The Effect of Various Physiological Conditions on Hg(II) Uptake in *Escherichia coli* and *Vibrio anguillarum.*

By

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A Thesis Submitted to
the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the
Degree of

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FACULTY OF GRADUATE STUDIES

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The Effect of Various Physiological Conditions on Hg(II) Uptake in

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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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Abstract

The fate of mercury (Hg(II)) in aquatic ecosystems is highly dependent on a number of different bacterial mediated transformations. Of greatest environmental concern is the transformation of Hg(II) into methylmercury (MeHg), which is not only a more toxic form of Hg(II), but is also readily bioaccumalated up the aquatic food chain. Sulfate reducing bacteria residing within the anoxic/oxic interface of sediments are the primary methylators of Hg(II). The proposed enzymes responsible for this transformation are located in the cytoplasm and therefore, Hg(II) must first be taken up into the cell before it can be methylated. For bacteria not containing or expressing the *mer* operon, it was assumed that the mechanism of Hg(II) uptake was via passive diffusion. On the basis of this assumption, aquatic Hg(II) research has focused predominantly on correlating the formation of neutral Hg(II) species formed in the bulk solution with bioavailability. However, passive diffusion has only been documented in the presence of mM concentrations of $HgCl_2$ across eukaryotic-based lipid bilayers. Therefore, it was unknown whether this passive diffusion model could also predict Hg(II) bioavailability in the presence of pM-nM Hg(II) concentrations, which is commonly encountered in the environment, and in Gram-negative bacteria, whose cell structure differs substantially from the lipid bilayers.

Currently the only method available that distinguishes "bioavailable" from non-available forms of a metal is the use of bioreporters. We therefore obtained the *mer-lux* bioreporter (pRB28), which is a plasmid that contains a fusion of the regulatory region of the Tn*21 mer* operon, *mer*Ro/p, to a promoterless *lux* operon, *lux*CDABE, from *Vibrio fischeri*, for the purpose of studying Hg(II) bioavailability

under anaerobic and aerobic conditions. Using this plasmid construct in two facultative anaerobes, *Escherichia coli* and *Vibrio anguillarum*, we demonstrated that *lux* was a viable reporting system under specific anaerobic conditions for the detection of pM concentrations of Hg(II). While neither *E. coli* or *V. anguillarum* were capable of methylating Hg(II), they do serve as model organisms for the study of Hg(II) uptake in methylating bacterial cells.

Investigations into the mechanism of trace concentrations of Hg(II) uptake in *V. anguillarum* and *E. coli* did not coincide with the current model of passive diffusion. Alternatively, differences observed in the accumulation of Hg(II) under aerobic and anaerobic conditions, the enhancement of Hg(II) uptake in the presence of specific organic acids, the indiscriminate uptake of $Hg(OH)_2$, $HgCl_2$, and $Hg(NH_3)_2^{2+}$, the enhanced uptake of Hg(II) under acidic conditions, and the mitigation of Hg(II) uptake by competing metals and CCCP, all provided evidence consistent with facilitated mechanisms of Hg(II) uptake. Attempts to identify the transport system(s) involved in the "accidental" uptake of Hg(II) could not be elucidated from this study, but results obtained using the *mer-lux* bioreporter were consistent with the co-transport of Hg(II) with organic acids and/or metal transport systems specific for another essential metal.

Facilitated mechanism(s) for the uptake of a variety of different species of Hg(II) implies that speciation models focusing only on the formation of neutral Hg(II) species in the bulk solution need to be reconsidered. In addition to studying the bulk solution chemistry of Hg(II), one also needs to consider that differences in water chemistry would also alter the speciation and physiology of the bacterial cell. For

example, under different environmental conditions there would also be differences in the expression of various proteins and metabolites, which could ultimately have significant effects on Hg(II) uptake, intracellular trafficking, or sequestration. Therefore, a complete understanding of the mechanism(s) of Hg(II) uptake into microbial cells and how different environmental parameters affect this process(es) is crucial before accurate predictions regarding the fate of Hg(II) in aquatic ecosystems are made.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

CVAFS = cold vapor atomic fluorescence spectrometer
Cam = chloramphenicol
CCCP = carbonyl cyanide m-chlorophenylhydrazone
CPM = counts per minute
DOC = dissolved organic carbon
EDTA = ethylenediaminetetraacetic acid
ELA = Experimental Lakes Area
FIAM = free ion activity model
GMM = glucose minimal medium
Hg = mercury
Hg^{2+} = "free-ion" of mercury
Hg(0) = elemental mercury
Hg(II) = inorganic divalent mercury, including Hg^{2+}
Kan = kanamycin
K_{ow} = octanol water distribution coefficient
LB = Luria-Bertani
LPS = lipopolysaccharides
MeHg = methylmercury
MNNG = N'-methyl-N'-nitro-N-nitrosoguanidine
NTA = nitrilotriacetic acid
OM = outer membrane
PMF = proton motive force
ROS = reactive oxygen species
SRB = sulfate reducing bacteria
UV = ultraviolet

Chapter 1

Literature Review

1.1 Introduction

Mercury (Hg) is a group IIB heavy metal that possesses unique metallic properties in comparison to other heavy metals, and exists in three different oxidized states; Hg(II), Hg(I), and also as a vapor, Hg(0), at standard temperature and pressure (Summers 1986). Since primordial times Hg(0) has been released to the atmosphere from natural sources such as volcanic emissions, geothermal vents, and the reduction of Hg(II) from terrestrial and aquatic ecosystems (Rasmussen 1994). However, since the onset of the industrial era, Hg concentrations in the atmosphere has increased two to five times above those of pre-industrial times through additional anthropogenic sources (EPA 1997), including metal processing, chloralkali production, waste incineration, coal combustion and medicinal purposes (Lindqvist 1991). These anthropogenic sources can contribute up to 70% of the current total Hg input in the atmosphere (Schuster et al. 2002).

1.2 Cases of epidemic Hg Poisoning

For a number of years Minamata Bay, Japan, was heavily polluted from the discharge of waste products involved in the production of vinyl chloride. For the production of vinyl chloride the Chisso Company used Hg as a catalyst. It has been estimated that for every ton of vinyl chloride produced, 500 - 1000g of Hg was expended in the waste water (D'Itri and D'Itri, 1977). In approximately four decades nearly 150 tons of Hg were discharged into the bay (Graeme and Pollack, 1998). The addition of Hg into the waterway ultimately resulted in the biomagnification of

methylmercury (MeHg) up the food chain. Human consumption of the MeHg-contaminated seafood, especially fish, from the bay resulted in numerous neurological effects, congenital deformations, and in some cases death (Lofroth, 1970; D'Itri and D'Itri, 1977; Takeuchi and Eto, 1977). In addition to the human tragedy, much of the wildlife in the surrounding area was also greatly affected.

