3.6E-08	8.0E-08	2.9E-08	2.1E-09		
Ni	3.4E-09	1.5E-07	3.6E-08	4.3E-08	3.0E-07	7.5E-08		
P	6.5E-07	1.6E-05		9.0E-01	8.7E-01	3.7E-05	1.9E-06	
K	1.3E-06			3.2E-05	1.9E+00	2.0E-04	5.9E-06	
Rb	2.3E-09	3.5E-09		4.7E-09	4.9E-05	1.4E-08		
Se	2.5E-08	2.8E-04	1.1E-05	1.5E-05	4.8E-04	1.8E-05		
Si	7.1E-06			2.8E-07				
Ag	1.9E-09				5.7E-08	3.7E-08		
Na	2.2E-07	1.9E-05	1.7E-05	1.0E+00	1.8E-03	9.7E-01	4.3E-06	1.6E-06
Sr	1.1E-09	1.0E-08	1.9E-08	4.0E-08	9.7E-08	3.8E-07		
Te	3.9E-09		7.8E-10	3.9E-09	2.4E-09	1.6E-09		
Tl	4.9E-10				1.4E-08	9.8E-10		
Sn	1.7E-09	2.6E-08	5.1E-09	1.1E-07	1.7E-08			
Ti	1.0E-08	2.9E-08	5.3E-06	5.0E-06	5.5E-06	2.8E-07		
W	1.1E-09			7.6E-09	2.7E-09			
U	4.2E-10	9.2E-07			8.4E-09			
V	2.0E-08				2.4E-08			
Zn	7.6E-08	1.2E-07	2.4E-06					
Zi	2.2E-09			5.0E-08	2.5E-06	1.6E-08		

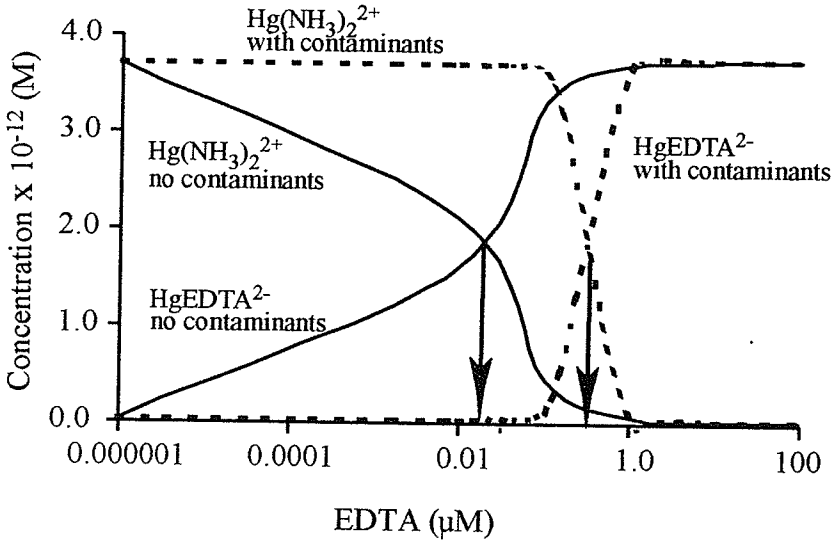

Figure 9.3 Thermodynamic speciation modeling output to illustrate the effect of contaminant metals on the efficacy of EDTA in complexing Hg(II). Model input included 9.1 mM $(NH_4)_2SO_4$, 3 mM phosphate, 0.75 ng L^{-1} Hg (3.7 pM), varying concentrations of EDTA, 8.4 x 10^{-10} M Cu, 2.5 x 10^{-8} M Zn, 1.4 x 10^{-7} M Al, 4.7 x 10^{-7} M Ca, 1.6 x 10^{-8} M Mg, and 3.2 x 10^{-6} M Cl, at pH 7.

Chapter 4

BIOAVAILABLE HG IN AQUEOUS SAMPLES ITS SOURCES TO & BEHAVIOUR IN LAKES AT THE ELA

Abstract

Bioavailable Hg measurements using a *mer-lux* bioreporter were made for the first time on a variety of aquatic samples, cumulatively known as "input sources", as a preliminary assessment of source strengths of bioavailable Hg entering lakes at the Experimental Lakes Area. All input sources had detectable bioavailable Hg. In precipitation, through-fall and run-off, bioavailable Hg represented on average 1 to 2.5% of the total Hg, and in snow, nearly 50% of the total Hg. Bioavailable Hg was not detectable in lakes.

The *mer-lux* bioreporter was also used to examine the behaviour of Hg(II) in four distinctly different lakes at the Experimental Lakes Area by conducting trace level Hg addition experiments and measuring subsequent changes in its bioavailability. Up to 90% of the added Hg was immediately bound, due primarily to complexation in the dissolved phase. The remaining Hg(II) that was bioavailable decreased to ~5% over the course of up to three hours, and to <5% over 24 hours. When higher Hg concentrations were added (up to 20 ng L^{-1} Hg or 100 pM), the proportion of bioavailable Hg increased with increasing Hg concentration. This behaviour was likely due to the saturation of functional groups on dissolved organic carbon (DOC) and other dissolved constituents, as more Hg was added.

Introduction

The global nature of Hg pollution is due primarily to its predominance (≥ 80%) in the atmosphere as elemental Hg vapour (Hg^0) (Lindqvist and Rodhe, 1985), and to the existence of other reactive Hg species that have more regional deposition patterns (Lindberg and Stratton, 1998). The residence time of Hg^0 vapour in the atmosphere is estimated to be ~1 year (Lindqvist and Rodhe, 1985) due to its relative chemical stability and low water solubility (Schroeder and Munthe, 1998) thus permitting its dispersion in the atmosphere over long distances. Over time, atmospheric Hg^0 is oxidized to a more water soluble form, e.g. divalent Hg (Hg(II)), and scavenged from the atmosphere by wet and dry deposition (Brosset, 1981). The wide distribution of Hg via atmospheric processes, and subsequent deposition to terrestrial and aquatic ecosystems, including those in remote areas far from point source pollution, has gained Hg the title of a toxic global pollutant.

In freshwater environments, Hg(II) is the substrate for two important microbial processes; the reduction of Hg(II) to Hg^0 and the methylation of Hg(II) to methyl Hg, MeHg (Osborn *et al.*, 1997). In oceans, Hg(II) is the substrate for the microbial production of dimethyl Hg, $(CH_3)_2Hg$ (Pongratz and Heumann, 1998). Both Hg^0 and $(CH_3)_2Hg$ readily evade from lake and ocean surfaces back to the atmosphere (Mason *et al.*, 1995; Pongratz and Heumann, 1998). MeHg, on the other hand, is a highly toxic form of Hg that biomagnifies in aquatic food chains. Fish that live in relatively pristine environments far from point sources of Hg emissions have been found to have elevated levels of MeHg in their tissues. This has led to fish consumption guidelines and health advisories in Scandinavia, North America, and elsewhere (Grigal, 2002) including the

Arctic where elevated levels of MeHg have been reported in marine mammals (Wagemann *et al.*, 1995). For Hg(II) to become microbially transformed, however, it must first enter the bacterial cell or be "bioavailable". Identifying the sources of bioavailable Hg and understanding the factors that control the concentration of bioavailable Hg in lakes is thus important to understanding the microbially mediated processes contributing to the biogeochemical cycling of Hg in aquatic ecosystems.

During the past fifteen years, there have been many advances in the measurement of trace levels of inorganic Hg in environmental samples by chemical methods (Bloom and Crecelius, 1983; Bloom and Fitzgerald, 1988; Stratton and Lindberg, 1995). However, these methods do not provide information on the fraction of Hg(II) that is bioavailable to bacteria and therefore has the potential to be biotically transformed to other Hg species. A new analytical method, using genetically engineered bacteria that produce light when Hg(II) enters their cells (Selifonova *et al.*, 1993), has provided a way to gain insight into the fraction of Hg(II) that is bioavailable, and into factors controlling its bioavailability.

The objectives of this research were to determine the concentration of bioavailable Hg in aquatic environmental samples at the Experimental Lakes Area, including in precipitation, snow, through-fall, run-off, and lake waters, and to explore the nature of Hg(II) bioavailability in ELA lakes, which are typical of those in boreal regions impacted by Hg pollution. The fate of Hg(II) entering lakes as atmospheric precipitation was simulated by adding trace amounts of Hg to water from four lakes of differing DOC concentration.

Methods

The research described in this chapter was carried out at the Experimental Lakes Area in northwestern Ontario, Canada. A general description of the area and lakes is presented in Chapter 2.

Input Sources. The protocol used for sampling input sources of bioavailable Hg (snow, precipitation, run-off, and through-fall) was also described in Chapter 2. To allow the direct comparison between sample types, only those samples analyzed at pH 7 are presented in the 1999 data set. In 2000, the run-off samples were analyzed at a pH more closely resembling the natural pH of the sample and were, therefore, presented separately from the 1999 input source data.

Lake "Time Series" Experiments. Each time series assay included 16 samples: 8 standards and 8 water samples from one of the four study lakes. For each set of water samples, 1.71 ml (or 1.55 ml for lake 661) of unfiltered or filtered (0.45 μm) lake water was dispensed into luminometer tubes immediately prior to the Hg additions. Hg was added for a final concentration of 5 ng L^{-1} (25 pM) for lakes 240, 224 and 239 and 10 ng L^{-1} (50 pM) for lake 661 at different times between 0 and 180 minutes before commencing the assay. The assay began with the addition of cells to the standards and samples.

The Hg solution used to spike the samples was prepared fresh for every spike addition, and each lake was assayed twice. During the Hg addition period prior to starting the assay, samples were covered to protect them from light, which causes a substantial DOC related chemiluminescence (Figure 6, Chapter 3). Additional assays were also carried out with water from lakes 224 and 240 except additions were continued

over the course of approximately 24 hours. It was confirmed through other experiments in milli-Q water that sorption of Hg to the sides of the luminometer tubes does not occur over a four hour period (data not shown).

Lake "Concentration Series" Experiments. For the "concentration series" experiments, the relationship between the amount of Hg added to lake water samples and bioavailable Hg was examined. In 1998, unfiltered lake water samples (from the same four lakes as the "time series" experiments) received Hg additions resulting in final concentrations between 0 and 20 ng L^{-1} (100 pM), and were assayed immediately after the additions. In 1999, this experiment was repeated with unfiltered water from lakes 661, 240 and 224.

Hg(II) Volatilization Experiments. To determine if volatilization of Hg(II) to Hg^0 was contributing to the overall loss of bioavailable Hg over time, duplicate lake water samples (125 ml) received additions of Hg for a final concentration of 5 ng L^{-1} (25 pM) for lakes 239, 240 and 224 and 10 ng L^{-1} (50 pM) for lake 661. One of the duplicates from each lake was then acidified immediately and the second of the duplicates was acidified approximately three hours later; the same length of time as in the "time series" experiments. Prior to acidification, samples were gently shaken, opened and closed three times to allow any gaseous Hg^0 that may have formed to leave the sample bottles. All samples were then analyzed for total Hg concentrations.

When lake water samples were acidified immediately upon addition of Hg, over 90% of the addition was recovered in all lakes (Table 1). When spiked samples sat for three hours and were then acidified, between 85 and 94% of the spike was recovered (Table 1). The less than 100% recovery observed in these samples may be due to either

Table 1.4 Percent of the total Hg recovery following addition of $HgCl_2$. Samples were either immediately acidified or acidified after three hours following the Hg addition. $HgCl_2$ additions were 5 ng L^{-1} for lakes 224, 240 and 239 and 10 ng L^{-1} for 661.

Lake	% total Hg recovery Time (0)	% total Hg recovery Time (3 hours)
224	94	94
239	92	90
240	91	85
661	91	86

analytical error or to a small immediate loss by volatilization as Hg^0. The majority of the added Hg, however, was present for the duration of these experiments.

Results & Discussion

Input Sources of Bioavailable Hg and Total Hg

This study represents the first attempt to quantify bioavailable Hg in aquatic samples from a remote, "pristine" ecosystem. Bioavailable Hg measured in 1999 was detectable in all of the input sources including rain, snow, run-off and through-fall samples (Figure 1A). Interestingly, none of the 24 lake or reservoir samples analyzed had detectable bioavailable Hg without adding Hg. Therefore, based on the detection limit of ~0.05 ng L^{-1} or 0.25 pM (scintillation counter), the maximum percentage that *could* have been bioavailable in the surface waters of these lakes was less than ~5% of the total Hg concentrations. With the exception of snow, the highest concentrations of bioavailable Hg were in through-fall, followed by rain, and run-off (Figure 1A, Table 2). Concentrations of total Hg followed the same general trend as bioavailable Hg; highest in through-fall samples, followed by precipitation and run-off, and lowest in lakes (Figure 1B, Table 2). The percentage of the total Hg that was bioavailable was low; ~1% for through-fall and run-off, and 2.5% for precipitation (Figure 1C). It appears that regardless of its source, bioavailable Hg represents a very small and fairly consistent proportion of the total Hg pool.

Measurements of bioavailable Hg in run-off were continued in 2000, increasing the number of sampling sites to include the lake 658 streams (Figure 2). The concentration of bioavailable Hg in the upland stream at lake 658 does appear to be

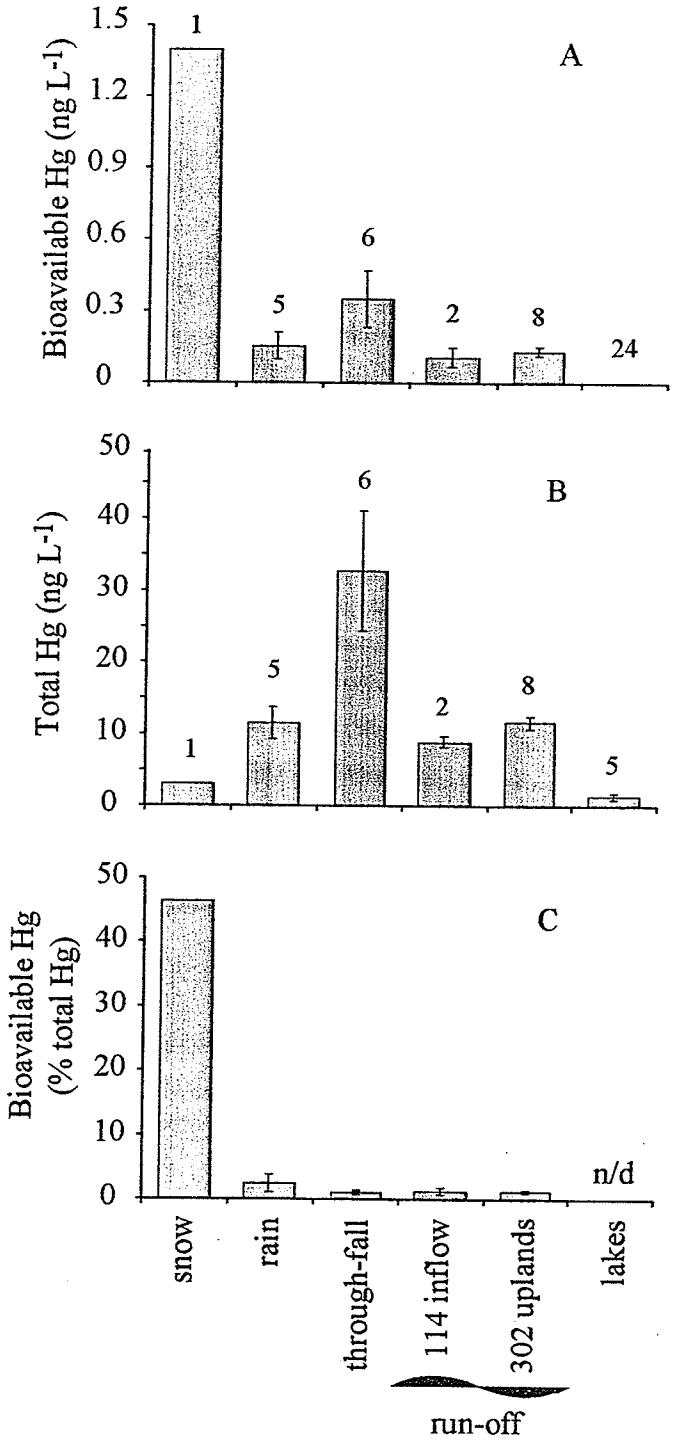

Figure 1.4 Bioavailable and total Hg concentrations in aqueous samples at the Experimental lakes Area in 1999. Replicates are above the bars in top and middle panels and represent samples analyzed from different precipitation events. Total and bioavailable Hg were analyzed from the same events and used to determine % bioavailable (bottom). n/d = not detectable

Table 2.4 Bioavailable and total Hg concentrations in input sources at the Experimental Lakes Area, 1999. All bioavailable Hg samples were analyzed at pH 7 with 5 mM glucose, 9.1 mM $(NH_4)_2SO_4$, and 3 mM phosphate. SD = standard deviation

	BioHg ng L^{-1} (± SD)	n	Total Hg ng L^{-1} (± SD)	n
Snow	1.4	1	3.02	1
Rain	0.15 ± 0.13	5	11.6 ± 4.93	5
Through-fall	0.35 ± 0.29	6	32.7 ± 20.5	6
Run-off 114 inflow	0.11 ± 0.06	2	8.97 ± 1.07	2
Run-off 302 uplands	0.13 ± 0.05	8	11.7 ± 2.47	8
Lakes	not detectable	24	1.38 ± 0.85	5

consistently higher than the wetland stream (Figure 2). However, compared with the other run-off sites, all of the concentrations fall within a fairly narrow range despite the very distinct catchment characteristics. The lake 114 in-flow catchment, for example, is characterized by Precambrian bedrock overlain with thin glacial drift composed of gravel and sand (Brunskill and Schindler, 1971) and forested with dense stands of jack pine and paper birch (St. Louis *et al.*, 1996). The 302 upland catchments, on the other hand, are characterized by exposed and lichen-covered bedrock with only interspersed patches of treed soil deposits (Allan *et al.*, 1993). One would expect differences between catchment types and perhaps with improved detection limits and a more refined analysis, distinct differences will be discerned in the future.