Hg was also commonly used as a fungicide, which subsequently resulted in the largest reported epidemic of Hg poisoning. In 1970 a trade agreement was established between the Iraqi government and the USA, in which nearly 100,000 tons of Hg treated wheat and barley were shipped to Iraq (D'Itri and D'Itri, 1977). Despite poison labels on the treated seeds, repeated broadcast warnings from the Iraqi government not to consume the wheat and barley, and the known toxicological effects of Hg, this epidemic still arose. The Hg treated seed was used to produce mainly bread and its consumption resulted in 6530 hospitalizations and 500 deaths, which were officially acknowledged by the Iraqi government (Bakir et al., 1973, D'Itri and D'Itri 1977). Unofficial estimates however, claim that up to 60,000 people were potentially affected. As with Minamata Bay, much of the surrounding wildlife was also affected from the consumption of the contaminated seeds, especially domestic animals that were directly fed with the Hg-treated barley.

These two epidemics provided the strongest link of human/wildlife fatalities and neurological diseases to the toxicity of Hg. The toxicity of Hg to human and wildlife has since been studied in great detail and has been reviewed extensively (Lofroth 1970, Skerfving and Vostal 1972, D'Itri and D'Itri 1977, Takeuchi and Eto 1977, Clarkson 1994, Graeme and Pollack 1998, Wolfe et al. 1998, Langford and

Ferner 1999, Ozuah 2000, and Satoh 2000). The problem faced today is that Hg in the biosphere cannot be destroyed and the total amount present is not expected to decrease readily. Although Minamata Bay and Iraq are two examples of point source contaminated sites, the threat of Hg contamination is a global concern that extends far beyond point sources of contamination and into "pristine" locations. For example, a large study conducted in the northeastern United States and eastern Canadian provinces looked at Hg levels in more than 5000 lake and stream fish species (NESCAUM, NEWMOA, NELWPCC, CEMAN, 1998). Results from this study indicated Hg levels > 0.5 ppm in one out of every 10 fish examined, a level on which many countries post fish consumption warnings.

1.3 The Hg(II) cycle and bacterial mediated transformations of mercury

On a global scale, the atmosphere plays a major role in the biogeochemical cycling of Hg. Hg(0) released to the atmosphere is relatively stable. This stability can result in the dispersion of mercury thousands of kilometers away from a point source of emission (Lindqvist 1994), thereby extending mercury contamination globally. In the atmosphere, Hg(0) is oxidized to Hg(II) through interactions with ozone and water (Schroeder et al. 1991). Hg(II) is water soluble and highly reactive allowing it to be reintroduced back to the biosphere as wet and dry deposition (reviewed in Downs et al. 1998). The primary concern with increasing atmospheric mercury is the contamination of non-point source remote aquatic ecosystems (Fitzgerald et al. 1998). Hg contamination of these habitats can result in detrimental effects both directly within the food webs of impacted systems, as well as linkages to

human health concerns associated with Hg-contaminated fish supplies (Wolfe et al. 1998, Graeme and Pollack 1998, Mahaffey 1999, Boening 2000, Ozuah 2000, Lutter and Irwin 2002).

The fate of Hg introduced to aquatic ecosystems via atmospheric deposition (Fitzgerald et al. 1998) is highly dependent on a number of different bacterial mediated transformations of Hg (Barkay et al. 1992). Hg methylation, demethylation, reduction and oxidation are the four primary bacterial mediated transformations that make up this cycle (Figure 1.1). Some of these reactions are specific for Hg, while others appear to mistake Hg as an essential substrate in metabolic pathways. Of greatest environmental concern are the bacteria that are capable of methylating Hg. MeHg is the most toxic form of Hg(II) (Clarkson 1994, Wolfe et al. 1998, Ozuah 2000) and is readily bioaccumalated up aquatic food chains (Watras et al. 1998). The methylation of Hg occurs predominantly in the anoxic sediment of aquatic ecosystems (Compeau and Bartha 1984) and the bacteria primarily involved are specific and diverse strains of sulfate-reducing bacteria (SRB) (King et al. 2002, Macalady et al. 2000, Pak and Bartha 1998, Gilmour et al. 1992, Compeau and Bartha 1985).

To date the mechanism of mercury methylation has only been proposed for the sulfate-reducing bacterium *Desulfovibrio desulfuricans* LS (Choi et al. 1994a, Choi et al. 1994b). In this bacterium the methylation of Hg involves the acetyl-CoA synthase pathway. In this pathway it is proposed that methyltransferase I transfers the methyl group from CH_3-tetrahydrofolate to cobalt in a corrinoid protein. A second enzyme, methyltransferase II, is then responsible for transferring the methyl group

from the corrinoid protein to Hg(II) (Choi et al. 1994a). Interestingly, bacteria such as acetogens, acetate-oxidizing sulfate reducers, and methanogens are not capable of methylating Hg(II), despite containing 100- to 1000-fold higher levels of the enzymes of this acetyl-CoA-synthesis/breakdown pathway (Choi et al. 1994b). Therefore, the ability to methylate Hg seems to belong to a small number of distantly related strains of SRB, but for reasons that remain to be determined. One possibility is that *D. desulfuricans* LS contains an "atypical" form of methyltransferase that is capable of binding Hg as an alternate substrate, which is then subsequently methylated.

In addition to the acetyl-CoA synthesis pathway however, four incomplete oxidizing strains of SRB that do not utilize the acetyl-CoA pathway, but are still capable of methylating Hg(II), have also been recently identified (Ekstrom et al. 2003). Therefore, additional pathways for Hg(II)-methylation exist in these incomplete oxidizing strains of SRB, or the corrinoid model proposed for *Desulfovibrio desulfuricans* LS might not represent the actual mode of transfer.

The mechanisms of the bacterial methylation of the metalloid selenium, resulting in the formation of dimethyl selenide and dimethyl diselenide, have been well characterized. In comparison to methylmercury, the methylated forms of selenium are volatile, less toxic, and not bioaccumulated up the food chain (Atkinson et al. 1990). The enzyme thiopurine methyltransferase was originally identified as the enzyme responsible for selenium methylation in *Pseudomonas syringae* (Ranjard et al. 2002) and a freshwater *Pseudomonas anguiliseptica*-like strain (Ranjard et al. 2003). However, a novel selenium methyltransferase, *mmt*A, was later identified from the freshwater bacterium *Hydrogenophaga* sp. Esa.33, which shared strong

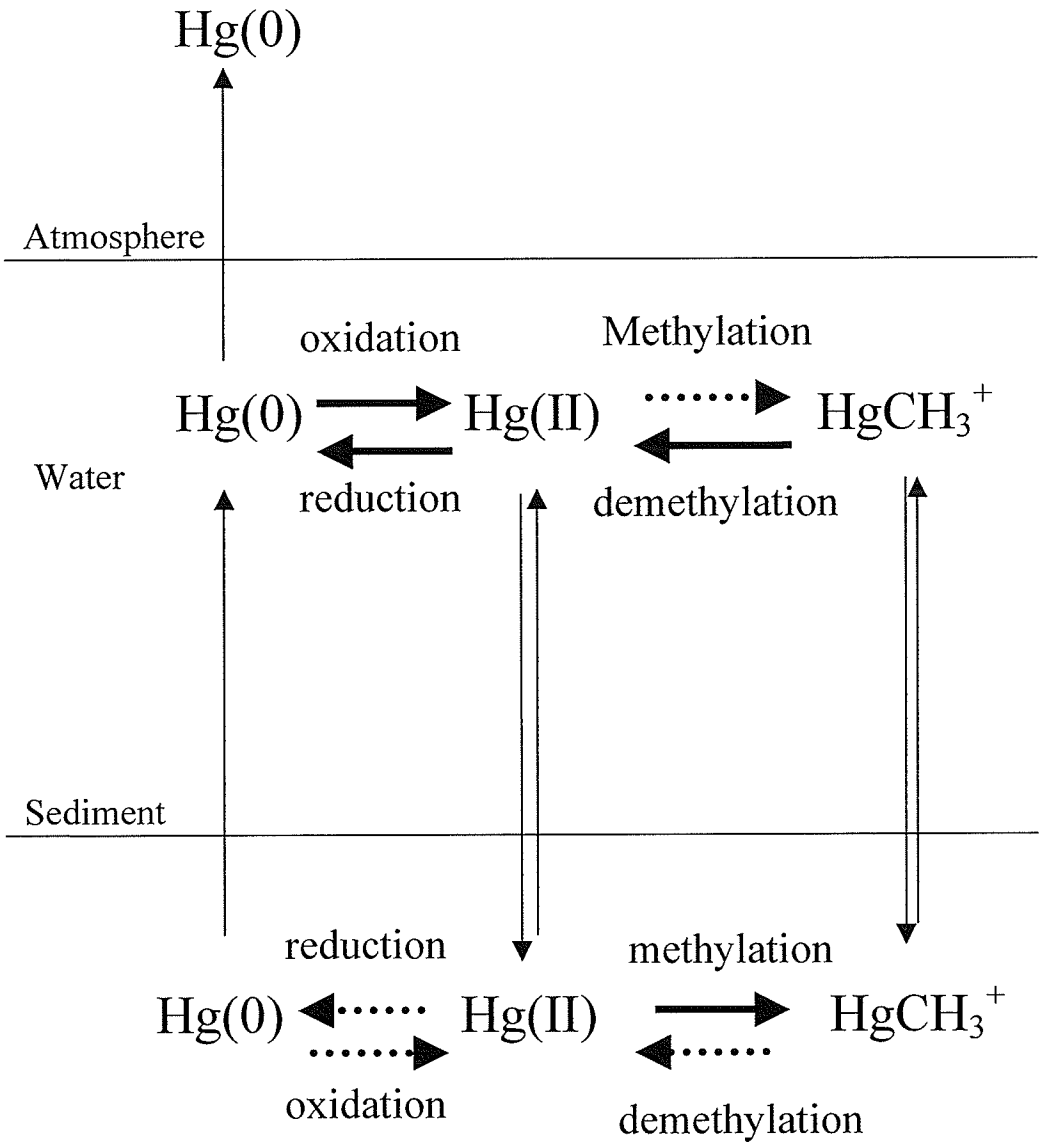

Figure 1.1. The bacterial mediated cycling of Hg in aquatic ecosystems. Mercury methylation, demethylation, oxidation, and reduction are the four main transformations mediated by bacteria. The bold arrows represent processes that appear to contribute significantly, and the dashed arrows represent bacterial processes might contribute only slightly, to the biogeochemical cycling of Hg.

similarities with the calicheamicin methyltransferase (Ranjard et al. 2004). The existence of alternative selenium methylation pathways could be used to explain the differences in the aforementioned Hg(II) methylating bacteria pathways amongst the SRB. Further studies involving the mechanism of Hg(II) methylation and the identification of the enzymes involved would greatly improve our understanding of this methylation process.

Not all bacterial transformations of Hg are ecologically detrimental. Bacteria containing the *mer* operon are capable of detoxifying organic and inorganic species of Hg. The *mer* operon encodes mercuric reductase, which reduces Hg(II) to the highly volatile and non-reactive Hg(0) species that passes freely back to the atmosphere (Nazaret et al. 1994). The bacteria capable of demethylating methylmercury contain an additional *mer*B gene in the operon, which encodes organomercurial lyase (Walsh et al. 1988). This enzyme catalyzes a protonolytic fragmentation of the carbon-mercury bond, yielding Hg(II) and methane (Walsh et al. 1988). The Hg(II) is then directly transferred from MerB to MerA, which is then reduced to Hg(0) (Benison et al. 2004). The overall detoxification mechanism, genetic variation, regulation and occurrence of the *mer* operon will be discussed in more detail in the subsequent section (section 1.4).

In aquatic ecosystems, microbial contributions to the oxidation of Hg(0) have never been considered significant. However, a high tendency for bacteria to oxidize Hg(0) was recently demonstrated (Smith et al. 1998). Bacteria can oxidize Hg(0) to Hg(II) by the enzyme hydroperoxidase-catalase, which is commonly found in a variety of bacteria (Smith et al. 1998). In surface water, photochemically produced

H_2O_2 induces the expression of this enzyme, which could explain the significant decrease in dissolved gaseous mercury during the day (Siciliano et al 2002). The total concentration of Hg in all its various forms in a given environment is dependant on the rates of abiotic and bacterial mediated processes, such as the rates of oxidation, reduction, and methylation of Hg and the degradation rates of MeHg (Schaefer et al. 2004, Hintelmann et al. 2000). All of these processes would ultimately have an impact on the total concentration of MeHg available for bioaccumulation in higher organisms.

1.4 Hg(II) specific uptake in bacteria containing the *mer* operon

Unlike the bacterial detoxification strategies for other metals, which commonly involve eliminating intracellular metals through an ATPase efflux pump (reviewed in Nies 2003, Nies 1999), bacterial resistance to Hg requires transport of the metal into the cytoplasm. In the cytoplasm the detoxification strategy involves Hg(II) reduction to the elemental form Hg(0). This is an effective system because of the unique properties of Hg, such as the redox potential and low vapor pressure of Hg(0). The elemental form is a non-toxic volatile species that is relatively inert and is therefore capable of diffusing freely out of the cell. The proteins involved in this Hg detoxification process are all encoded in the *mer* operon. The genes commonly found in Hg resistant bacteria include *mer*A encoding mercuric reductase, *mer*T and *mer*P encoding a Hg(II) transport system, and *mer*R encoding the regulatory protein of the operon. These genes are considered to be essential for efficient enzymatic Hg resistance.

Comparisons of sequences from many isolated Hg resistant bacteria have revealed variations in either the composition or in the gene arrangement within the *mer* operon (Liebert et al. 2000, Liebert et al. 1999, Bogdanova et al. 1998). Some notable variations were the presence or absence of the following genes; *mer*C, *mer*E, and *mer*F encoding putative transmembrane proteins involved in Hg(II) transport, *mer*D encoding a down regulatory protein of the operon, and *mer*B encoding organomercurial lyase.