Snow seems to be an anomaly of sorts with nearly 50% of the total Hg being bioavailable (Figure 1, Table 2). No mass balance for bioavailable Hg was determined in this study. However, in a study of Ontario lakes and catchments, Mierle (1990) found that more than half of the annual stream load of total Hg was supplied during the spring runoff period, and suggested that most of the Hg was derived from melting snow. Similar findings in Boreal catchments in Sweden showed that the monthly output of both total Hg and MeHg peaked during spring snowmelt (Lee *et al.*, 2000). Given these findings and the remarkable difference in the bioavailability of Hg between snow and other source inputs, a substantial pulse of bioavailable Hg could be entering ELA lakes in the springtime.

The snow analyzed from the ELA prompted an exciting investigation into the bioavailability of Hg(II) associated with "Mercury Depletion Events" (MDEs) in the

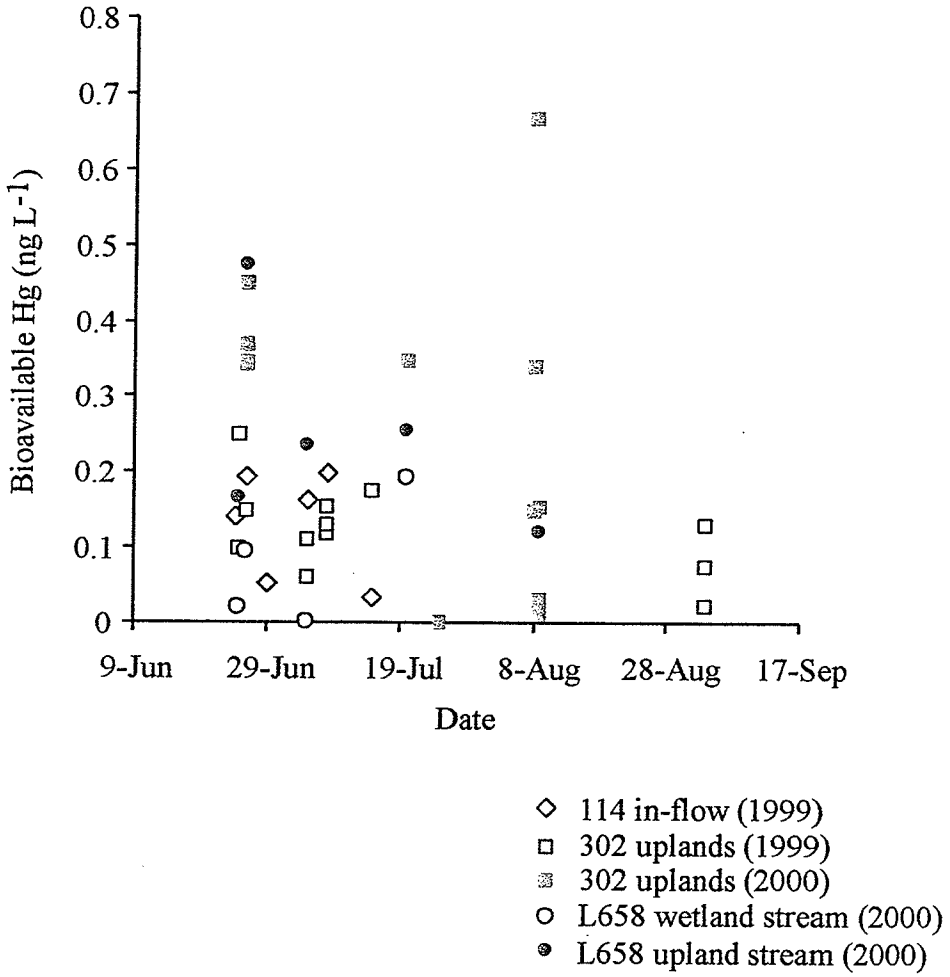

Figure 2.4 Bioavailable Hg concentrations in run-off samples from three different catchment areas at the Experimental Lakes Area. Samples were collected in 1999 and 2000.

Arctic (in collaboration with Dr. S. Lindberg (ORNL) and Dr. S. Brooks (NOAA)), which further supported the ELA findings. The detailed results from the Arctic campaign will not be included in my thesis, however, I encourage those interested to read Scott (2000) and Lindberg *et al.,* (2002) for details of the first year of the study. Pertinent to this discussion is that MDEs, recently discovered in the Canadian Arctic, are caused by a sunlight-induced oxidation of Hg^0 that is associated with ozone depletion, halogens, and polar sunrise (Schroeder *et al.*, 1998). The Hg species produced by this mechanism have not been well characterized to date but do appear to have a shorter residence time in the atmosphere than their gaseous Hg precursor, Hg^0 (Schroeder *et al.*, 1998). This means that they will leave the atmosphere more readily, potentially entering the biosphere. I examined the bioavailability of Hg in snow collected in Barrow, Alaska (before and after polar sunrise) and found that up to 80% of the total Hg associated with MDEs was bioavailable (Figure 3). Moreover, concentrations were unprecedented, reaching nearly 25 ng L^{-1} (125 pM) (Figure 3). Among other things, this supports the finding that snow is a comparatively important source of bioavailable Hg. The question of WHY bioavailable Hg is so high in snow remains to be answered.

Of importance now, in both Arctic and Boreal ecosystems, is determining the fate of bioavailable Hg in run-off during the spring melt period. Lalonde *et al.*, (2002) noted that the average total Hg levels in Quebec snow never decreased below 1 pM (0.2 ng L^{-1}) possibly due to a refractory fraction of Hg that was not photo-reduced. Some indirect evidence from the Barrow study suggests that bioavailable Hg was less readily photo-reduced than the total Hg pool since the total Hg concentrations decreased during the

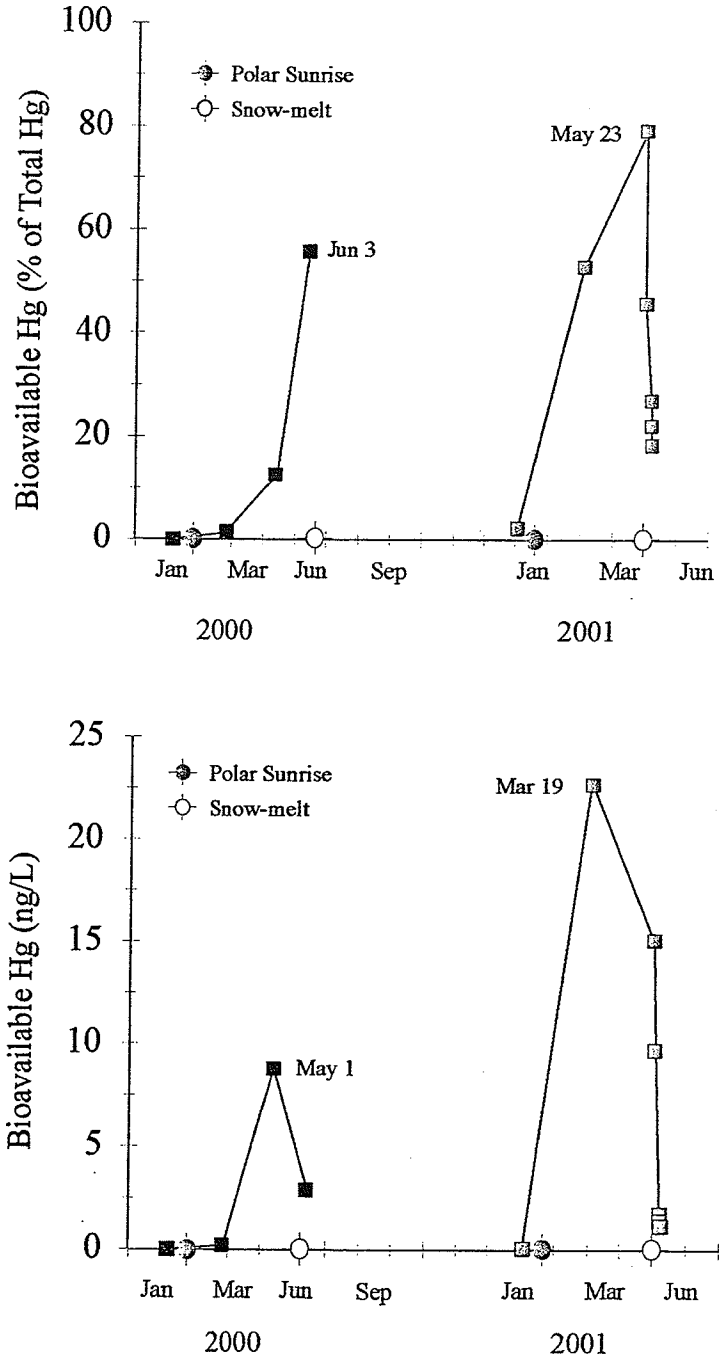

Figure 3.4 Bioavailable Hg in snow collected in Barrow, Alaska and associated with Mercury Depletion Events. Top panel is bioavailable Hg as a percentage of the total Hg in snow. Bottom panel shows the concentration of bioavailable Hg in snow.

spring melt more rapidly than did the bioavailable Hg concentrations (see Lindberg *et al.*, 2002). It appears, therefore, that within the total Hg pool, the photo-reducable fraction is not the same as the bioavailable fraction although some Hg species could be available for both processes. Indeed, it is highly probable that a Hg species such as $HgCl_2$, which is not appreciably photo-reducable (Xiao *et al.*, 1994), represents the refractory yet highly bioavailable fraction in snow. The fate of Hg after the melt period is extremely important because the longer that bioavailable Hg is able to persist, the more chance it has to become microbially transformed.

To conclude, bioavailable Hg was detectable in all aquatic source inputs. It appears, therefore, that bioavailable Hg is entering lakes from the atmosphere via direct precipitation and also via the terrestrial environment in throughfall and run-off. Furthermore, snow could be a particularly important source of bioavailable Hg. What happens to bioavailable Hg once it enters a lake? This question is all the more intriguing given that bioavailable Hg is not detectable in lake water. The next section of this chapter explores this question using the Hg addition approach previously described in Chapter 3.

Lakes

All lakes studied were of low alkalinity, sulfate and chloride (Table 3). On the basis of results from a previous study (see Chapter 6), the concentration of chloride in these lakes would not be a significant factor influencing the bioavailability of Hg(II). There were marked differences, however, among the lakes in DOC concentrations (Table 3), which ranged from a low of 270 μmol L^{-1} (lake 224) to a high of 1080 μmol L^{-1} (lake 661). Lakes 239 and 240 had concentrations of 580 and 550 μmol L^{-1}, respectively. All lakes were near 7 in pH except for lake 661, which is a naturally acidic brown water lake with adjacent bog areas. Lake 661 was also the highest in total Hg, (2.85 ng L^{-1} or 14.3 pM); lake 224 the lowest, (0.26 ng L^{-1} or 1.3 pM). Most of the Hg was associated with the "dissolved" fraction, i.e., filtered Hg values were 65 to 88% of unfiltered, whole lake values (Table 3).

"Concentration Series" Experiments". The behaviour of each lake in response to trace level additions of Hg was distinct (Figure 4). In 1998, when light was measured with a luminometer (detection limit was ~0.25 ng L^{-1} or 1.25 pM), lake 224 required an addition of 3 ng L^{-1} (15 pM) to detect bioavailable Hg. Additions of 5 ng L^{-1} (25 pM) Hg were required to detect bioavailable Hg in lakes 240, 239 and 10 ng L^{-1} in lake 661 (Figure 4A). Despite the improved detection limit in 1999 (0.05 ng/L or 0.25 pM), lake 224 still required an addition of 1.5 ng L^{-1} (7.5 pM) and lakes 240 and 661 required ~2.5 ng L^{-1} or 12.5 pM (Figure 4B). Lake 239 was not analyzed in 1999. Thus, the capacity of each lake to bind Hg differed. Another difference among lakes and sampling dates was the concentration of added Hg at which the percent bioavailable Hg reached an

Table 3.4 Water chemistry of lakes on dates sampled.

Year	Lake	Date	DOC $\mu mol\ L^{-1}$	POC $\mu g\ L^{-1}$	Chloride $mg\ L^{-1}$	Sulfate $mg\ L^{-1}$	Cond. $\mu mohs$	Alkalinity $\mu eq\ L^{-1}$	pH	Total Hg $ng\ L^{-1}$ (unfiltered)	Total Hg $ng\ L^{-1}$ (filtered)
1998	239	Aug. 6th	580	900	0.35	3.21	30	179	7.33	0.86	0.56 (65%)
	224	Aug. 20th	270	640	0.22	2.88	27	94	7.07	0.26	0.23 (88%)
	661	Aug. 27th	1080	860	0.14	0.57	19	127	5.72	2.85	n/a
	240	Sept. 19th	550	670	0.33	2.70	27	148	7.02	0.85	n/a
1999	224	Aug. 16th	300	710	0.3	2.63	19	89	7.02	0.54	n/a
	224	Oct. 24th	320	640	0.34	2.74	20	95	6.67	n/a	n/a
	240	Oct. 21st	600	630	0.39	2.77	27	149	6.85	n/a	n/a
	114	Aug. 17th	680	2990	0.19	1.69	14	48	6.34	1.81	1.01 (56%)

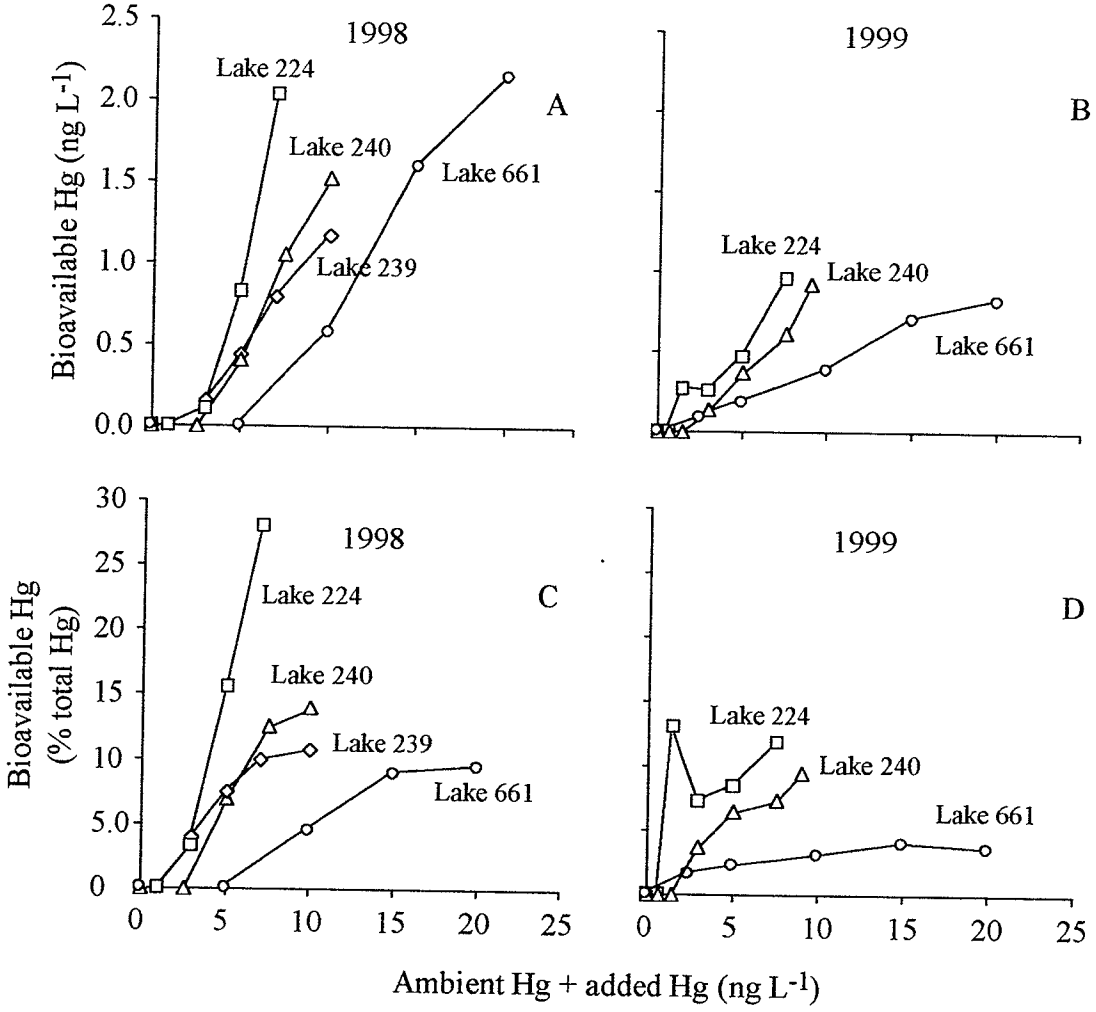

Figure 4.4 "Concentration series" experiments. Bioavailable Hg in unfiltered water from lakes 224, 240, 239, and 661 over a range of added Hg concentrations from 1 to 20 ng L^{-1}.

apparent plateau (Figure 4C & D). In lake 661, this plateau was reached at 15 ng L^{-1} (75 pM) in 1998 and at <5 ng L^{-1} (<25 pM) in 1999. In lakes 240 and 239, the plateau was reached at ~10 ng L^{-1} (50 pM) added Hg. In lake 224, no plateau was reached in 1998, but 7 ng L^{-1} (35 pM) was the highest addition that was tested. At the cell density used for these experiments, the *mer-lux* system becomes saturated with bioavailable Hg at concentrations exceeding ~2-3 ng L^{-1} (~10-15 pM). Additions of Hg greater than 7 ng L^{-1} (35 pM) to lake 224 water, therefore, would have resulted in concentrations of bioavailable Hg exceeding the saturation limit for the *mer-lux* system, thus higher addition levels could not be studied.