Bacteria possessing only the essential genes exhibit "narrow spectrum" resistance to only Hg(II). However, bacteria possessing the essential detoxification genes and *mer*B exhibit "broad-spectrum" resistance to both organomercurials and mercuric ion compounds. The *mer* operon of transposons Tn*21* and Tn*501* are two of the best-studied systems of Hg detoxification. The DNA sequence of the Tn*21 mer* operon (Barrineau et al. 1984) has revealed eight open reading frames, two of which are putative (Liebert et al. 1999). The gene structural order of the operon is *mer*RTPCADorf1(E)orf2. The composition of the Tn*501 mer* operon is very similar, with the exception that it does not contain *mer*C (Misra et al. 1984). The Tn*21* and Tn*501 mer* operons both lack *mer*B, and so only exhibit narrow spectrum resistance to mercuric ion compounds.

1.4.1 Roles of MerT, MerP, and MerC in Hg(II) transport Hg toxicity primarily stems from the very high binding strength of sulfhydryl and amino ligands for Hg. Therefore it seems counterintuitive that the cell takes up Hg for detoxification. However, the presence of high affinity transport systems would actually prevent non-

specific Hg binding with proteins in the periplasm and cytoplasmic membrane of bacterial cells. In addition, the uptake of Hg into the cytoplasm is required for Hg(II) to be reduced to a non-toxic volatile form by mercuric reductase. This reaction must occur in the cytoplasm to avoid exporting the $FADH_2$ and NADPH cofactors required by mercuric reductase (Hobman and Brown 1997). Nakahara et al. (1979) provided the first evidence that there was a mercuric ion transport system encoded by the Tn*21 mer* operon. Minicell polypeptide analysis had shown two inner-membrane polypeptides and one periplasmic polypeptide encoded by the Tn*21 mer* operon (Jackson and Summers 1982). These three polypeptides were found to be the products of the first three structural genes of the *mer* operon, MerTPC.

MerT is an inner membrane protein with three transmembrane α-helices and is involved in Hg(II) transport (Summers 1986). A conserved pair of vicinal cysteine residues, Cys24 and Cys25, appears in the first transmembrane helices. A second pair of cysteine residues, Cys76 and Cys82, is located in a cytoplasmic loop. In MerT, Cys24 and Cys25 are essential for Hg(II) binding and transport. Mutations of Cys24Ser and Cys25Ser eliminated hypersensitivity in strains lacking MerA and the loss of resistance in strains expressing MerA, whereas Cys76Ser and Cys82Ser mutations reduced, but did not abolish mercury hypersensitivity or resistance (Morby et al. 1995). Although the whole *mer*TPC is required for full Hg resistance, MerT was determined to be the most important component for the transport of Hg (Hamlett et al. 1992).

MerP is located in the periplasm (Summers 1986) and has a βαββαβ fold with the two α-helices overlaying a four-strand antiparallel β-sheet (Steele et al. 1997). The

sequence of *mer*P has a heavy metal binding motif, GMTCXXC, and shares homology with a CadA Cd(II)-ATPase (Nucifora et al. 1989) and copper transport proteins from both prokaryotes and eukaryotes (Bull and Cox 1994). These conserved cysteine residues are required for specific Hg(II) binding (Sahlman and Johnson 1992, Sahlman and Sharfstad 1993). NMR spectroscopy shows that Hg(II) binding is bicoordinate with the cysteine side chain ligands (Steele et al. 1997). In the absence of Hg(II) one of the cysteine residues is surface exposed and the second cysteine residue is buried inside the protein. Upon Hg binding both cysteine residues become exposed to the surface (Steele et al. 1997). This structural change may be important for the "baseball glove" model of Hg(II) transport (Foster 1987). This model proposes that Hg(II) is transferred from the periplasmic MerP protein to the cytoplasmic membrane-bound MerT transport protein, and then directly transferred to MerA. This was recently supported in cells containing mercuric reductase, in which the expression of both MerP and MerT resulted in an approximate 20% increase in the amount of Hg volatilized, in comparison to cells expressing only MerT (Wilson et al. 2000).

However, additional evidence proposes another function for MerP. For example, it was shown that the presence of MerP did not significantly increase the uptake of Hg(II) in vesicles containing MerT (Sahlman et al. 1997). In addition, the expression of MerT alone resulted in hypersensitivity in cells lacking mercuric reductase, but the expression of both MerP and MerT decreased hypersensitivity, suggesting a possible sequestering/protective role for MerP (Morby et al. 1995). These results contradict both the "baseball glove" model of Hg(II) transport and the periplasmic binding-

protein dependant systems. In such systems, transport is dependent upon the periplasmic proteins' ability to scavenge low levels of nutrients and present them to the inner membrane transport protein. In the absence of a periplasmic protein, minimal transport should occur. However, none of these studies were performed using trace concentrations of Hg(II). Therefore, because of the high concentrations of Hg used (0.1-100 μM), Hg might not require MerP to bind to the inner membrane transporters. Faced with trace concentrations of Hg (pM) it is likely that the role of MerP would be more significant. MerP should then increase specific Hg(II) transport by sequestering Hg(II) in the periplasm and transferring it to MerT, thereby increasing the likelihood that Hg will bind specifically to MerT and not other cellular ligands in the periplasm. The proposed mechanism of Hg(II) transfer from MerP to MerT involves temporary 3-co-ordinate bridging structures between cysteine residues in MerP and cysteine residues from MerT (Figure 1.2).

MerC shares homology with MerT, but the exact role of MerC in the detoxification of Hg(II) remains unclear. Earlier studies showed that the deletion of *mer*C from the Tn*21 mer* operon did not have an affect on Hg(II) transport or resistance (Hamlett et al. 1992). In addition, the Tn*501 mer* operon, which does not contain *mer*C, exhibits Hg(II) resistance equivalent to that of the Tn*21 mer* operon (Lund and Brown 1987). However, bacteria isolated from Hg-contaminated sediments have shown a high occurrence of *mer*C transcripts suggesting a functional role for MerC (Osborn et al. 1997). A *mer* operon identified in *Thibacillus ferooxidans* was shown to contain *mer*C, but there was no gene corresponding to *mer*T (Inoue et al. 1990). The expression of this *mer*C gene in *E. coli* demonstrated

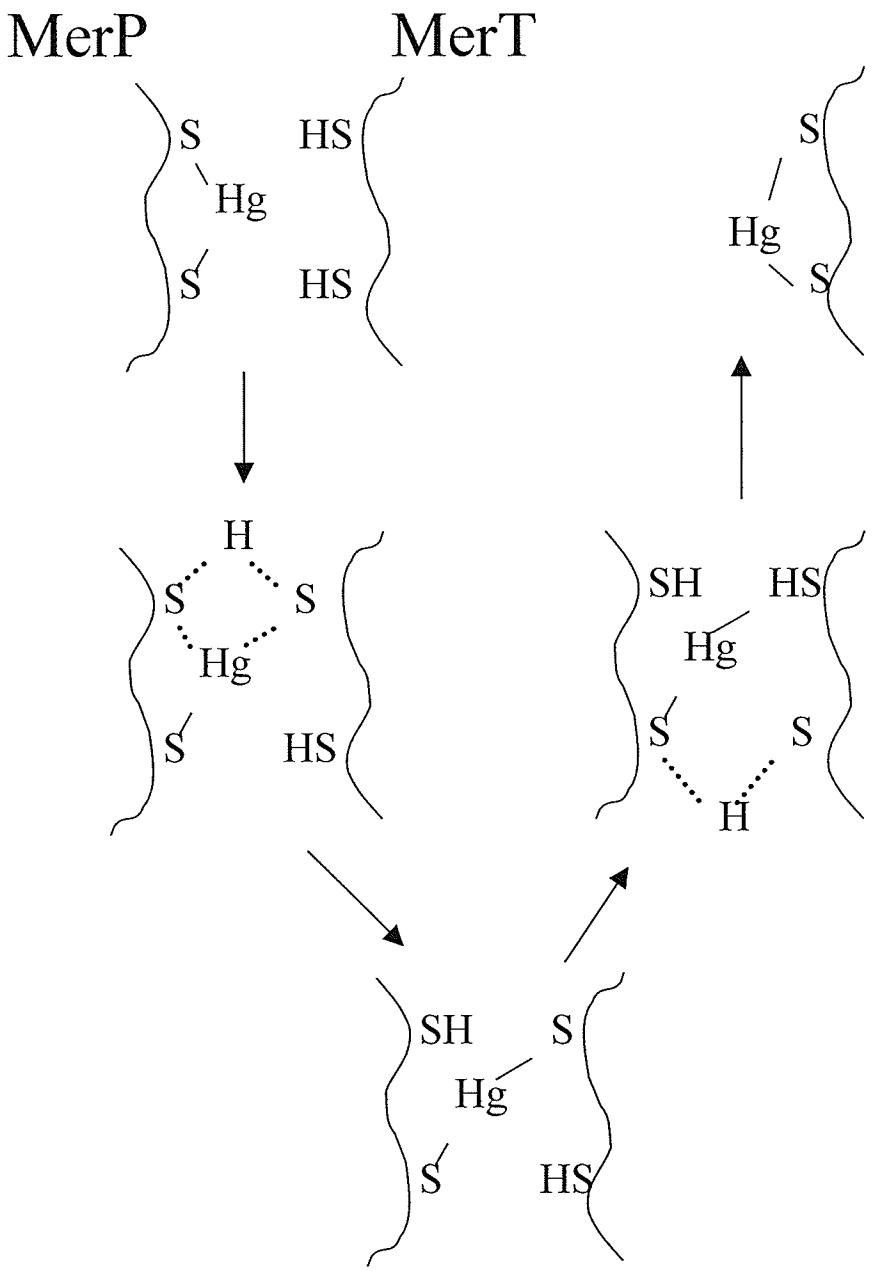

Figure1. 2. The proposed mechanism of thiol-ligand exchange of Hg(II) from the periplasmic MerP metallochaperone to the inner membrane transport protein MerT. Adapted from Brown et al. (2002).

increased Hg uptake and hypersensitivity in the absence of mercuric reductase, suggesting that MerC was involved in transport (Kusano et al. 1990, Inoue et al. 1996). In light of these results, MerC from Tn*21* was reinvestigated. Initial studies involving vesicles demonstrated that MerC was capable of facilitating Hg(II) uptake in the presence of excess cysteine, but the Hg remained bound to MerC and did not appear to be released into the interior of the vesicle (Sahlman et al. 1997). However, the expression of MerC in the presence of mercuric reductase resulted in increased volatilization in comparison to an isogenic strain lacking MerC, suggesting that MerC of Tn*21* is involved in Hg(II) transport (Wilson et al. 2000). The observation that Hg only seemed to bind MerC in the vesicle studies might be related to the absence of mercuric reductase, which might be required to remove Hg(II) from the tight-binding cysteine residues of MerC (Sahlman et al. 1999, Sahlman et al. 1997).

In contrast to MerT, the volatilization of Hg in cells containing mercuric reductase did not increase when MerP was co-expressed with MerC, suggesting that MerP only interacts with MerT (Wilson et al. 2000). As with MerT, MerC contains two pairs of highly conserved cysteine residues, one pair is located in the membrane, Cys22 and Cys25, and the other on the cytoplasmic side of the membrane, Cys127 and Cys132 (Sahlman et al. 1997). Site-directed mutagenesis of either Cys22 or Cys25 to alanine abolished Hg uptake, whereas mutations of Cys127 or Cys132 did not significantly affect uptake of Hg in vesicles (Sahlman et al. 1999). The cysteine residues of MerC located in the membrane are therefore involved in Hg binding and