These results suggest that there is at least one type of functional group or 'class of ligand' that binds the added Hg. At least 1.5 ng L^{-1} (7.5 pM) of added Hg was required in this study for detection of bioavailable Hg (Figure 4A & B) suggesting the presence of a functional group for which Hg(II) has a high affinity such as sulfur. Hg is a 'soft' Lewis acid/metal and should therefore interact strongly with 'soft' Lewis bases such as thiols, sulfide, and other sulfur-containing ligands. Indeed, calculated stability constants for complexes formed between Hg and H_2S and thiol are very high (Dryssen and Wedborg, 1991) and there is growing evidence suggesting that Hg(II) actually preferentially binds to thiol groups in organic matter. Xia and co-workers (1999), for example, experimentally demonstrated the preference of Hg(II) for reduced sulfur-containing functional groups (i.e. thiol, disulfide/disulfane) over other functional groups in a soil humic acid using X-ray absorption spectroscopy. Recently, Haitzer and coworkers (2002) suggested that the binding of Hg to DOC under natural conditions (that is low Hg

to DOC ratios) was controlled by a small fraction of DOC molecules containing a reactive thiol functional group.

Equally significant is that these results illustrate the importance of working at environmentally relevant concentrations of Hg for studies of both bioavailability and sorption. The response of a high affinity, low capacity binding site that essentially controls the bioavailability of Hg(II) could be masked entirely with Hg additions that marginally exceed ambient Hg concentrations.

"Time Series" Experiments". The presence of more than one functional group was suggested by the "time series" experiments, which demonstrated that the bioavailability of Hg changed over time. In this set of experiments, Hg additions of 5 ng L^{-1} (25 pM) were used for all lakes except lake 661 (10 ng L^{-1} or 50 pM)). These concentrations were sufficient for detection of bioavailable Hg with the field luminometer, but were below the concentration at which the plateau commenced (Figure 4C & D). In unfiltered water, between 3 and 10% was bioavailable (Figure 5) and in filtered lake water, only about 6 to 12 % of the added $HgCl_2$ was bioavailable at time 0 (Figure 5). The initial percentage available was higher in lake 661 (12 to 16%), but 10 ng L^{-1} (50 pM) was added to this lake water because 5 ng L^{-1} (25 pM) was not detectable (Figure 3). In all four lakes, therefore, approximately 90% of the added Hg was immediately bound and unavailable for uptake (Figure 5) suggesting the presence of functional group(s) for which Hg(II) had a high affinity. The less rapid binding that followed the initial loss in bioavailability (Figure 5) may have been due to a different functional group with a comparatively lower affinity for binding Hg or possibly to a less accessible functional group(s) owing to the heterogeneous nature of DOC molecules

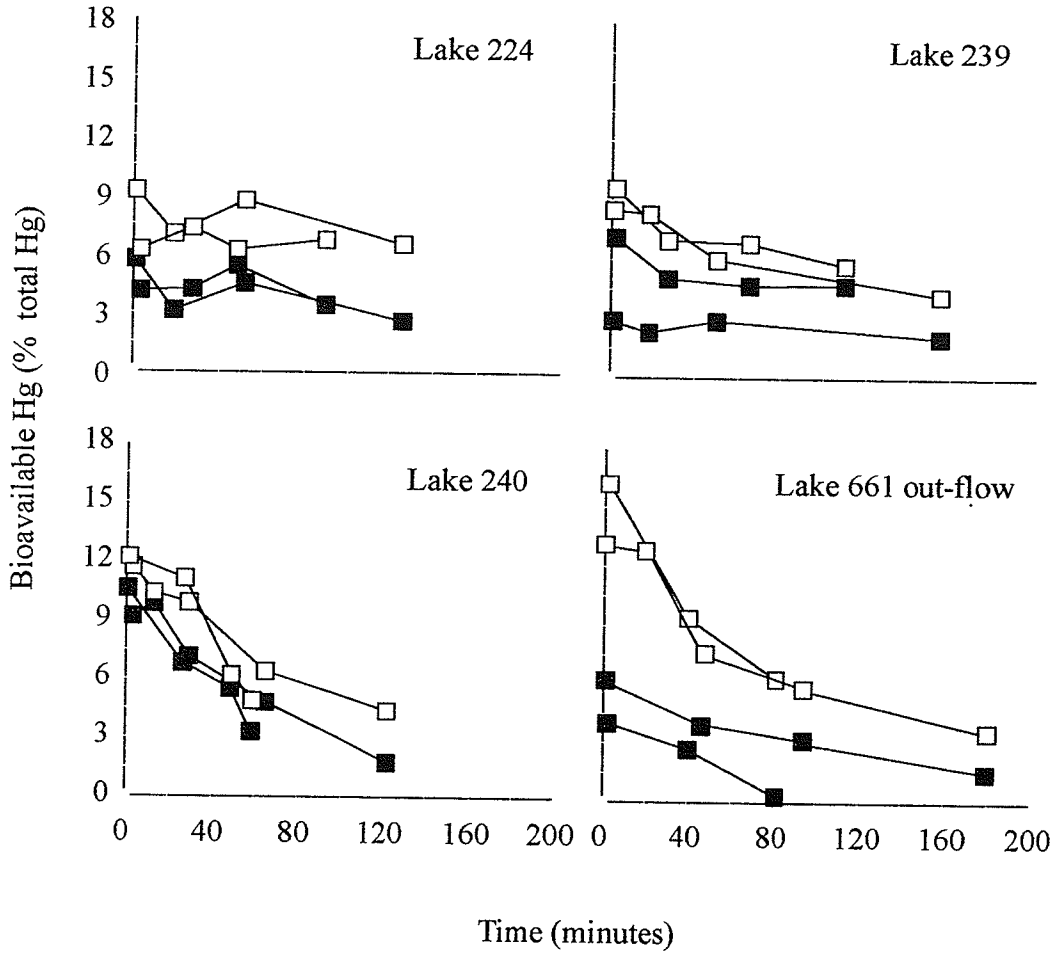

Figure 5.4 "Time series" experiments. Bioavailable Hg after additions of $HgCl_2$ to filtered (□) and unfiltered (■) lake water over time periods of up to three hours. Each lake was assayed on two different dates for both filtered and unfiltered samples. $HgCl_2$ additions were 5 ng L^{-1} for lakes 224, 240, and 239 and 10 ng L^{-1} for lake 661 out-flow.

(Thurmann, 1985).

The extent to which bioavailable Hg decreased over the course of three hours varied between lakes (Figure 5). The decrease was small and variable for lakes 224 and 239, with no clear difference between filtered and unfiltered samples on a given date. Lakes 240 and 661, on the other hand, showed a more pronounced decrease over time on the two dates sampled. This decrease was greater in filtered water than in unfiltered water for lake 661, but there was no difference between filtered and unfiltered water from lake 240 suggesting that particulates were less important in binding Hg in lake 240. Differences in the change in bioavailability of Hg over time between lakes 239 and 240 (Figure 5) is particularly interesting since these two lakes had similar concentrations of DOC (Table 3). The water renewal time for lake 239, however, is approximately five times greater than for lake 240 (Brunskill and Schindler, 1971) so microbial and solar degradation would be more extensive. This could affect the size and chemical structure of the DOC molecules, which would in turn likely affect its bioavailable reactivity with Hg.

In 1999, the time series was extended to 24 hours to determine if some of the added Hg remained bioavailable over a longer term. After 24 hours, bioavailable Hg was still detectable in filtered and unfiltered water from lake 224 and filtered water from lake 240 (Figure 6). This is important because it demonstrates that complexation equilibrium is not reached even after 24 hours. One of the difficulties in determining the aqueous speciation of Hg(II) for modeling purposes is slow kinetics of complexation which may prevent Hg(II) from attaining complexation equilibrium (Hudson *et al.*, 1994). Hudson and co-workers exemplified slow kinetics by an initial rapid binding of Hg(II) to

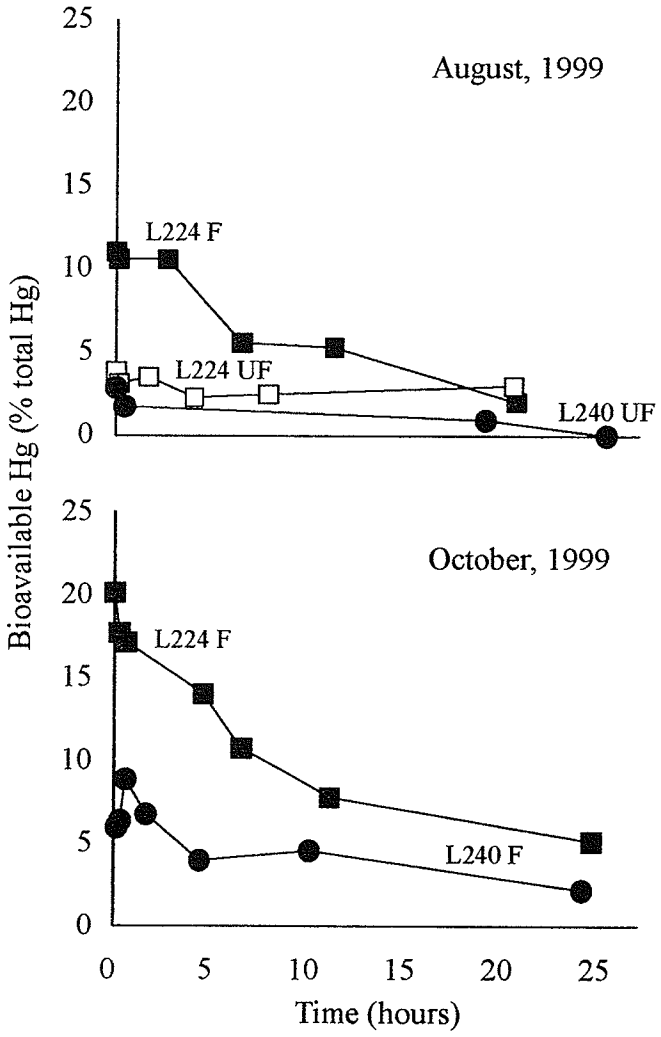

Figure 6.4 "Extended time series" experiments. Bioavailable Hg after additions of $HgCl_2$ to filtered (F) and unfiltered (UF) lake water over time periods up to 25 hours. $HgCl_2$ addition was 5 ng L^{-1}.

numerous weak humic sites followed by slower and stronger complexation. Although the opposite appears to be true in this study, (rapid binding to low capacity, high affinity sites), equilibrium was nonetheless, clearly not attained (Figures 5 and 6). Other processes in addition to atmospheric deposition to a lake, (as was simulated in this study), may be subject to kinetic effects as well. For example, the mixing of run-off with lake water, or freshwater with estuarine or seawater would result in changes in Hg(II) and ligand concentrations in addition to Hg(II) speciation. The kinetic aspects of the behaviour of Hg(II) entering an aquatic system may have significant effects on its ecological fate especially when the rate of re-establishment of chemical equilibrium is slower than the rates of competing biological processes such as uptake by bacteria (Ma *et al.*, 1999). The approach taken here using the *mer-lux* bioreporter is a start to understanding the kinetic behaviour of Hg. However, further characterization is required. Until then, chemical speciation calculations used in thermodynamic equilibrium models should be interpreted with caution (Hudson *et al.*, 1994).

In comparison to the overall loss of bioavailability observed in the time series experiments, the difference of a few percent between filtered and unfiltered lake water samples was notable but negligible (Figure 5). This suggests that in these lakes, the chemistry of the "dissolved" phase affected bioavailability more than did the particulate fraction. The "dissolved" phase is operationally defined as the <0.45 μm fraction, which may include inorganic ions, colloids, and DOC or humic substances. It is well established that Hg has a very high affinity for DOC (Kerndorff and Schnitzer, 1980), however, the importance of other constituents in the dissolved phase must not be overlooked. For example, inorganic complexes such as oxyhydroxides of Fe or Mn,

which may exist in particulate, colloidal and dissolved forms bind Hg (Meili, 1997). Furthermore, organic coatings on the surfaces of inorganic complexes are present in most natural waters. The strong association of oxyhydroxides with both Hg and organic matter, therefore, may contribute to the apparent importance of humic substances (Meili, 1997). Further characterization of the "dissolved" fraction would help better define the bioavailable reactivity of the DOC, in addition to the role of other dissolved constituents, on the complexation of Hg(II) and subsequent effects on its bioavailability.

Conclusions

An important source of Hg to lakes at the Experimental Lakes Area is the atmosphere, either through direct deposition or indirect via through-fall and run-off from the surrounding watershed (St. Louis *et al.*, 1995; 1996; 2001). The fraction of the total Hg in these input sources that was bioavailable to the *mer-lux* bacteria was detectable but generally low. Furthermore, bioavailable Hg was never detectable in any of the 24 lakes and experimental reservoirs without the addition of Hg. Based on these measurements and the Hg addition experiments, it seems likely that much of the Hg entering lakes rapidly becomes bound (and unavailable for uptake) by a functional group or ligand class for which Hg has a high affinity, such as reduced sulfur. Indeed, even though the amount of reduced sulfur in humic substances is low, it can be more abundant than many of the reported natural levels of Hg in the environment (Xia *et al.*, 1999). Thus, not only is this scenario stoichiometrically feasible, it stresses the importance of working at concentrations of Hg(II) that are environmentally relevant. Despite the rapid binding that occurs when Hg enters a lake, the extended "time series" experiments (Figure 6)

demonstrate that complexation equilibrium is not reached even after a period of 24 hours. The kinetic aspects of the behaviour of Hg(II) entering an aquatic system need further characterization since they may have significant effects on its ecological fate. The approach taken here using the *mer-lux* bioreporter is a start to understanding the kinetic behaviour of Hg.

Scientific evidence relating ecosystem response to reduced emissions of Hg^0 to the atmosphere is scant. Although a very complex problem, the results presented here showing Hg(II) proportionately more bioavailable at higher addition levels suggests that if inputs of Hg to the atmosphere increase, the change in bioavailable Hg entering lakes could be more than expected if the complexation capacity of the high affinity ligands is exceeded. Conversely, the beneficial effects of a reduction in Hg loading to ecosystems by imposition of emission controls could have a greater than one-to-one impact. A better understanding of bioavailability in both the oxic and anaerobic compartments of a lake is crucial to our understanding of the biogeochemical cycling of Hg(II). The *mer-lux* bioreporter will prove a useful tool in furthering our understanding of the behaviour of Hg in aquatic environments.

Chapter 5

EFFECT OF DOC CHARACTER ON THE BIOAVAILABILITY OF Hg(II) IN FRACTIONATED WATERS FROM THE FLUDEX RESERVOIRS

Abstract

The bioavailable reactivity of dissolved organic carbon (DOC) with inorganic divalent Hg, Hg(II), was investigated using a *mer-lux* bioreporter in conjunction with trace level ultrafiltration methods and XAD-8 chromatography for the characterization of DOC. Study sites included three experimental reservoirs and a boreal lake at the Experimental Lakes Area, Canada. There was a natural preference for Hg to bind or partition to the higher molecular weight DOC (>10 kDa) likely due to a comparatively higher capacity to bind Hg(II) than the lower molecular weight DOC. Consequently, the bioavailability of Hg(II) increased with decreasing molecular weight of DOC. The strongest correlation (negative) was between absorbance at 254 nm and bioavailable Hg ($R^2 = 0.903$). Further characterization of the DOC isolates is required to better define the bioavailable reactive components responsible for absorbance at this wavelength. Weak correlations were observed between bioavailable Hg and parameters such as DOC concentration ($R^2= 0.48$) and SUVA or molar absorptivity ($R^2 = 0.66$) suggesting that a much more specific parameter than carbon is needed to characterize the bioavailable reactivity of DOC. The character of DOC, rather than the quantity of DOC should be considered in attempts to understand implications of environmental change on the biological fate of Hg(II).

Introduction

The major source of Hg to remote ecosystems has been identified as atmospheric, originating from the long-range transport of Hg^0 from distant industrial areas and natural sources (Mierle, 1990). Hg(II) has also been implicated as the predominant species of Hg leaving the atmosphere and entering remote lakes (Brosset, 1981). Despite the regional homogeneity in loading implied by an atmospheric source, Hg levels in fish of the same species are highly variable from one lake to another within small geographical areas. Among the factors thought to be contributing to this difference is the interaction between Hg and dissolved organic carbon (DOC) (Driscoll *et al.*, 1995).

Studies have shown that the biogeochemical cycling of Hg is coupled to organic matter in both terrestrial and aquatic ecosystems. This strong interaction is believed to be an important factor in the mobility and transport of Hg from watersheds via streams to lakes (Mierle and Ingram, 1991), where organic complexes largely dominate its speciation (Allard and Arsenie, 1991). Furthermore, up to 95% of Hg(II) in lakes can be bound by DOC (Meili, 1997). Although there is evidence that DOC-bound Hg is not bioavailable (Barkay *et al.*, 1997), the extent to which this is true in natural systems remains untested (COMERN).