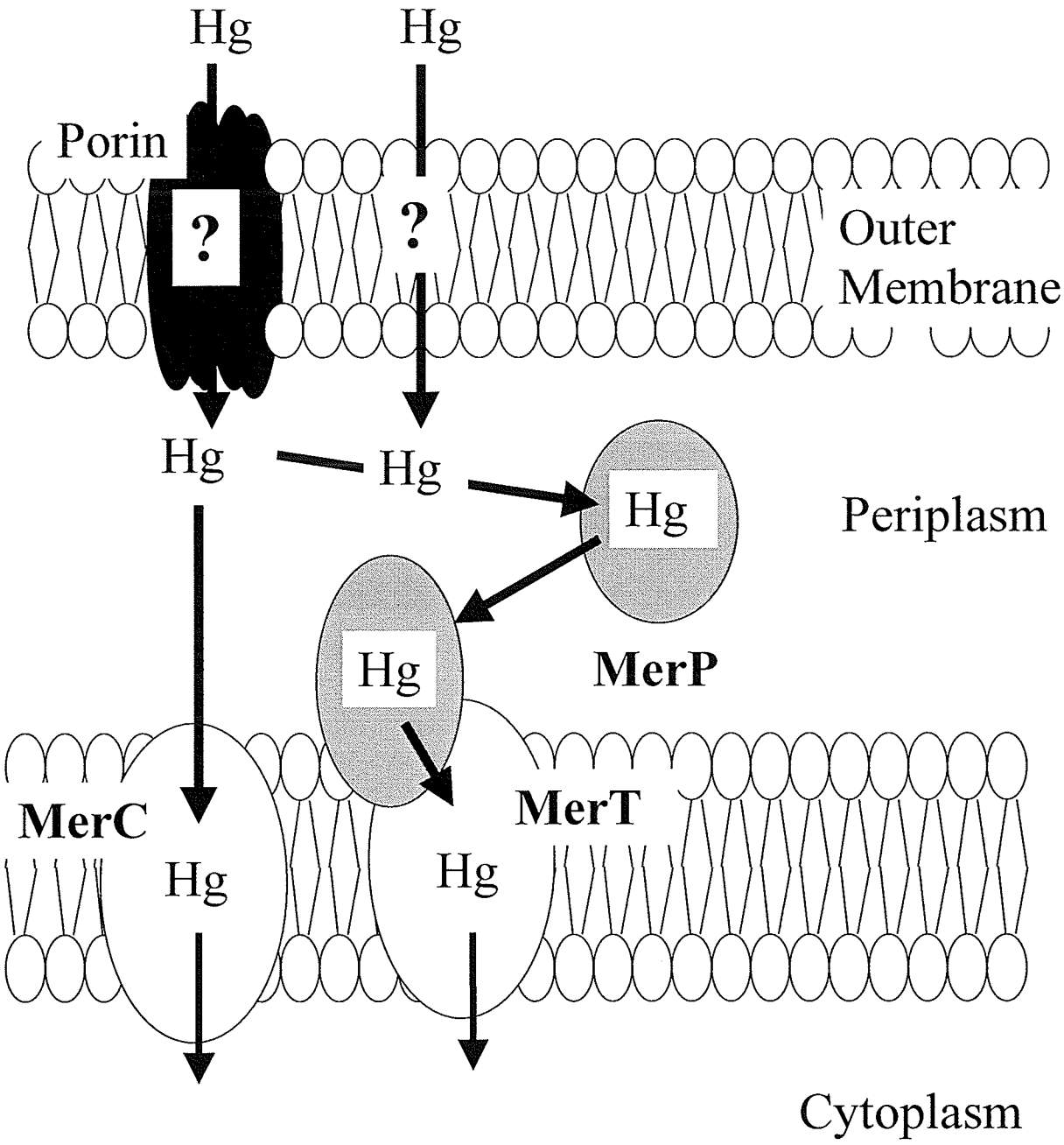

Figure 1.3. Overview of the roles of MerTPC in Hg transport. It remains unclear how Hg penetrates through the outermembrane. See section 1.4 for details.

uptake, similar to what was observed for MerT (Morby et al. 1995). The current model of Hg(II) transport via MerTPC is outlined in Figure 1.3.

The exact role of the conserved cysteine residues located in the cytoplasmic loop of MerT and MerC remains unclear. One possibility is that they are necessary for interactions with mercuric reductase. Recent evidence for this was obtained using a 23-amino acid synthetic peptide (ppMerT) corresponding to the cytoplasmic loop of MerT. In this study, ppMerT was shown to bind one Hg(II) atom per molecule, which was specifically transferred to the N-terminal extension of MerA (MerAa) (Rossy et al. 2004). Another suggestion is that due to the small size of these proteins it may be possible that they form multimers in the membrane, by intermolecular disulphide bond formation between the non-essential cysteine residues (Sahlman et al. 1999). Another possibility is that MerC and MerT form distinct structures with different functions, since multimer formation is not required for the uptake of Hg(II) (Summers 1986). In either case it appears that MerT or MerC can facilitate Hg(II) transport.

1.4.2 Role of MerA in Hg(II) detoxification All bacteria containing the *mer* operon have the *mer*A gene, encoding the homodimeric enzyme mercuric reductase (Fox and Walsh 1982). MerA is a cytoplasmic protein capable of efficient reduction of Hg(II) to Hg(0) (Robinson and Tuovinen 1984). Hg(0) is highly volatile and passes freely across biological membranes, out of the cell, and potentially back into the atmosphere (Barkay et al. 1992). For Hg resistant bacteria this is the last step in the Hg detoxification pathway. Mercuric reductase was found to belong to a class of

pyridine nucleotide-disulfide oxidoreductases sharing homology with glutathione reductase and lipoamide dehydrogenase (Williams et al. 1982, Brown et al. 1983).

The enzymatic reduction of Hg(II) by NADPH requires FAD and a redox-active pair of cysteine residues, Cys135 and Cys140 (Foster 1987). Site directed mutagenesis of either cysteine residue to serine or alanine virtually eliminates the enzyme's capability to reduce Hg(II) (Walsh et al. 1988). In addition to the redox-active cysteine residues, a homologous pair of cysteine residues, Cys557 and Cys558, is located at the C-terminus in all known mercuric reductases (Miller et al. 1989). These cysteine residues lie near the redox-active cysteine residues in the active site of the opposite monomer (Figure 1.4). Site directed mutagenesis of the C-terminal cysteine residues to alanine disrupted the catalytic reduction of $Hg(SR)_2$ substrates, and also resulted in a hypersensitive phenotype in vivo (Moore and Walsh 1989). However, this same mutant exhibited rates of Hg reduction equivalent to that of the wildtype enzyme when $HgBr_2$ was used as a substrate (Engst and Miller 1999). These results indicate that small labile ligands, such as $HgBr_2$, can access the active site directly in the absence of the C-terminal cysteine residues, but those with large bulky ligands, such as $Hg(Cys)_2$, require the C-terminal cysteine residues for rapid access (Engst and Miller 1999). However, the direct access of small substrates with high affinity, such as $Hg(CN)_2$, can lead to formation of an inhibited complex in the absence of the C-terminal cysteine residues. Therefore, the C-terminal cysteine residues play a critical role in removing high affinity Hg ligands before Hg(II) reaches the redox-active disulfide group in the inner active site (outlined in Figure 1.5) (Engst and Miller 1999).

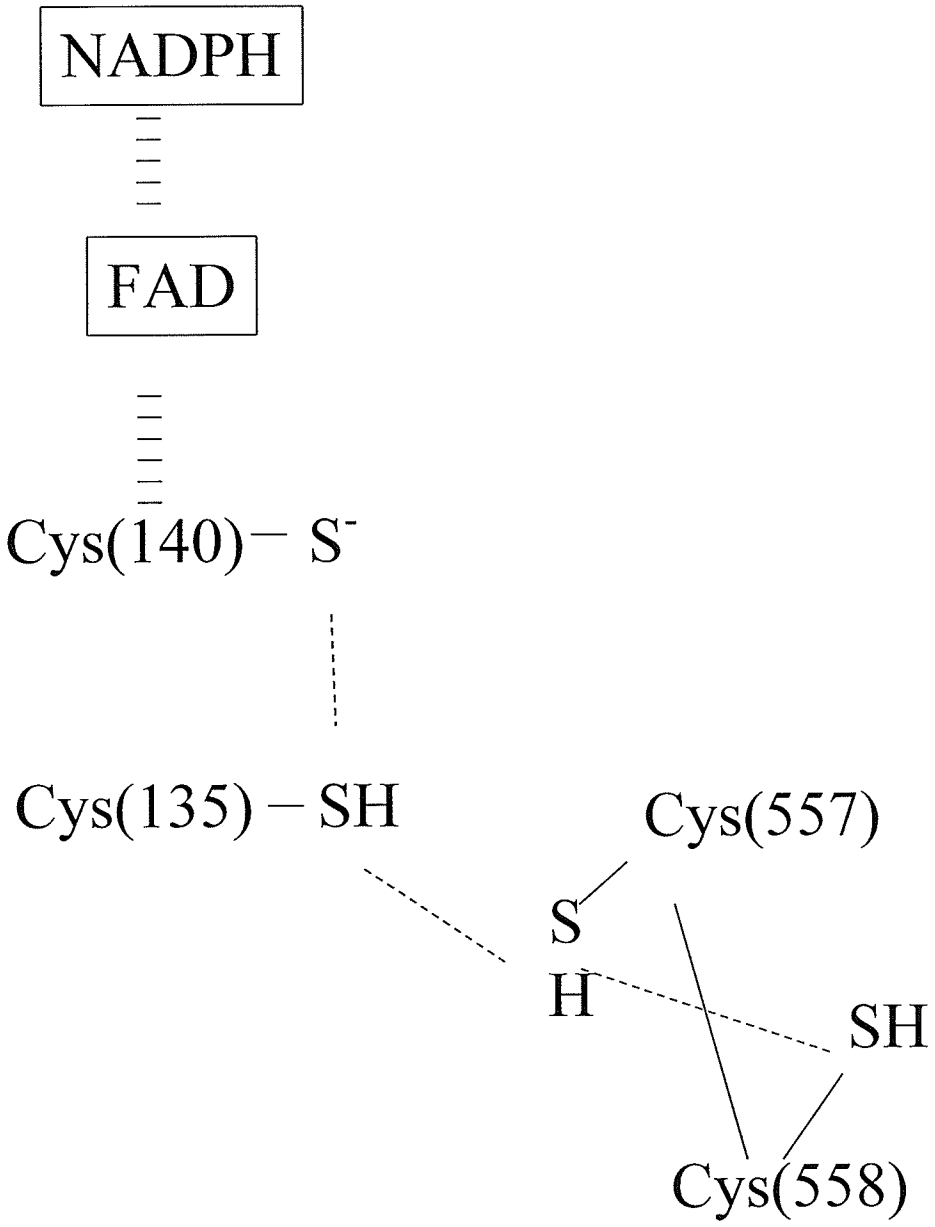

Figure 1.4. Organization of the cysteine residues in mercuric reductase. Adapted from Engst and Miller (1999).

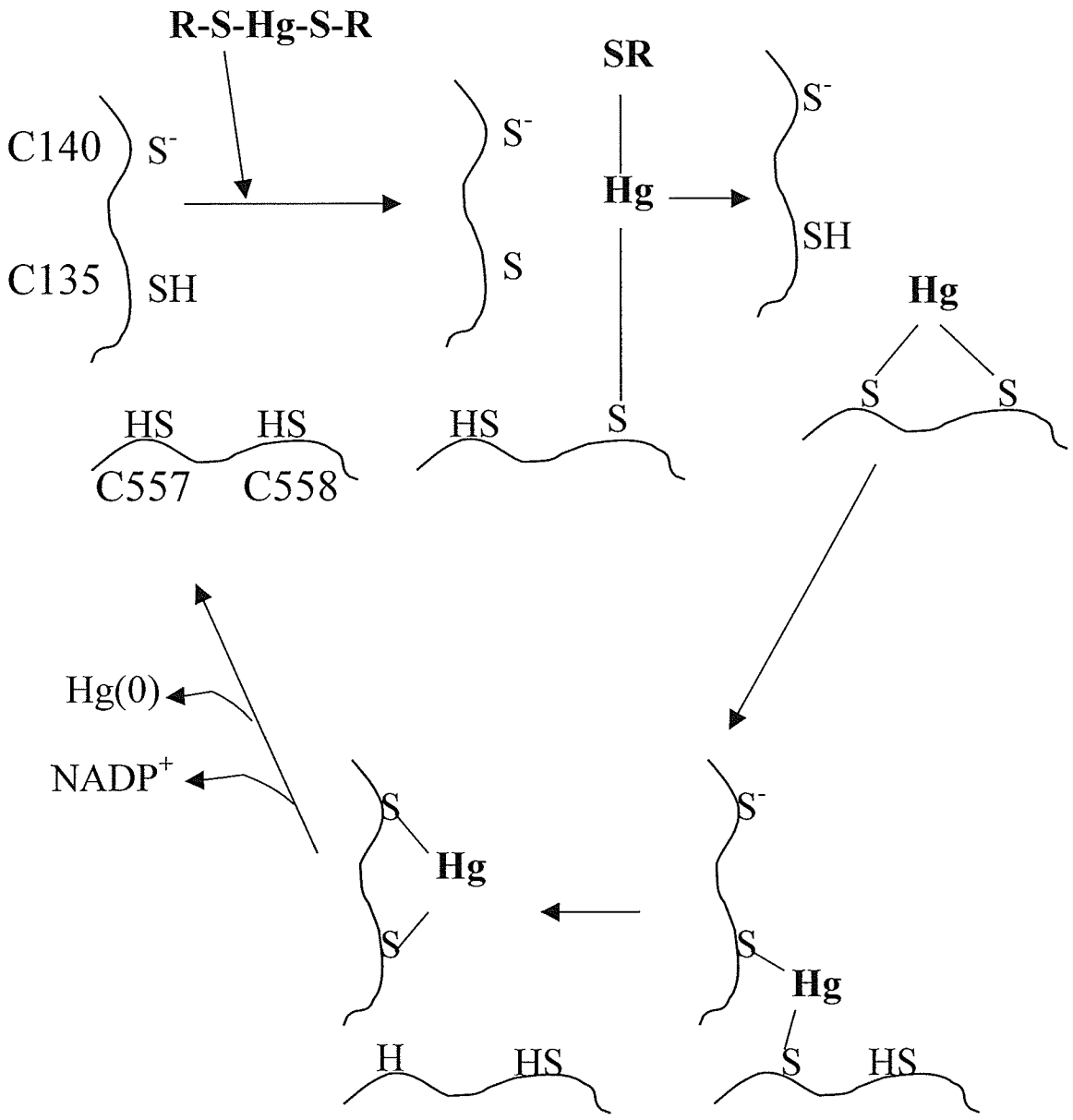

Figure 1.5. The role of the C-terminal cysteine residues of mercuric reductase in the removal of Hg(II) ligands, and the subsequent transfer into the active site. R-S represents any sulfhdyrl group. Adapted from Engst and Miller (1999).

1.4.3 Regulation of the mer operon The mercury responsive protein, MerR, maintains transcriptional regulation of the *mer* operon. MerR is a 144 amino acid polypeptide (O'Halloran and Walsh 1987) whose structure consists of an N-terminal helix-turn-helix motif (Summers 1992) and a C-terminal Hg(II) binding domain (Ross et al. 1986). In the Tn*21* and Tn*501 mer* operons, *mer*R is transcribed divergently from the structural genes (Misra 1984). MerR forms a dimer and binds to a region of dyad symmetry overlapping between the -35 and -10 RNA polymerase recognition hexamers of the promoter for the structural genes (Heltzel et al. 1987). Since this symmetrical stretch of DNA overlaps the +1 transcriptional start site of *mer*R, MerR represses its own transcription (Foster and Brown 1985). In the absence of Hg(II) MerR binds and induces a double kink in the DNA, thus repressing transcription of the *mer* operon (Ansari et al. 1992, Ansari et al. 1995). It was originally suggested that repression was due to MerR preventing access of RNA polymerase to the promoter of the structural genes. However, it was later shown that RNA polymerase still binds to the –35 site of the structural gene promoter when MerR is bound (Frantz and O'Halloran 1990, Heltzel et al. 1990, Caslake et al. 1997) and actually sequesters RNA polymerase at the inactive promoter (Kulkarni and Summers 1999). In mutant studies in which MerR is deleted, only low levels of structural gene expression is apparent (Ni'Bhriain et al. 1983). This low level of expression, ~ 1-2 %, can be accounted for by an unusual 19 base-pair interhexamer spacer (Lund and Brown 1989). Site-specific insertion or deletions of one or two base pairs within the interhexamer spacer either prevented induction or caused the promoter to be highly reactive in the presence or absence of Hg(II) (Parkhill and Brown 1990).