To more accurately assess the role of DOC in the behaviour of Hg in lakes, a more specific parameter than "DOC concentration" is needed. However, DOC is a heterogeneous mixture of organic compounds for which no single structural formula exists. Furthermore, its molecular weight (MW) distribution extends over several orders of magnitude and studying the environmental significance of DOC in whole water samples is often hampered by this inherent complexity (Aiken *et al.*, 1992). An approach

to reduce this complexity is to isolate functionally distinct DOC fractions from water to determine fundamental chemical properties of each fraction (Aiken *et al.*, 1992). Although the heterogeneity of DOC precludes determining exact structures, some generalizations can be made concerning the origin, chemical and structural characteristics of the DOC. For example, DOC originating in terrestrial plant and soil systems, where it is leached by interstitial water of soil into rivers and streams (allochthonous DOC), is aromatic in nature due to the lignin in plant tissues (Malcolm, 1992). Carbon molecules of this origin are also larger and more polydisperse than those from waters that receive little inputs of terrestrially derived organic carbon (Chin *et al.*, 1994). Aromatic acids entering the aquatic environment are, therefore, useful as source indicators of carbon from terrestrial sources. Conversely, DOC originating from within the lake or ocean (autochthonous DOC) is typically derived from such processes as extracellular release and leachate of algae and bacteria and is more aliphatic in character than allochthonous DOC (McKnight *et al.*, 1994).

To better understand how the chemical and structural characteristics of DOC influence its bioavailable reactivity with Hg(II), we used chromatographic fractionation using hydrophobic resins (XAD-8) and "Hg-clean" ultrafiltration in conjunction with a *mer-lux* bioreporter for the determination of bioavailable Hg(II). The study was conducted at the Experimental Lakes Area as part of the FLooded Uplands Dynamics EXperiment in 1999 in collaboration with Drs. Jim Hurley, Kris Rolfus, and Chris Babiarz, (all from the University of Wisconsin Water Resources Institute in Madison, Wisconsin) and Dr. George Aiken (United States Geological Survey, Colorado). Their contributions are specified below as they are described. We found that the chemical

nature of DOC exerts a strong control on its reactivity with Hg(II), thereby affecting its bioavailability.

Methods

Characterization of DOC

Ultrafiltration. The ultrafiltration method is based on a physical separation process using a membrane of a given pore size. DOC molecules smaller than the membrane pores pass through while those greater are retained and concentrated resulting in two size fractions known as the "permeate" and the "retentate", respectively. In this study, the permeate represented DOC with molecular weights less than 10 kDa, often considered "truly dissolved". The majority of the DOC in the retentate fraction was >10 kDa. However, this fraction may have also contained smaller MW molecules due to the isolation procedure. In addition, there was the <0.45 μm fraction which included both the retentate and the permeate.

XAD-8 Resin Columns. The procedure followed for the XAD-8 chromatography was based on that in Thurman and Malcolm (1981) and Aiken *et al.*, (1992). Unlike ultrafiltration, the separation of DOC using XAD-8 resin columns is based more on a chemical than a physical separation although MW does play a role. XAD resin is a nonionic methylmethacrylate polymer that adsorbs organic matter from water by hydrophobic bonding (Thurmann, 1985). The pKa of organic acids is between 4 and 5. Therefore, at a pH of 2, large MW hydrophobic organic acids (HPOA) adsorb onto the XAD resin, while the lower MW organic acids and inorganic compounds are not retained (XAD eluent). With the addition of NaOH, the HPOA are desorbed from the resin. Thus, instead of permeate and retentate, this characterization includes the eluent

and HPOA, or what was retained on the resin and back eluted with NaOH. The fraction that is not removed with NaOH from the resin is called hydrophobic neutrals (HPON) and can be calculated by mass balance.

A comparison of these two methods is valid and justifiable, especially for the type of DOC (high MW, hydrophobic) found in the low ionic strength waters of the ELA (Jim Hurley, pers. comm.). The >10 kDa fractions were highly coloured which is normally due to the presence of chromophores with multiple double bonds, possibly conjugated, aromatics, and phenolic functional groups which absorb at the blue end of the spectrum and impart a yellow brown hue to some waters (Kirk, 1994). Organic acids with these properties are also hydrophobic so the >10 kDa fraction is likely primarily HPOA. However, the HPOA may not all be >10 kDa in size. Along the same lines, the <10 kDa fraction was not highly coloured, therefore likely contained fewer chromophores, and was less hydrophobic, not unlike the XAD-8 eluent. Nonetheless, it may have also contained low MW hydrophobic molecules as well. So, there are differences between the fractions separated by ultrafiltration and the isolates resulting from XAD-8 resin separation, but there are also important characteristics, which justify a comparison. Furthermore, the 10 kDa molecular weight cut-off is the lowest that can be effectively used for trace metals at low ionic strengths (Jim Hurley, pers. comm.).

Sampling and Analyses

General. In July, August and September 1999, Jim Hurley and Kris Rolfhus sampled surface water from each of the three reservoirs and Roddy lake. For the ultrafiltration, each of the reservoir samples was composited by taking one third of a sample from three

different locations (rafts) within a given reservoir. For the XAD-8 characterization, samples were taken from each raft and were not composited. All samples were filtered in the field using conventional 0.4 μm Meissner filtration capsules.

For the September samples only, Chris Babiarz carried out the ultrafiltration using ultraclean techniques as described in Babiarz *et al.*, (2001). I did the bioavailable Hg analyses (described in detail below) on each of the size fractions (<0.45 μm, retentate and permeate) from the three reservoirs and Roddy lake in the Hg clean lab at the ELA. Kris Rolfus analyzed the <0.45 μm and the permeate fractions for total Hg in Madison, Wisconsin using cold-vapour atomic fluorescence spectroscopy detection as described in Gill and Fitzgerald (1987) and Liang and Bloom (1993). The concentration of total Hg in the retentate fraction was calculated by difference because of the concentration effect during the ultrafiltration procedure.

Dissolved Organic Carbon. Filtered (<0.45 μm) samples were sent to George Aiken in Boulder, Colorado for XAD-8 characterization, which included XAD-8 effluent, HPOA, and HPON. In addition, he determined the DOC concentration, percent carbon on a mass balance basis, UV absorbance at 254 nm, and specific UV absorbance (SUVA) on each of these fractions. SUVA is the UV absorbance at 254 nm normalized for total organic carbon content, and is essentially equivalent to molar absorptivity but with different units (mg C L^{-1}). Both measures are considered to be a simple indicator of the aromatic content of the DOC.

A number of trends have been observed between structural characteristics of humic substances and their spectroscopic properties. Chin and others (1994) have shown that the molar absorptivity at 280 nm of aquatic fulvic acids increases with molecular

weight and aromaticity. Using the equation derived from this latter correlation (equation 1), the percent aromaticity in each of the isolates was calculated.

$$\text{aromaticity} = 0.05\varepsilon + 6.74 \quad (1)$$

where ε is the molar absorptivity (L mol^{-1} cm^{-1}) (equation 2).

$$\varepsilon = \text{absorbance @ 254 nm/molar concentration of DOC} * \text{path length} \quad (2)$$

Different wavelengths have been used to represent aromatic compounds, likely because of their rather complex absorbance spectra. A common wavelength, 254 nm, was used in this study; others have used 270 nm (Traina *et al.*, 1990) and 280 nm (Chin *et al.*, 1994).

The XAD-characterization was performed on the reservoir samples collected in July, August and September 1999 but only in July for the Roddy lake samples. Between July and September, the HPOA concentration in the reservoirs decreased on average 5%; the HPON decreased ~9%; the XAD-8 eluent increased ~14% (data not shown). A feature of many lakes is the relatively small amount of change in concentration of DOC with depth or season within a lake (Steinberg and Muenster, 1985). Given that Roddy lake is considerably larger in volume and size than the reservoirs (Tables 1 & 2, Chapter 2), it would likely experience even smaller seasonal changes in the DOC concentrations. So the difference in the DOC concentrations between Roddy and the reservoirs likely reflects differences due to watershed and in-lake processes rather than to the time of sampling. For this reason, the July XAD-characterization data for Roddy was used even though the ultrafiltration and Hg analyses (total and bioavailable) were carried out in September.

Bioavailable Hg. Bioavailable Hg was not detectable in any of the size fractions without the addition of Hg. This was not surprising since bioavailable Hg has never been detected in any lakes or reservoirs at the ELA (see Chapter 4). Therefore, 1 ng L^{-1} (5 pM) Hg was added to the <0.45 μm and retentate fractions and 0.75 ng L^{-1} (3.75 pM) was added to the permeate. These concentrations were experimentally determined by conducting Hg additions on the retentate and permeate fractions from sites 1 and 2 over a range of Hg concentrations (similar to the addition experiments in the previous chapter). The permeate fractions from both reservoirs showed a response suggesting that saturation of the *mer-lux* system was occurring at Hg additions of ~1.0 to 1.5 ng L^{-1} (5 to 7.5 pM) total Hg (Figure 1). This saturation is not to be confused with saturation of functional groups on the DOC molecules as discussed in Chapter 4. Therefore, to avoid working too closely to this limit, 0.75 ng L^{-1} (3.75 pM) Hg additions were used for the experiments involving the permeate fractions. In contrast, the behaviour of the retentate allowed (and required) a slightly higher concentration of Hg (1 ng L^{-1} or 5 pM) since it showed a linear response with Hg additions as high as 12 ng L^{-1} (60 pM) (Figure 1). The two concentrations of Hg that were ultimately added to the respective fractions did not exceed the ambient Hg concentration in the filtered, whole water samples. This is very important because very few studies involving the partitioning behaviour or bioavailability of Hg have been conducted at concentrations of Hg that are environmentally relevant.

Since the concentration of bioavailable Hg changes with the concentration of Hg added (Figure 1), it is difficult to quantitatively compare the bioavailability between fractions when two different concentrations of Hg are used. To overcome this difficulty and allow a direct comparison between the permeate, retentate and <0.45 μm fractions,

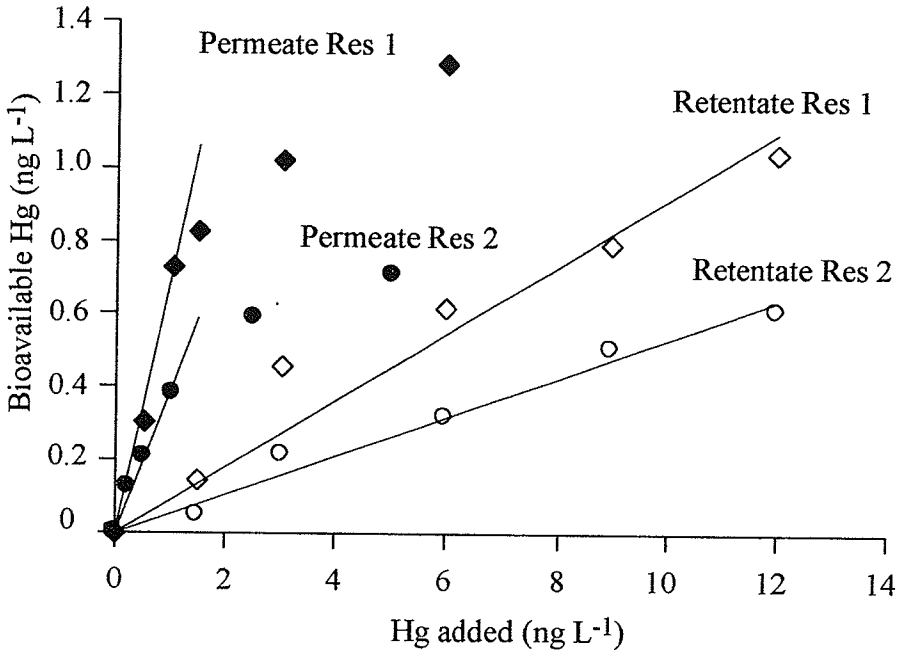

Figure 1.5 Hg additions to the permeate and retentate fractions of the FLUDEX reservoirs 1 and 2, Experimental Lakes Area.

the concentration of bioavailable Hg in the permeate was calculated through extrapolation to 1 ng L^{-1} (5 pM) Hg, the same concentration added to the retentate and <0.45 μm fractions. If the extrapolated concentration exceeded the highest standard for a particular assay, its value was not used. The bioavailable Hg data was also 'normalized' to account for differences in the ambient total Hg present in each fraction. Normalization was achieved by dividing the concentration of bioavailable Hg by the total Hg (ambient + the added Hg) in the sample; essentially representing the proportion of the total Hg(II) pool that was bioavailable.

The concentrations of reagents used in the bioassay were 5 mM glucose, 9.1 mM $(NH_4)_2SO_4$ and 3 mM phosphate in the cell suspension. All assays were run at pH 6, the average pH of the reservoirs. The retentate samples were reconstituted with Milli-Q water by the factor to which they were concentrated during the ultrafiltration procedure; Roddy lake (5.9x), Site 1 (6.45x), Site 2 (6.4x) and Site 3 (7.3x). Bioassays for each size fraction were repeated between 2 and 8 times depending on the total volume of sample available. Standardization was by linear regression and the student's t-test was used to determine the significance of the differences between size fractions and between reservoirs.

Results & Discussion

The concentration of total Hg in the whole, filtered samples ranged from a low of 0.74 ng L^{-1} (3.7 pM) in Roddy lake to 5.23 ng L^{-1} (26.2 pM) in reservoir 2. The retentate (>10 kDa fraction) represented 59 to 84% of the total Hg (Table 1), while in the permeate (<10 kDa fraction), the concentration of total Hg was less than 1 ng L^{-1} (5 pM) at all sites

Table 1.5 Concentrations of total Hg in the whole, filtered water samples and in the fractionated water from the three FLUDEX reservoirs and Roddy Lake.

Location	Hg_T (ng L^{-1})			Hg_T (%)	
	<0.45 μm	>10 kDa	<10 kDa	>10 kDa	<10 kDa
Roddy lake	0.74	0.51	0.23	68.9	31.1
Reservoir 1	1.93	1.13	0.80	58.5	41.5
Reservoir 2	5.23	4.39	0.84	83.9	16.1
Reservoir 3	2.39	1.79	0.60	74.9	25.1

representing 16 to 41% of the total Hg (Table 1). There appears, therefore, to be a natural preference for Hg to bind or partition to the higher molecular weight DOC (Table 1). A greater tendency of binding to higher molecular weight fractions has been observed before (Lodenius, 1987).

The 10 kDa molecular weight cut off also delineated distinct differences in the bioavailability of Hg(II) (Figure 2). With the exception of reservoir 1, the lowest bioavailability was observed in the <0.45 μm fraction, followed by the retentate (>10 kDa) and then the permeate (<10 kDa) (Figure 2, Table 2). The differences in bioavailable Hg between size fractions within a given site were statistically significant with t-values at $\alpha = 0.05$. One must keep in mind that the retentate (>10 kDa) contained some of the <10 kDa fraction due to the isolation procedure; thus, the observed signal for bioavailable Hg(II) in the >10 kDa isolate could be associated in part with the lower MW fraction. This would increase even further the differences in the bioavailability of Hg(II) between the permeate and retentate fractions.

When increasing concentrations of Hg were added to the permeate and retentate fractions (reservoirs 1 and 2), saturation of the *mer-lux* biosensor occurred in the low MW isolates but not in the higher molecular weight fractions despite the addition of considerably higher concentrations of Hg (Figure 3). (Saturation typically occurs at ~0.75 ng L^{-1} or 3.75 pM *bioavailable Hg* at pH 6 with 1.2 x 10^{-5} cells ml^{-1}.) This suggests that >10 kDa fraction had a comparatively higher capacity to bind Hg(II) than the lower MW DOC resulting in lower bioavailability in the higher molecular weight fraction.

This is, of course, assuming that DOC bound to Hg(II) is not able to enter the

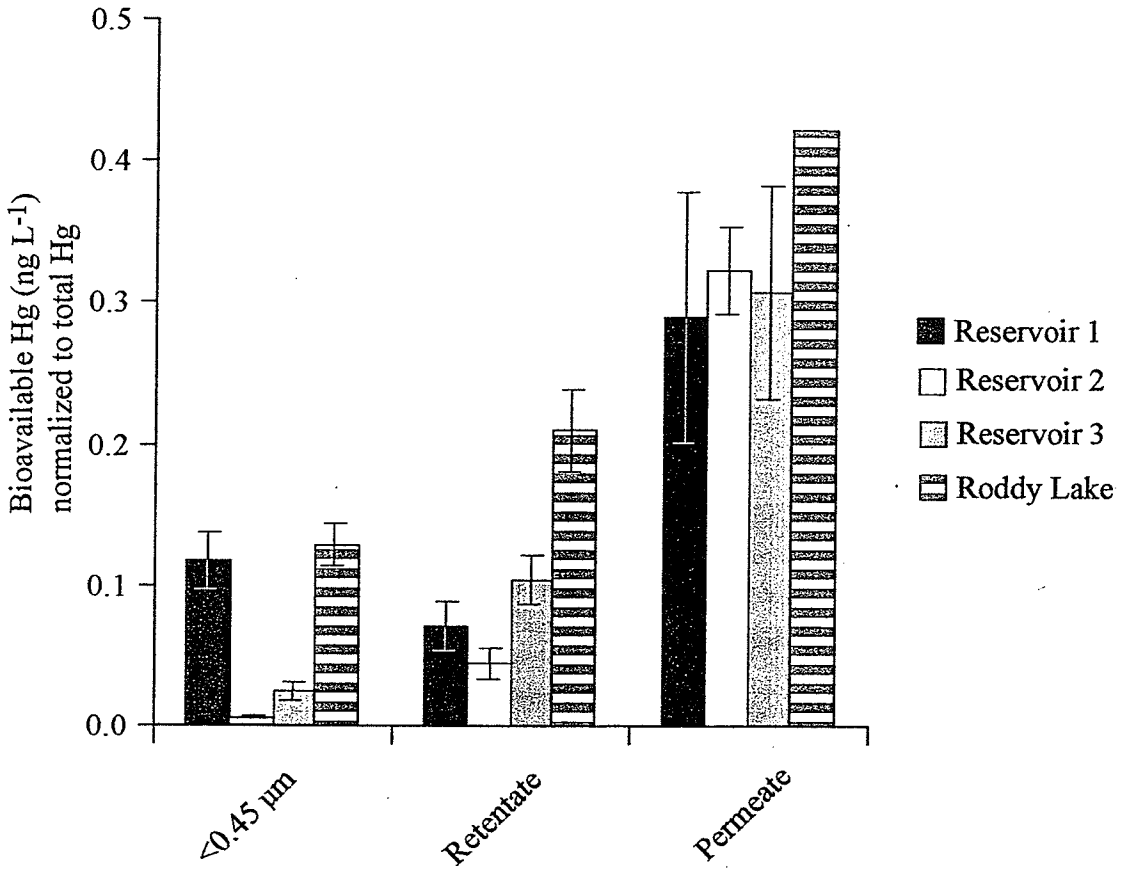

Figure 2.5 Bioavailable Hg in fractionated surface waters of the three FLUDEX reservoirs and Roddy lake, Experimental Lakes Area. Error bars are standard error.

Table 2.5 Bioavailable Hg in fractionated surface waters from the three FLUDEX reservoirs and Roddy lake. (Standard error, replicate assays)

Site	*Bioavailable Hg in ng L^{-1} (SE, n)*		
	<0.45 µm	*Retentate*	*Permeate*
Roddy	0.13 (0.02, 4)	0.21 (0.03, 5)	0.42 (--, 1)
Reservoir 1	0.12 (0.02, 4)	0.07 (0.02, 6)	0.29 (0.09, 2)
Reservoir 2	0.005 (0.0005, 2)	0.045 (0.009, 7)	0.32 (0.03, 3)
Reservoir 3	0.02 (0.006, 4)	0.11 (0.02, 8)	0.31 (0.08, 2)

bacterial cell. However, DOC bound Hg(II) could conceivably enter the cell if the DOC molecules were small enough. Aiken and Malcolm (1987) found that the molecular weight of aquatic fulvic acids was ~400-650 Da. This size is comparable to siderophores, and, therefore, capable of passing cellular membranes (Aiken and Malcolm, 1987). Alternatively, if the affinity of Hg(II) associated with the low MW fraction is low, uptake could conceivably occur by ligand exchange with a high affinity cell membrane protein.

Fulvic and humic substances also have a surfactant-like structure, containing both hydrophilic (carboxylic and phenolic groups) and hydrophobic domains (aliphatic and aromatic moieties) (Vigneault *et al.*, 2000). Consequently, humic substances can adsorb on a large number of natural surfaces, including biological membranes, potentially changing the structure and fluidity of the lipid bilayer. Vigneault and co-workers (2000), for example, showed an increase in the cell membrane permeability resulting in increased passive diffusion of fluorescein diacetate by a green alga. This type of interaction could have implications for the passive uptake of $HgCl_2$ or other neutral, nonpolar Hg(II) complexes as well. It is, therefore, important to consider not only the metal-complexing properties of natural DOC but also its direct metabolic and physiological influences on the organism (Parent *et al.*, 1996). Potential changes in cell membrane integrity imposed by a particular size fraction of DOC should be reflected in a change in light production by the constitutive *lux* strain *V. anguillarum* pRB27. This was not apparent, however, since samples did not cause a decrease in light production relative to the distilled water control (Figure 4). In fact, light production was slightly stimulated in most samples. This suggests that the fractionated water samples used in this study did not cause disruption of the cellular membrane or undo physiological stress to the *mer-lux* organisms.

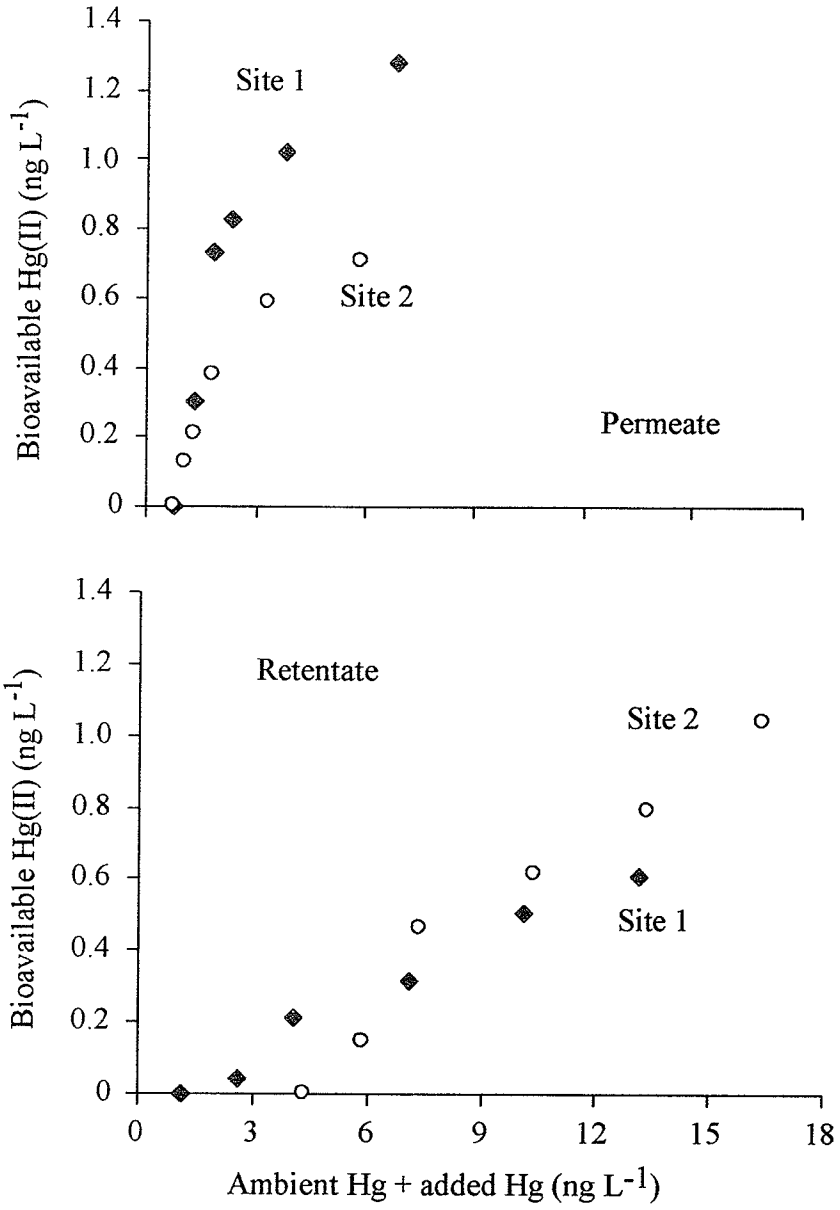

Figure 3.5 Bioavailable Hg concentrations in the retentate and permeate fractions of reservoirs 1 and 2 of the FLUDEX reservoirs at the Experimental Lakes Area.

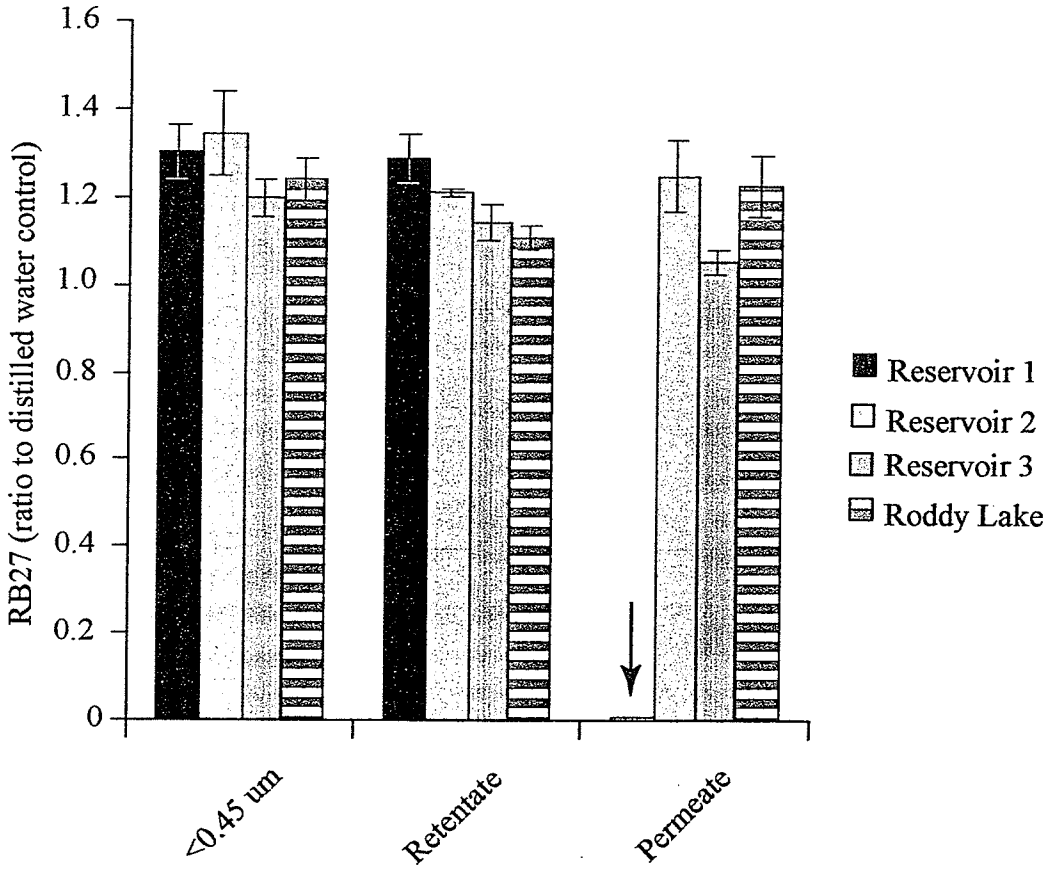

Figure 4.5 Relative differences in light production by the constitutive control organism *V. anguillarum* pRB27 exposed to fractionated FLUDEX reservoir samples and Roddy Lake at the Experimental Lakes Area. Error bars are standard error, n = 3.

Furthermore, the differences in the response of the constitutive *V. anguillarum* pRB27 between sites and size fractions were small (with the exception of the permeate from site 1, see below) and could not account for the observed trends in the Hg(II)-dependent assays using *V. anguillarum* pRB28 (Figure 2).

The only exception to the observations described above was the permeate fraction from reservoir 1 (Figure 4). This sample was most unusual because no light whatsoever (apart from background) was produced by the constitutive *V. anguillarum* pRB27 except when the cell density was doubled (data not shown). This may have been due to a low MW compound, present only in reservoir 1, which was toxic or otherwise inhibitory to the light-emitting reaction. For example, long chain alcohols inhibit bioluminescence by blocking the aldehyde binding site (Makemson, 1986). Biomass production was also comparatively low in reservoir 1 (M. Paterson, unpubl. data) supporting the possibility that there was something inherently different with this reservoir that was affecting the biota.

The tendency for the distribution of the total Hg pool to the higher molecular weight fraction of DOC (Table 1) and the lower bioavailability of Hg(II) in that size fraction (Table 2, Figures 2 & 3) suggest that complexation of Hg(II) by DOC is the primary factor affecting the bioavailability of Hg(II) in this study. Furthermore, the characteristics of the DOC, defined by a 10 kDa molecular weight cut-off, significantly affected the bioavailability of Hg(II). To explore the relation between the chemical and structural characteristics of DOC and the bioavailability of Hg(II), whole (filtered) samples from the reservoirs and Roddy lake were characterized using XAD-8 resins. Two isolates resulted in addition to the <0.45 μm fraction; the hydrophobic

organic acids (HPOA) and the XAD eluent. As discussed in the Methods, these two isolates are comparable to the retentate and permeate fractions, respectively. The hydrophobic neutrals (HPON) were calculated by mass balance.

Roddy lake, which served as the source of water to fill the reservoirs, was characteristically distinct in terms of the DOC content in the various fractions (Table 3). The concentrations of DOC in the <0.45 μm filtered water and in the XAD-8 eluent were 5.5 mg L^{-1} and 1.9 mg L^{-1}, respectively, whereas in the three reservoirs, concentrations were higher (Table 3). The HPOA concentration was similar at all sites. However, due to the lower concentration of DOC in the <0.45 μm filtered water from Roddy lake, the HPOA represented a greater percentage (62%) of the total carbon than it did in the reservoirs. The HPON concentrations were low in samples from all of the sites, representing no greater than 3.5% of the total carbon.

It is interesting that the HPOA and XAD-8 eluent comprised nearly 50% each of the total carbon in the reservoirs (Table 3) yet there were significant differences in both total Hg and bioavailable Hg associated with the size fractions separated by ultrafiltration (Tables 1 & 2, Figure 2). This finding suggests that the behaviour of the DOC in terms of its reactivity with Hg(II) is determined less by the *quantity* of DOC than by its chemical and structural characteristics.

UV absorbance at 254 nm ranged from 0.020 to 0.165 with the lowest values occurring in the XAD-8 eluent and the highest in the <0.45 whole, filtered sample (Table 4). The absorbance measured in isolates from Roddy lake was lower than those from the reservoirs. SUVA and molar absorptivities are considered useful parameters for describing DOC in terms of its general chemical characteristics. For example, they have

Table 3.5 Dissolved organic carbon (DOC) in the XAD-8 isolates. Reservoirs were sampled in September, 1999 while Roddy lake was sampled in July, 1999. HPOA and HPON are Hydrophobic Organic Acids and Neutrals, respectively and XAD-8 is the eluent fraction as determined by XAD-8 chromatography.

Site	***DOC mg L^{-1} (% Carbon)***			
	<0.45 µm	***HPOA***	***HPON***	***XAD-8***
Reservoir 1	8.05 (100)	3.90 (48.5)	0.25 (3.5)	3.90 (48)
Reservoir 2	8.13 (100)	3.95 (48.7)	0 (0)	4.20 (52)
Reservoir 3	7.60 (100)	3.42 (45)	0.27 (3.5)	3.91 (52)
Roddy	5.50 (100)	3.41 (62)	0.19 (3)	1.90 (35)

been shown to be strongly correlated with percent aromaticity in a variety of organic matter isolates as determined by ^{13}C NMR (Chin *et al.*, 1994). The absorptivities of the reservoir samples and Roddy lake ranged from 108 to 343 L mol^{-1} cm^{-1} (Table 4). Again, the lowest values were observed for the XAD-8 eluent, somewhat lower than Lake Fryxell, Antarctica fulvic acid (150 L mol^{-1} cm^{-1}) which characteristically have little or no terrestrial organic matter (Chin *et al.*, 1994). This suggests that the DOC comprising the permeate fractions was of autochthonous origin. The highest absorptivities were found in the HPOA isolates and were comparable to the fulvic acids reported in Chin and co-workers (1994) but lower than Suwannee River and Aldrich humic acids (Chin *et al.*, 1997, Chin *et al.*, 1994). SUVA followed the same trend as molar absorptivity with values ranging from 0.009 to 0.03.

Based on the relationship derived in Chin *et al.* (1994), a first approximation of aromaticity was also calculated (Table 4) for each of the isolates in this study. The presence of aromatics suggests a greater terrestrial influence with more allochthonous DOC derived largely from precursors of higher plants (Wetzel, 1983). It is not surprising, therefore, that the percent aromaticity in the whole, filtered samples from the reservoirs was greater than in Roddy lake. The HPOA isolates had the highest percent aromaticity (up to 23.9%) and again, Roddy was distinctively lower than the reservoirs. Interestingly, the aromaticity in the XAD-8 eluent was comparable between all sites including Roddy lake, ranging from 12.1% (site 3) to 13.3% (site 1). This means that the lower aromaticity in the HPOA isolate from Roddy lake accounts for the overall lower aromaticity from that site.

Table 4.5 Spectroscopic characterization of DOC isolates separated by XAD-8 columns from the three FLUDEX reservoirs and Roddy Lake, Experimental Lakes Area. Parameters include UV absorbance at 254 nm, specific UV absorptivity (absorbance normalized by total organic carbon), molar absorptivity, and % aromaticity.

Site	*Isolate*	*UV abs (A_{254} nm)*	*SUVA*	*Molar Absorptivity*	*Aromaticity¥ (%)*
Res 1	XAD-8	0.045	0.011	137	13.6
	HPOA	0.110	0.028	343	23.9
	<0.45 μm	0.164	0.021	245	19.0
Res 2	XAD-8	0.044	0.010	126	13.0
	HPOA	0.110	0.027	338	23.7
	<0.45 μm	0.165	0.020	243	18.9
Res 3	XAD-8	0.035	0.009	108	12.1
	HPOA	0.090	0.030	317	22.6
	<0.45 μm	0.132	0.018	208	17.2
Roddy	XAD-8	0.020	0.010	126	13.1
	HPOA	0.077	0.022	269	20.2
	<0.45 μm	0.092	0.017	201	16. 8

¥ calculated based on the relationship in Chin *et al.* (1994).

Trends were apparent between the spectroscopic properties of the isolates and the bioavailability of Hg(II). A weak relationship was observed between bioavailable Hg (normalized to total Hg) and the concentration of DOC (R^2= 0.48). Further, the correlation between bioavailable Hg and SUVA/molar absorptivity was also only weakly positive (R^2 = 0.66). This was not unexpected since DOC measures all sources of carbon including humic and fulvic matter as well as carbohydrates and proteins (Mierle and Ingram, 1991), some of which could potentially enter the cell. A carbon-based normalization, therefore, would tend to dilute the actual parameter that is determining the bioavailable reactivity of the DOC. Moreover, if the ratio of carbon to the functional group components (e.g. C:O, C:S, C:N) binding Hg(II) is inconsistent between isolates, normalizing for carbon would weaken the relationship with bioavailable Hg.

Along the same lines, had direct measurements of aromaticity been made using ^{13}C-NMR analysis, the correlation between aromaticity and bioavailable Hg would likely not have improved over that found in this study (R^2 = 0.63). ^{13}C-NMR analysis is limited to providing information on the number or distribution of specific types of carbon atoms, namely sp^2 hybridized carbon atoms bonded to other carbon atoms (Westerhoff *et al.*, 1999). The resonances attributed to, for example, carbonyl groups (>C=O) would not be included in the same region of the spectra and would, therefore, exclude some important functional groups such as carboxylic acids, amides, and esters, which typically bind metals. It is possible that the weak positive correlation that was observed between aromaticity and bioavailable Hg may have been due to another parameter to which aromaticity is well correlated, such as molecular weight, since larger molecules characteristically have a greater percent aromaticity (Chin *et al.*, 1994). This is assuming

that Hg(II) does not interact with aromatics through hydrophobic bonding or increased van der Waal's interactions like many hydrophobic organic contaminants do (Gauthier *et al.*, 1987).

In this study, the strongest correlation (negative) was between absorbance at 254 nm and bioavailable Hg normalized to total Hg (Figure 5) for all sites including Roddy lake ($R^2 = 0.903$). This logarithmic relationship improved further when reservoir 1 was omitted from the correlation ($R^2 = 0.95$). Omitting this site could be justified given the unusual response of the constitutive organism *V. anguillarum* pRB27 to reservoir 1 permeate (Figure 4). Absorbance in the UV range is dependent on the electronic structure of the molecule and concentration of the sample. At ~254 nm, absorbance will increase with an increasing number of molecules undergoing $\pi \rightarrow \pi^*$ electron transitions, typically conjugated molecules (Westerhoff *et al.*, 1999) including some chromophores and substituted aromatics. Interestingly, Kern (1953) demonstrated the photolysis of Hg mercaptides (thiols) to elemental Hg (Hg^0) in benzene at 2537 angstroms (~254 nm). Although not at all environmentally relevant, his work does nonetheless suggest that something as specific as a Hg-mercaptide bond may also absorb in this region. Similarly, Xiao *et al.* (1994) found that HgS_2^{2-} absorbed strongly at 285 nm (they did not go below this wavelength) with the production of Hg^0 and hypothesized that $HgS(SH)^{2-}$ and $Hg(SH)_2$ would have similar light absorption and photochemical behaviour.

The strong correlation between bioavailable Hg and absorbance at 254 nm could conceivably reflect the specific presence of reduced sulfur functional groups to which Hg is bound and consequently, unavailable for biological uptake. The affinity of Hg(II) for biological sulfur groups is well recognized, and there is growing evidence to support the

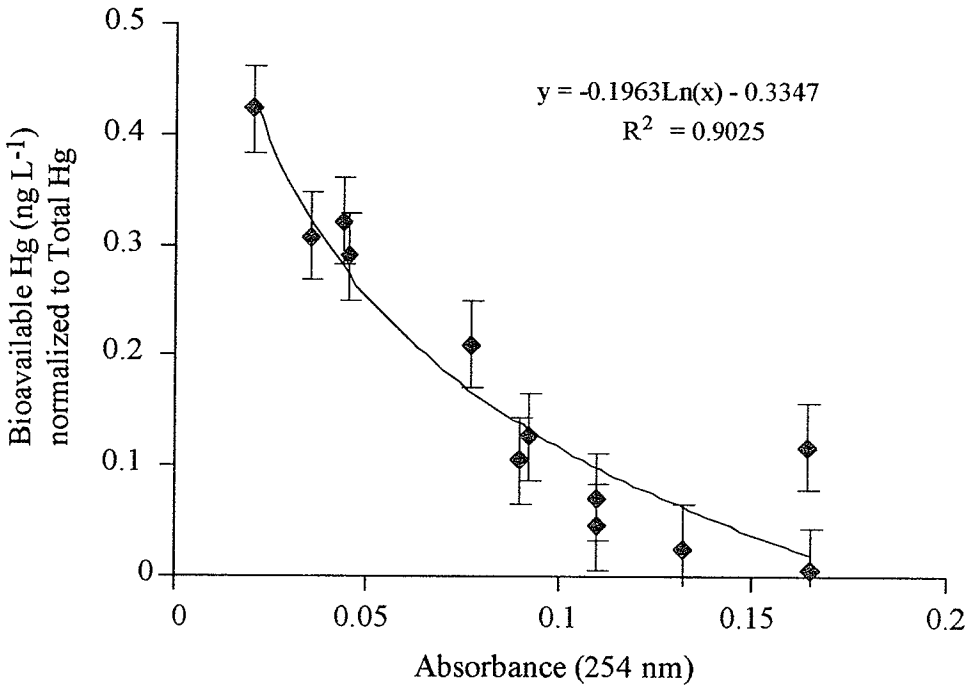

Figure 5.5 Relationship between bioavailable Hg normalized to total Hg (ambient and added Hg) and absorbance at 254 nm. Data points represent the mean of bioassays conducted on the <0.45 µm, retentate and permeate fractions of the FLUDEX reservoirs and Roddy Lake at the Experimental Lakes Area.

hypothesis that Hg(II) preferentially binds to thiol groups in soils (Xia *et al.*, 1999; Bloom *et al.*, 2001) and aquatic organic matter (Bloom *et al.*, 2001; Haitzer *et al.*, 2002). Furthermore, reduced forms of sulfur have been shown to be dominant in the hydrophobic fraction of both biosolids (Hundal *et al.*, 2000) and surface waters in the Florida Everglades (Aiken *et al.*, 2001). To reproducibly describe the chemical nature of DOC, normalizing for carbon and expressing absorbance as SUVA or molar absorptivity is necessary and can provide useful information on the origin of the precursor material. It falls short, however, in describing the biological reactivity of DOC. Although carbon may form the backbone of DOC, normalizing absorbance (at 254 nm) for reduced sulfur may prove a more useful approach for characterizing DOC in terms of its effects on the bioavailability of Hg(II).

Conclusions

An important finding of the research presented here is the demonstration that the size of DOC molecules is important in determining the biological fate of Hg(II) to bacteria. The higher MW fraction of DOC was largely responsible for the binding of Hg(II) and the subsequent reduction in its bioavailability. Despite similar concentrations of DOC in the respective size fractions, Hg associated with the lower MW DOC appeared to be more bioavailable than the higher MW DOC, likely due to a lower capacity to bind Hg(II) and possibly a lower affinity as well. Since extremely low concentrations of Hg were used in this study, a low capacity to bind Hg(II) could result from fewer high affinity functional groups, such as thiols. Furthermore, if the affinity of Hg(II) for the smaller size fraction is low, Hg(II) could possibly dissociate from the complex by ligand exchange with a

surface membrane protein and subsequently enter the cell. Alternatively, it is conceivable that the low MW DOC was small enough for Hg-DOC complexes to enter the cell in a similar fashion as siderophores.

The chemical characteristics of the DOC showed that the higher MW fraction was more typical of allochthonous carbon, whereas the low MW DOC was more autochthonous in character. This, together with the associated differences in the bioavailability of Hg(II) between these fractions, has interesting environmental implications. For example, Donahue and coworkers (1998) characterized the DOC in archived lake water samples from experimentally acidified lakes at the ELA. They found that lake water acidification had caused a change in what they termed the "quality" of the DOC to more "autochthonous-like" (microbially derived), compared with the allochtonous (terrestrially derived) "quality" that was characteristic of the control lakes. Given the findings presented here, acid-induced changes in the quality of DOC could cause an increase in the bioavailablility of Hg(II). In another whole ecosystem experiment carried out at the ELA, phosphorus fertilization in Lake 226N caused a dramatic increase in the autochthonous production of DOC and a concomitant utilization of allochthonous DOC (Schindler *et al.,* 1992). Again, a change of this nature in the character or "quality" of the DOC could result in greater bioavailability of Hg(II). Understanding how the qualitative differences in DOC affect the biological fate of Hg(II) in lakes will be useful in predicting potential impacts of environmental perturbations, natural or anthropogenic, on the behaviour of Hg(II). This work represents a beginning to a better mechanistic understanding of the relationships that exist between the nature of DOC and its bioavailable reactivity with Hg(II).

Chapter 6

CONTROLS ON THE BIOAVAILABILITY AND UPTAKE OF Hg(II)

Abstract

Inorganic divalent Hg (Hg(II)) is the substrate for two important microbially-mediated processes of the Hg cycle, both of which are intracellular: the reduction of Hg(II) to elemental Hg (Hg^0), and the methylation of Hg(II) to the potent neurotoxin methyl mercury (MeHg). Understanding how Hg(II) is taken into cells and the relationship of uptake on the aqueous chemical speciation of Hg(II) is essential for understanding and modeling the fate of Hg in the environment. The current model for Hg(II) uptake is by passive diffusion of neutral species such as $HgCl_2$, while many other metals such as Cd^{2+} and Cu^{2+} have been shown to be taken up as the free ion. Using a highly sensitive and specific genetically-engineered *mer-lux* bioreporter bacterium, this study demonstrates that the observed relationship of Hg(II) uptake to the aqueous chemical speciation of Hg(II) was inconsistent with a mechanism of passive uptake of neutral Hg(II) complexes. These results suggest that, despite the absence of specific Hg transport genes associated with the *mer* operon (*merP*, *merT*), Hg(II) enters the bacterial cell by an, as yet, undescribed facilitated uptake mechanism. Furthermore, our results do not conform to the free ion activity model (FIAM) frequently applied to metal uptake. This holds even when competition for uptake sites between Hg^{2+} and H^+ is considered since bioavailability increases, rather than decreases, with decreasing pH. Based on these findings, a greater variety of biologically-labile Hg(II) species is likely available to bacteria than previously believed.

Introduction

Metals are of environmental interest as limiting nutrients (Fe, Zn, Mn, Cu, Co, Mo, Ni) and as toxicants with little or no known biological function (Hg, Cd, Pb, Ag, Sn, Cr) (Sunda and Huntsman, 1998; Nies, 1999). To exert physiological or toxic effects at low levels, most metals have to enter cells (Nies, 1999). Essential metals are generally taken up into cells by specialized membrane proteins specifically adapted and regulated for intracellular transport. It is often found, however, that these metal transporters are not completely specific for the targeted essential metal and that they can mistakenly transport toxic metals (Cd and Cu) and metalloids (As and Sb) (Sunda and Huntsman, 1998; Nies, 1999). Nonetheless, the inadvertent facilitated uptake of Hg(II) has never been observed in aquatic (micro)organisms. Rather, it is generally thought that with the exception of microbial Hg resistance determinants that encode their own Hg(II) transport systems, the mechanism of Hg(II) uptake is passive diffusion of neutral lipophilic complexes (Gutknecht, 1981; Bienvenue *et al.*, 1984; Mason *et al.*, 1996; Benoit *et al.*, 1999).

The uptake of Hg(II) and chemical factors controlling Hg(II) bioavailability was investigated using a highly sensitive and specific *mer-lux* bioreporter, a genetically-engineered bacterium that produces light in proportion to the amount of Hg(II) that enters the cytoplasm of the cell (Selifonova *et al.*, 1993). An advantage to this approach is that bioreporters measure intracellular Hg(II) only, not surface-bound Hg(II), an important factor often neglected in uptake studies (Hudson, 1998). Furthermore, the bioreporter approach provides the opportunity to study the uptake of Hg(II) at environmentally realistic concentrations, well below those required to elicit a toxic response or nutritional deficiency.

Methods

The bioassays conducted in this chapter followed the cell preparation protocol described in Chapter 3. Standardization for the experiments exploring the relative differences in bioavailability between "high" and "low" ammonia was by the 'Method of Ratios' also described in Chapter 3. For the EDTA experiments, the EDTA and Hg were always added at least 10 minutes before the assay medium components (N, P, C) to allow the complexation of Hg with EDTA without interference. The thermodynamic speciation modeling for these experiments included a number of other metals that were identified as contaminants in the reagents. The concentrations used in modeling efforts are listed in the Figure captions. The full list of metals in the stock reagents can be found in Table 2, Chapter 3.

Interestingly, even though EDTA is a synthetic compound, its presence (and persistence) in natural aquatic systems, especially rivers, is not uncommon. This is due to anthropogenic sources and resistance to degradation (Breault *et al.*, 1996). So even though the intent for using EDTA was not to represent natural systems, it may nonetheless serve as a model ligand for some aquatic environments close to anthropogenic sources of EDTA.

Results & Discussion

To explore the effect of chemical speciation on Hg(II) bioavailability and uptake, bioassays were conducted using a defined medium with 15 pM (3 ng L^{-1}) total Hg, and varying concentrations of NaCl (up to 0.1 M). Maximum uptake occurred at NaCl concentrations between 0 and 2 mM followed by a gradual decrease with increasing NaCl

concentration (Figure 1A). Speciation calculations predicted that only three Hg species followed a similar trend as the bioreporter response; Hg^{2+}, $Hg(NH_3)_2^{2+}$, and $Hg(OH)_2$ (Figure 1B). $Hg(OH)_2$ was the only neutral species but represented a maximum of only ~1% of the total Hg at low NaCl (Table 1). The concentration of Hg^{2+} was extremely low while the majority (~99%) was the Hg-ammonia complex, $Hg(NH_3)_2^{2+}$, which carries an electrochemical charge (Table 1). Cell membranes are virtually impermeable to charged or highly polar neutral species (Sunda and Huntsman, 1998) so $Hg(NH_3)_2^{2+}$ was initially assumed to be unavailable for uptake. However, experimentally reducing the concentration of $(NH_4)_2SO_4$ by two orders of magnitude in the assay medium did not result in a corresponding increase in the uptake of Hg(II) at either 3 or 67 mM phosphate despite the accompanying predicted speciation changes showing a predominance of neutral species (Table 2), notably $Hg(OH)_2$. In fact, at 3 mM phosphate, uptake was reduced in the "low N" treatments relative to the "high N" treatments (Table 2).

Early studies using the *mer-lux* bioreporter *E. coli* pRB28 (Barkay *et al.*, 1997) over a range of NaCl concentrations were carried out with 75 μM $(NH_4)_2SO_4$ (not 9.1 mM as in our experiments). Consequently, the presence of a charged species dominating the assay medium was not an issue and, therefore, not addressed in that study. Regardless, an important result in the study by Barkay *et al.*, (1997) was that the response of the biosensor was the same at 0.001 mM and 1 mM NaCl and they concluded that the predominant species (the neutral $HgCl_2$ and $Hg(OH)_2$) must, therefore, be equally bioavailable. This is in agreement with our findings (Figure 1). However, the octanol-water partition coefficient of $Hg(OH)_2$ is more than an order of magnitude lower than that of $HgCl_2$ (0.05 vs 3.33, respectively from Mason *et al.*, 1996). Also, clear differences in

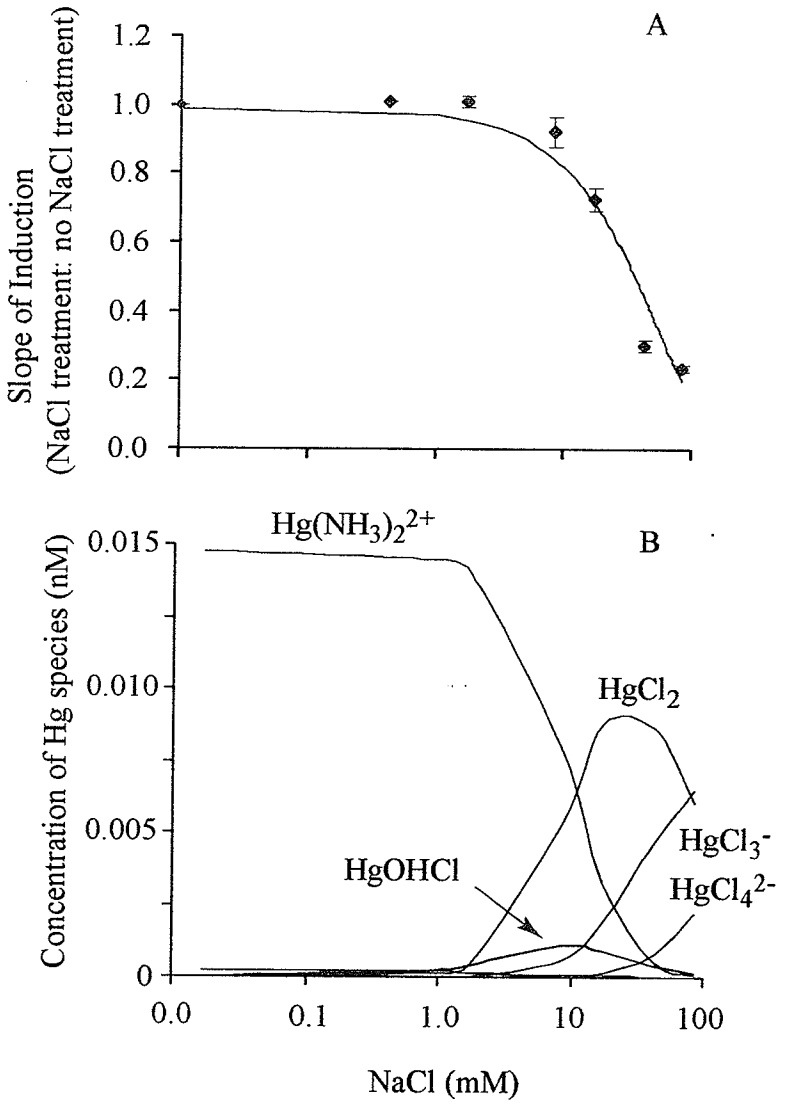

Figure 1.6 Panel A -the effect of NaCl (up to 0.1 M) on the bioavailability of 15 pM Hg(II) (3 ng L^{-1}) in defined medium containing 9.1 mM $(NH_4)_2SO_4$, 67 mM phosphate, 5 mM glucose at pH 7. Assays were repeated up to eight times depending on the salt concentration. Error bars are for standard error. Panel B -speciation calculations run under the same conditions as the bioassay.

Table 1.6 Percent Hg species as a function of NaCl concentration determined by thermodynamic speciation modeling. Input parameters included 15 pM Hg, 67 mM phosphate, 5 mM glucose, 9.1 mM $Hg(NH_3)_2^{2+}$, pH 7. Empty spaces represent concentrations that were <0.005%.

Hg species	***NaCl (mM)***						
	0.017	***0.856***	***1.71***	***8.56***	***17.1***	***42.8***	***85.6***
Hg^{2+}							
$HgCl^+$							
$HgCl_2$		0.65	2.5	35	58	58	40
$HgCl_3^-$		0.01	0.05	3.82	12.6	31.1	43.4
$HgCl_4^{2-}$				0.13	0.86	5.3	15
HgOHCl	0.03	1.4	2.6	7.4	6.1	2.4	0.84
$HgOH^+$							
$Hg(OH)_2$	1.1	1.1	1.1	0.61	0.25	0.04	0.01
$Hg(OH)_3^-$							
$HgNH_3^{2+}$							
$Hg(NH_3)_2^{2+}$	99	97	94	53	22	3.4	0.60
$Hg(NH_3)_3^{2+}$	0.07	0.07	0.07	0.04	0.02		
$Hg(NH_3)_4^{2+}$							
$HgPO_4^-$							
$HgHPO_4$	0.02	0.02	0.02	0.01			

Table 2.6 Relative difference in the bioreporter signal between "low" (0.09 mM) and "high" (9.1 mM) $(NH_4)_2SO_4$ treatments as determined by the Method of Ratios. Bioassays were carried out at pH 7 in a defined medium containing 0.09 and 9.1 mM $(NH_4)_2SO_4$, 67 and 3 mM phosphate, 5 mM glucose, 15 pM Hg (SD = standard deviation). Input parameters for the speciation calculations were the same as the conditions under which the bioassays were run with the addition of 0.032 mM Cl which is present in the reagents as a contaminant. Hg species are presented as % charged vs % neutral under "high" and "low" $(NH_4)_2SO_4$.

	Experimental	Calculated Speciation			
Phosphate	Bio Hg(II)	0.09 mM $(NH_4)_2SO_4$ "low" N		9.1 mM $(NH_4)_2SO_4$ "high" N	
(mM)	low N:high N (± SD)	% charged	% neutral	% charged	% neutral
67	1.05 (±0.09) n=7	0.80	99.2	98.8	1.19
3	0.50 (±0.15) n=8	0.50	99.5	98.4	1.60

the flux of the $HgCl_2$ and $Hg(OH)_2$ across membranes have been shown in studies using lipid bilayer membranes, which contained no transport proteins and where passive diffusion was the only uptake mechanism (Gutknecht, 1981). If passive diffusion of neutral species was occurring, as was assumed in the study by Barkay *et al.*, (1997), one would expect a relative increase in the bioreporter signal mirroring $HgCl_2$, not $Hg(OH)_2$ (and certainly not $Hg(NH_3)_2^{2+}$). In light of the fact that neutral species fail to explain the observed bioreporter responses under these experimental conditions, and that the predominance of the charged $Hg(NH_3)_2^{2+}$ complex did not reduce the uptake of Hg(II), an alternative explanation was sought by (1) more effectively controlling the speciation of Hg in the test solutions and then (2) exploring the possibility that Hg(II) uptake was occurring by a facilitated mechanism, likely in addition to passive diffusion.

In order to investigate controls on the uptake of Hg(II), bioassays were carried out in the presence of NaCl and the synthetic organic chelator EDTA. EDTA was used to create a well-defined and buffered concentration of various Hg(II) species (Simkiss and Taylor, 1995) thus reducing uncertainties associated with the stability constants of Hg-ammonia complexes which, in the presence of EDTA, no longer dominated the solution matrix. Increasing the concentration of NaCl in the assay medium to 17 mM (~1 g L^{-1}) caused a concomitant increase in the concentration of $HgCl_2$ of ~28 million times (from 8 x 10^{-23} M to 2.5 x 10^{-15} M), while the concentrations of all non-chloro Hg complexes (including Hg-hydroxy, Hg-ammonia, and Hg-EDTA complexes) and the free Hg^{2+} ion were maintained constant (Figure 2). If passive diffusion were the predominant uptake mechanism, one would expect an increase in the bioavailability of Hg with increasing $HgCl_2$ since this species is the most lipophilic, neutral complex and should therefore

cross biological membranes the most readily. The induction slope, however, remained remarkably unchanged over the NaCl concentration range examined (Figure 2) demonstrating that $HgCl_2$ was not directly correlated to uptake.

In the absence of chloride, $Hg(OH)_2$ should control the passive uptake of Hg(II) and one would, therefore, expect an increase in the bioreporter response with increasing hydroxide concentration (i.e. increasing pH). Again, there was no positive correlation between these parameters. In fact, the bioreporter signal decreased with increasing pH (Figure 3). Taken together, these data show that under well-defined and buffered conditions, neither of the predominant neutral species, $HgCl_2$ nor $Hg(OH)_2$, could have been solely responsible for the observed response of the *mer-lux* bioreporter, thus demonstrating that the uptake of Hg(II) is not consistent with the standard model of passive diffusion. Interestingly, the concentration of EDTA required to control the speciation of Hg(II) far exceeded that predicted by thermodynamic speciation modeling even when other metals present in the assay medium were included in the model. This suggests that even Hg-EDTA species, notably $HgEDTA^{2-}$ are available for uptake which further points in the direction that Hg(II) is entering the cell by a facilitated uptake mechanism.

Many bioassays conducted with unicellular organisms have demonstrated that the free ion concentration (or activity) of the metal is the key factor influencing dissolved metal uptake, toxicity, or nutrition (Tessier *et al.*, 1994). These observations have led to the formulation of the "free ion activity model" (FIAM), which postulates that the biological effect of metals on organisms is proportional to the free ion concentration rather than to the concentration of the total dissolved metal or of metal-ligand complexes

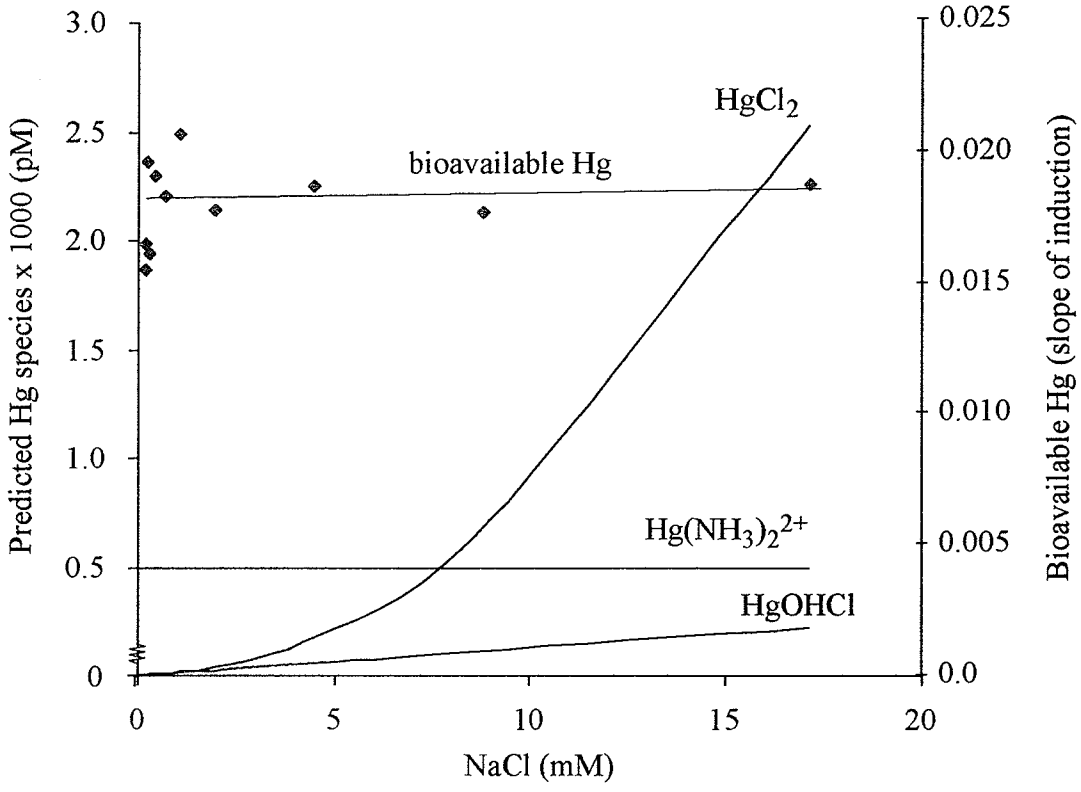

Figure 2.6 Effect of NaCl on the bioavailability of Hg(II) in a defined medium containing 100 μM EDTA, 9.1 mM $(NH_4)_2SO_4$, 3 mM phosphate, 5 mM glucose, 3.75 pM Hg at pH 7. Speciation calculations were run under the same conditions as the bioassay. Included in the model input was Cu (0.84 nM), Zn (25 nM), Al (140 nM), Ca (470 nM), Mg (16 nM), and Cl (0.032 mM) due to their presence as contaminants in the assay medium.

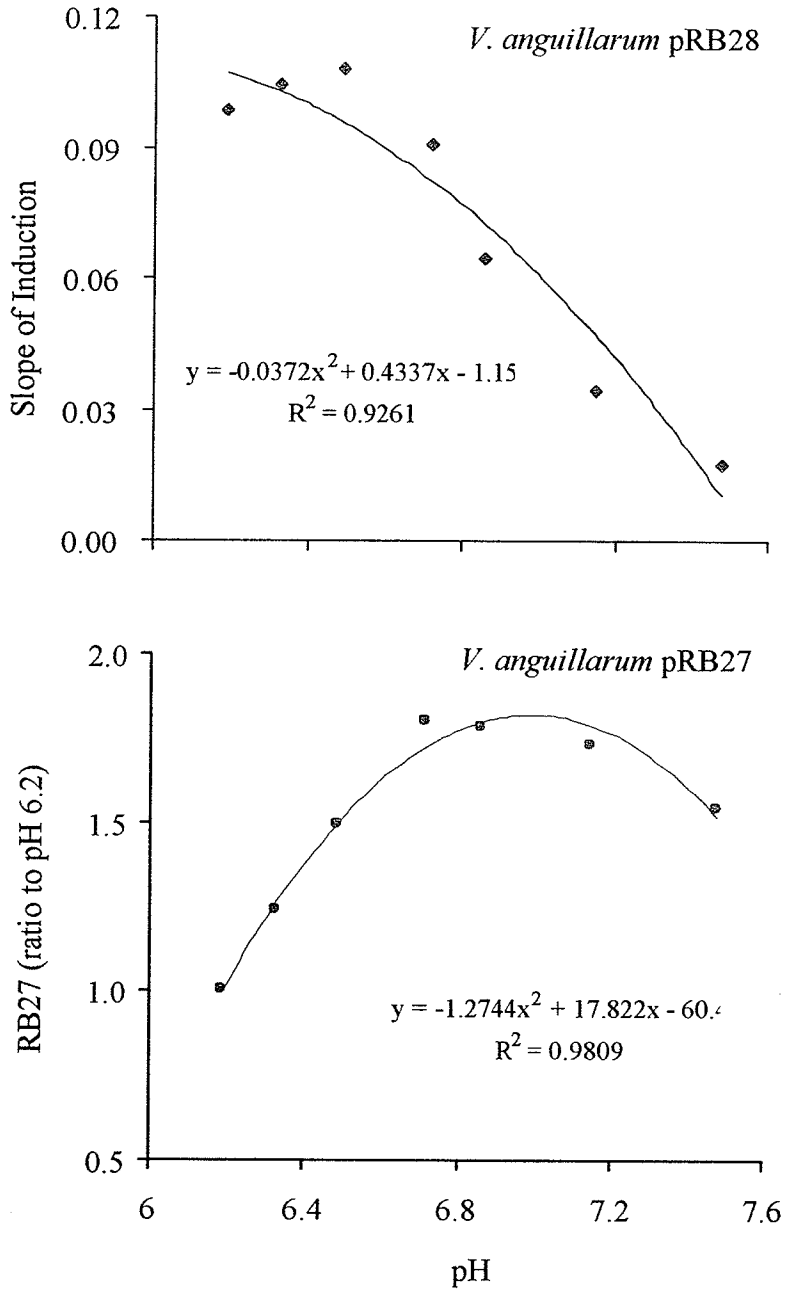

Figure 3.6 Effect of pH on the bioavailability of Hg(II) as measured with the *mer-lux* bioreporter *V. anguillarum* pRB28 and on light production by the constitutive control organism *V. anguillarum* pRB27. Bioassay conditions were 5 mM glucose, 9.1 mM $(NH_4)_2SO_4$, 3 mM phosphate, 2.5 pM Hg (0.5 ng L^{-1}), and varying concentrations of NaOH.

(Morel, 1983; Tessier *et al.*, 1994). The free ion concentration is determined by the total dissolved metal concentration and by the concentration and nature of the ligands in solution (Campbell, 1995). These ligands are assumed to be at equilibrium with the metal both in the external medium and at the organisms' surface transport sites (Tessier *et al.*, 1994), which can be thought of as ligands themselves participating in ligand exchange reactions (Playle, 1998). Uptake is considered to be under "equilibrium" or "thermodynamic" control when all metal species react with the transporter at the same rate. The biological response is then proportional to the concentration of the metal-surface complex, which will vary as a function of the free ion concentration (Campbell, 1995). Although organic complexes generally do not dissociate rapidly enough to directly donate a metal ion to the transport protein, they may still have an important effect on metal uptake by influencing the solution concentrations of free metal ions and inorganic complexes (Sunda and Huntsman, 1998).

The strongest experimental evidence for the FIAM has been obtained by demonstrating the same biological response from treatments containing different combinations of chelating agents and ligand concentrations while maintaining the calculated free metal-ion concentration constant (Stumm and Morgan, 1996; Hudson, 1998). To explore the facilitated uptake of Hg(II) and its possible relation to the free Hg^{2+} ion concentration, *mer-lux* bioassays were carried out in a defined medium with between 0 and 10 pM (2 ng L^{-1}) total Hg and varying concentrations of EDTA (Figure 4). The dependence of uptake on the free Hg^{2+} ion concentration in the bulk solution under these experimental conditions was clearly not evident (Figure 4). If it were under free ion control, one would expect the biological response to be the same at one Hg^{2+}

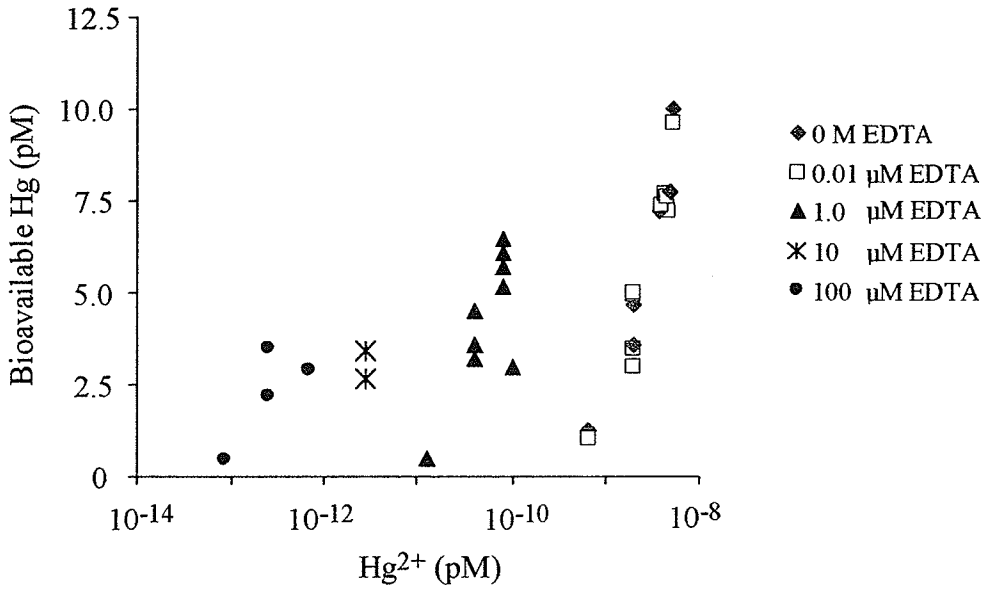

Figure 4.6 Bioavailability of Hg(II) as a function of the free Hg^{2+} ion concentration. Bioassays were carried out in 5 mM glucose, 9 mM $(NH_4)_2SO_4$, 3 mM phosphate, and up to 10 pM (2 ng L^{-1}) Hg at pH 7. Contaminant metal concentrations included in the speciation calculations were the same as those listed in Figure 2.

concentration and as a result, all EDTA treatments would fall on one line. It is important to note that bioassays using the constitutive control organism *V. anguillarum* pRB27 in the presence of EDTA showed that concentrations of EDTA up to 10^{-5} M resulted in no significant change in the light produced relative to a distilled water control (Figure 5). It is therefore highly improbable that the EDTA treatments were disrupting the cell membrane and enhancing uptake. At EDTA concentrations exceeding 10^{-4} M EDTA, however, the organism started to produce less light. Using concentrations as high as 10^{-3} M is not recommended (Figure 5).

Some studies have found that the FIAM more effectively describe metal uptake when competition between the free ion and hydrogen ions (H^+) for the same biological binding sites are taken into account (Hare and Tessier, 1996). This was not the case here since increasing the H^+ concentration increased rather than decreased the uptake of Hg(II) (Figure 3) so the relation between uptake and Hg^{2+} was not improved. In addition to potential competition between H^+ and the free ion, shifts in pH may also affect trace metal uptake due to changes in the metal speciation in solution. For example, at constant dissolved metal concentration, a shift in the hydrolysis equilibrium to the left (lower pH) would favour the formation of the free metal ion (Burggraff *et al.*, 1998). Under these experimental conditions, speciation calculations showed that the proportion of the Hg^{2+} does increase with decreasing pH (data not shown). However, as described above using similar experimental conditions, Hg^{2+} was not the species controlling the uptake of Hg(II) (Figure 4). Thus, Hg(II) uptake does not appear to conform with the FIAM even when competition between the free Hg^{2+} and H^+ is considered.

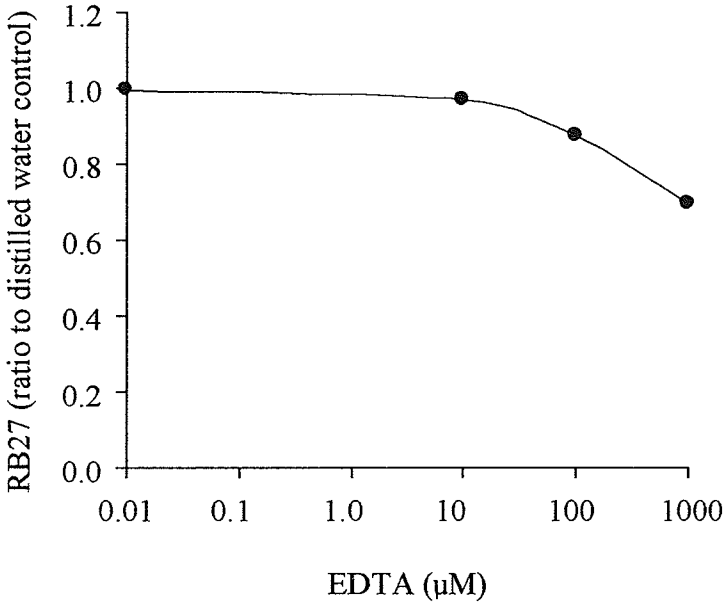

Figure 5.6 Effect of EDTA on the constitutive control organism *V. anguillarum* pRB27. Bioassay conditions were the same as those used for *V. anguillarum* pRB28 in Figure 4.

There are several cases where the FIAM, or equilibrium controlled uptake, will not apply. For example, if the reaction of the free ion (or labile Hg-ligand complex in equilibrium with the free ion) at the cell surface is slower than the internalization of the metal, uptake will be controlled by the kinetics of the complexation reaction rather than by thermodynamic considerations (Campbell and Stokes, 1985). The FIAM will also not apply if the transport of the metal to the cell surface proves to be the rate-limiting step (diffusion limitation). In cases of diffusion limitation, metal uptake depletes transported species at the cells' surface as well as throughout the cells' boundary layer. If other metal complexes (that are normally kinetically inert with respect to the transporter) are able to dissociate to form transportable species, the diffusion limitation is lifted (Hudson, 1998). Uptake rates, therefore, will be determined by the concentration of those labile inorganic complexes, not by the free ion concentration (Sunda and Huntsman, 1998). Lastly, the FIAM will not apply to metals that form stable ternary surface complexes (i.e. ligand-Hg-cell-surface site). If such complexes do form, and if they contribute to the biological response, then simple dependence of the response on the free ion concentration would not be observed.

The results presented here suggest that $Hg(OH)_2$, $HgCl_2$, $Hg(NH_3)_2^{2+}$ and possibly even Hg-EDTA exhibited direct and rapid ligand exchange with a membrane transport protein. In the case of Hg-EDTA, this exchange may have been via a ternary complex (i.e. EDTA-Hg-cell). Hg is well known to be highly specific for sulfhydryl groups, hence its attraction to proteins, and although Hg-sulfur bonds are thermodynamically stable, they are known to undergo rapid exchange reactions, especially for MeHg (Boudou *et al.*, 1991). If the Hg-EDTA complex binds to a thiol group at the cell surface, Hg^{2+} could be

released and taken up either by that protein or a nearby transporter. The free ion would be transported into the cell, however, the Hg-EDTA complex (or other predominant species in solution) would be the species controlling the rate of transport. Consequently, uptake would not conform to the FIAM.

Since the molecular size and electrochemical charge of the Hg-EDTA complex would likely not permit its direct entry into the cell, this scenario requires that a membrane protein is accessible extracellularly. Furthermore, the affinity of Hg for the surface protein must be greater than that for EDTA. That is, the membrane protein would require sulfhydryl groups arranged in such a manner that the resulting affinity for Hg(II) is greater than the log stability constant of 23.5 (Table 5, Chapter 2) for Hg-EDTA. This is, of course, assuming that the stability constant for Hg-EDTA is correct. The sodium-potassium-transporting adenosinetriphosphatase (Na^+/K^+-ATPase), a member of the P-type ATPase superfamily, is a membrane protein responsible for the transport of Na^+ and K^+ across the plasma membrane against their electrochemical gradients (Kotyk and Amler, 1995). Interestingly, it also belongs to the metal-binding proteins and has a particularly high affinity for Hg(II), presumed due to the formation of stable (and rare) bi- and tri-coordinate thio-Hg(II) complexes (Anner *et al.*, 1992a). Indeed, Hg compounds are known to be unspecific Na^+/K^+-ATPase inhibitors at μM concentrations (Imesch *et al.*, 1992). One of the mechanisms of this inhibition is believed to be due to Hg binding to an extracellular cysteine residue of the α-subunit of the protein (Wang and Horisberger, 1996). Given this high affinity for Hg(II) and the substantial similarity of the α-subunit both among species and with other P-type ATPases, including bacterial (Kotyk and Amler, 1995), a ubiquitous membrane protein of this sort may play a role in

drawing Hg(II) toward the cell surface and out-competing other ligands such as EDTA in solution.

Another requirement for Hg-EDTA to be available for uptake would be that EDTA not chelate Hg as effectively as it traditionally does other metals so that the surface protein or transporter can get access to it. This seems highly improbable given its reputation of rendering metals biologically inert. However, there are a number of reports in the literature, which support this possibility as well. Kungolos *et al.,* (1999) found that the addition of EDTA to growth medium did not reduce the toxicity of Hg to the yeast *Saccharomyces cerevisiae*. Also, EDTA had no significant effect on Hg^{2+} bioaccumulation by a genetically engineered strain of *Escherichia coli* (JM109) expressing metallothionein and a Hg^{2+} transport system (Chen and Wilson, 1997). On the other hand, Gutknecht (1981) used EDTA to effectively reduce the diffusion of $HgCl_2$ across bilayer lipid membranes, which contain no proteins. So it appears that the efficacy of EDTA in irreversibly binding Hg(II) is reduced when membrane proteins or other strong competing ligands at the surface of living cells are present.

The presence of a membrane protein with a high affinity for Hg(II) also explains the observed effect of pH on the response of the bioreporter. As mentioned above, the pH-dependent changes in the chemical speciation of Hg(II) do not explain the response of the bioreporter to changes in pH (Figure 3). This could possibly be due to increased adsorption of Hg(II) to the cell surface at lower pH thus increasing its chance of internalization. An interesting study by Ledin and co-workers (1997) showed that the adsorption of ^{203}Hg to the Gram-negative bacterium *Pseudomonas putida* increased with decreasing pH. Furthermore, only 20% was desorbed by EDTA demonstrating a strong

interaction between Hg(II) and the bacterium. This was in contrast to a number of other metals (Sr, Zn, Cd), which showed that adsorption to the same cells increased with increasing pH, while desorption by EDTA was more effective than it was for Hg(II). If adsorption of Hg(II) to the membrane of the *mer-lux* bioreporter increases at lower pH, internalization might also increase. The response of the constitutive organism *V. anguillarum* pRB27 (Figure 3) can also be explained by increased adsorption of Hg(II) to the cell. Luminescence is coupled to the respiratory electron transport chain, notably by $FMNH_2$ production via an NADH and FMN reductase couple (Ulitzur *et al.*, 1981). Thus, an environmental change such as the increased concentration of a metal bound to the membrane could indirectly decrease the production of light by the constitutive organism by affecting respiration, hence the opposite trend to the Hg(II)-induced response by *V. anguillarum* pRB28 (Figure 3). This possibility requires and warrants further investigation.

Conclusions

The results presented in this chapter show strong evidence to support the finding that, contrary to current thinking, Hg(II) uptake by our study organism does not conform to the standard model of passive diffusion. Rather, it is likely entering the cell by a facilitated uptake mechanism that does not appear to be under free ion control. The evidence using the synthetic chelator EDTA is particularly interesting for a number of reasons. EDTA provides a well-defined and buffered chemical matrix, thus allaying concerns regarding uncertainties in the stability constants of dominant Hg(II) species like $Hg(NH_3)_2^{2+}$. In addition, the presence of EDTA minimizes, if not eliminates the possibility of

interference from contaminant metals in the assay solutions. This is especially important since we do not know what their effects are on Hg(II) uptake, but could include competition with Hg(II) for cellular binding sites, transport or otherwise. Lastly, $HgEDTA^{2-}$ appears to be bioavailable possibly through ternary complex formation and ligand exchange. The possibility that this species could enter the cell by passive diffusion is essentially impossible unless perhaps if the cell membrane was disrupted. The constitutive control organism, *V. anguillarum* pRB27, showed no evidence of this at the concentrations used in this work. Overall, these findings suggest that more species of Hg(II) are available for uptake than previously believed. They also show that the uptake of Hg(II) is not purely a function of the chemistry of the medium but includes important interactions with the organism itself. These interactions need to be better characterized and included in attempts to model the behaviour of Hg in aquatic ecosystems.

Chapter 7
SUMMARY

The pervasive and global nature of Hg pollution is largely due to two reasons. The first is its wide distribution as Hg^0 via atmospheric processes, and subsequent deposition as Hg(II) to terrestrial and aquatic ecosystems, including those in remote areas far from point source pollution. The second is its complex behaviour in the environment as it binds to an assortment of ligands and transforms (cycles) between oxidation states. In essence, Hg travels and changes but never really goes away. Many advances have been made in the measurement and detection of a variety of Hg species (Bloom and Crecelius, 1983; Bloom, 1989; Gill and Bruland, 1990; Morrison and Watras, 1999), yet there remains a need for analytical methods that provide information on the fraction of Hg that is able enter the cell. In recognition of this, this dissertation was aimed to further develop the *mer-lux* bioreporter for the analyses and characterization of Hg(II) in aquatic samples from the Experimental Lakes Area (ELA) in northwest Ontario. The ELA is a Boreal ecosystem impacted by Hg pollution characterized by non-point source atmospheric deposition.

Bioavailable Hg measurements were made for the first time on a variety of aquatic samples, cumulatively known as "input sources", as a preliminary assessment of source strengths of bioavailable Hg entering lakes at the Experimental Lakes Area. These sources included snow, rain, through-fall and run-off, all of which had detectable bioavailable Hg. With the exception of snow, the fraction of the total Hg in these input sources that was bioavailable was generally low, representing on average 1 to 2.5% of the

total Hg, a small and fairly consistent proportion of the total Hg pool. In contrast, nearly 50% of the total Hg in snow was bioavailable. The question of WHY bioavailable Hg is so high remains to be answered. Nonetheless, a substantial pulse of bioavailable Hg is potentially entering ELA lakes in the springtime. It is also highly probable that areas of the Arctic may be similarly impacted as well.

Of importance, therefore, in both Arctic and Boreal ecosystems, is determining the fate of bioavailable Hg in run-off (as opposed to snow) during the spring melt period. It appears that within the total Hg pool, the photo-reducable fraction, which would evade back to the atmosphere, is not the same as the bioavailable fraction although some Hg species could be available for both processes. Indeed, it is highly probable that a Hg species such as $HgCl_2$, which is not appreciably photo-reducable (Xiao *et al.*, 1994), represents a refractory yet highly bioavailable fraction remaining in snow at the start of the spring melt period. The fate of this fraction after the melt period is extremely important because the longer that bioavailable Hg is able to persist, the more chance it has to become microbially transformed.

Of equal interest and importance is what happens to bioavailable Hg once it enters a lake from atmospheric sources. This question is all the more intriguing given that bioavailable Hg was never detectable in any of the lakes and experimental reservoirs analyzed at the Experimental Lakes Area without the addition of Hg(II). Complexation of Hg(II) occurred primarily in the dissolved phase. Furthermore, there appeared to be at least one type of functional group or 'class of ligand' binding the added Hg(II); a high affinity, low capacity site and possibly a low affinity, high capacity site. Given the extremely low concentrations of Hg(II) used in these studies, it seems probable that the

high affinity, low capacity site contained reactive thiol functional groups which controlled the bioavailability of Hg(II). The importance of working at environmentally relevant and realistic concentrations of Hg(II) for studies of both bioavailability and sorption can not be overstated. The response of a high affinity, low capacity binding site that likely controls the bioavailability of Hg(II) under natural conditions could be masked entirely with Hg additions that marginally exceed ambient/natural concentrations.

Despite the rapid binding and subsequent reduction in bioavailability that was shown to occur when Hg(II) was added to lake water, a very small percentage of bioavailable Hg persisted over time periods exceeding 24 hours thus demonstrating that complexation equilibrium was not reached. This could potentially have significant effects on the ecological fate of Hg(II) especially if the rate of re-establishment of chemical equilibrium is slower than the rates of competing processes such as uptake by bacteria (Ma *et al.*, 1999). Consideration of the kinetic aspects of this nature may need to be included in attempts to model the aqueous speciation of Hg(II) in natural systems.

To better understand how the character of DOC influences its bioavailable reactivity with Hg(II), ultrafiltration methods and XAD-8 chromatography were used in conjunction with the *mer-lux* bioreporter. There was a natural preference for Hg(II) to bind or partition to the higher molecular weight DOC (>10 kDa) which also had a comparatively higher capacity to bind Hg(II) than the lower molecular weight DOC. Consequently, the higher molecular weight DOC was largely responsible for the reduction in the bioavailability of Hg(II). Since extremely low concentrations of Hg were used in this study, the comparatively lower capacity of the low molecular weight DOC could have been a result of fewer high affinity functional groups, such as thiols. It is also

conceivable that the low MW DOC was small enough for Hg-DOC complexes to enter the cell in a similar fashion as siderophores. Regardless, the size of the DOC molecules clearly exerted a strong control on its reactivity with Hg(II), thereby affecting its microbiological fate.

The chemical characteristics of the DOC showed that the higher MW fraction was more typical of allochthonous carbon (derived largely from precursors of higher plants), whereas the low MW DOC was more autochthonous in character (typically derived from such processes as extracellular release and leachate of algae and bacteria). This, together with the associated differences in the bioavailability of Hg(II) between these fractions, could have interesting environmental implications. For example, acid-induced changes in the quality of DOC as described in Donahue *et al.* (1999) could increase the bioavailablility of Hg(II) in aquatic systems. Also, eutrophication resulting in an increase in the production of autochthonous DOC and a concomitant use of allochthonous DOC (Schindler *et al.,* 1992) could lead to increased bioavailability of Hg(II). The character of DOC, rather than the quantity of DOC, should be considered in attempts to understand implications of environmental change on the biological fate of Hg(II).

Although spectral properties (SUVA or molar absorptivity, aromaticity) have proved useful in describing the chemical nature of DOC and in providing information on the origin of the precursor material, they fall somewhat short in describing the actual biological reactivity of DOC with Hg(II). This is not unexpected since DOC measures all sources of carbon, including humic and fulvic matter as well as carbohydrates and proteins (Mierle and Ingram, 1991), some of which could potentially enter the cell. A carbon-based normalization, therefore, dilutes the actual parameter that is specifically

controlling the bioavailable reactivity of the DOC with Hg(II). Although further studies are required, the strong negative correlation between bioavailable Hg and absorbance at 254 nm (not normalized for carbon) could be due to the presence of Hg-mercaptide bonds, which not only appear to absorb in this region (Kern, 1953; Xiao *et al.*, 1994) but would also have a profound control over the bioavailability of Hg(II). Although carbon may form the backbone of DOC, normalizing absorbance for reduced sulfur instead of carbon may prove a more useful approach for characterizing DOC from differing systems in terms of its effects on the bioavailability of Hg(II).

Another important consideration in attempts to understand the biological fate of Hg(II) in lakes is the relationship of the uptake of Hg(II) by the organism on its aqueous chemical speciation. With the exception of microbial Hg resistance determinants and a recent study demonstrating facilitated uptake of Hg(II) under anaerobic conditions (Golding *et al.,* 2002), it is generally believed that the primary mechanism of Hg(II) uptake is passive diffusion of neutral lipophilic complexes (Gutneckt, 1981; Bienvenue *et al.*, 1984; Mason *et al.*, 1996; Benoit *et al.*, 1999). However, the results presented here show that under well-defined and buffered conditions, neither of the predominant neutral species, $HgCl_2$ nor $Hg(OH)_2$, could have been responsible for the observed response of the *mer-lux* bioreporter. Thus, the uptake of Hg(II) was not consistent with the standard model of passive diffusion, but rather appeared to be by an unknown facilitated uptake mechanism. The dependence of Hg(II) uptake on the free Hg^{2+} ion concentration was also not apparent suggesting that uptake did not conform with the Free Ion Activity Model either. Overall, these findings demonstrate that more species of Hg(II) are available for uptake than previously believed. They also show that the uptake of Hg(II) is

not purely a function of the chemistry of the medium but includes important interactions with the organism itself.

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