The MerR Hg(II) binding domain has three essential cysteine residues, two cysteine residues from one monomer and the third cysteine from the second monomer (Ross et al. 1989, Shewchuk et al. 1989). Together, these three cysteine residues coordinate the mercuric ion trigonally (Helmann et al. 1990, Wright et al. 1990, Zeng et al. 1998). Hg(II) binding to the MerR-DNA complex causes a conformational change in MerR and results in changes in MerR contacts at the promoter (O'Halloran et al. 1989, Frantz and O'Halloran 1990, Ansari et al. 1992, Summers 1992, Livrelli et al. 1993, Ansari et al. 1995, Parkhill et al. 1998, Miller 1999). These changes are believed to be responsible for an under winding of the DNA at the promoter, thereby compensating for the 19 base-pair spacer region, allowing RNA polymerase to bind to its –10 recognition site and form an open complex (Ansari et al. 1995) (outlined in Figure 1.6). The *mer* promoter is therefore critical for MerR binding, repression, and activation of transcription in response to mercuric ions.

Since MerR is responsible for the regulation of the Hg detoxification proteins it would be expected to exhibit both high affinity and high selectivity to mercuric ions. MerR activates transcription in vitro at concentrations as low as 3 nM Hg(II), with an apparent K_M of 10^{-8} M, in the presence of mM thiols added to mimic potential Hg(II) binding sites in the bacterial cell (Ralston and O'Halloran 1990). Metals such as Zn, Cd, Au, and Ag have been shown to activate the *mer* operon, but at concentrations 10^2 - 10^3 fold higher than Hg(II) (Ralston and O'Halloran 1990). It has been suggested that the high affinity and selectivity of Hg(II) for MerR lies in the unusual trigonal cysteine coordination of Hg(II) (Helmann et al. 1990).

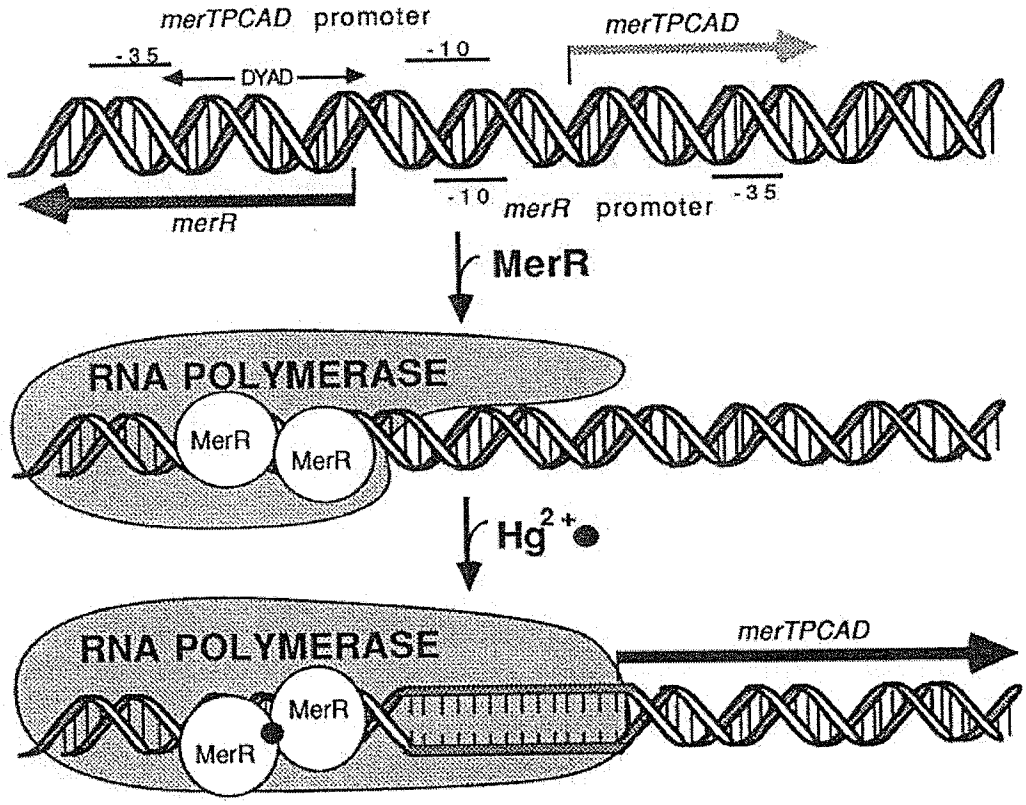

Figure 1.6. The role of MerR in the regulation of the *mer* operon (Condee and Summers 1992).

In addition to MerR, a second regulatory protein of the Tn*21 mer* operon, MerD, has been identified. MerD is cotranscribed with the structural genes of the *mer* operon (Lee et al. 1989). MerD shares homology with MerR in respect to the predicted N-terminal DNA binding domain (Brown et al. 1986). Partially purified MerD was shown to bind specifically, but with lower affinity than MerR, to the MerR operator site. However, MerD was not capable of inducing transcription of the structural genes in the presence of mercuric ions (Mukhopadhyay et al. 1991). A possible regulatory role was then deduced for MerD, which was confirmed using MerD null mutants that resulted in increased operon expression (Mukhopadhyay et al. 1991). This down regulation of the *mer* operon may represent a feedback inhibition mechanism for gene expression (Hobman an Brown 1997). An alternative role however, was recently proposed using the MerD protein isolated from *Ralstonia metallidurans* CH34 (Champier et al. 2004). In this study, using electrophoretic mobility shift assays and DNA footprinting experiments, MerD did not bind to DNA. Alternatively, Western analysis demonstrated that MerD forms a ternary complex with the *mer*o/p and MerR. The formation and stability of this complex were dependent on the relative concentration of the two proteins and modulated by the presence of Hg(II). It was then proposed that MerD displaces Hg(II)-bound MerR from the *mer* operator to allow new synthesis of metal-free MerR, which would then switch off the induction of the *mer* structural genes when the external mercury is exhausted (Champier et al. 2004).

Bacterial resistance to Hg compounds has been identified worldwide in both gram-positive (reviewed in Bogdanova et al. 1998) and gram-negative bacteria

(reviewed in Liebert et al 1997), and recently in the hyperthermophilic archaeon *Sulfolobus solfataricus* (Schelert et al. 2004). This widespread occurrence of Hg resistance is most likely due to the horizontal gene transfer of the *mer* operon (Mindlin et al. 2002), which is commonly associated with either class II transposons or broad host range plasmids (Osborn et al. 1997). The *mer* operon has been isolated from a vast number of different bacteria. It could be found in as many as 50 % of the culturable heterotrophic bacteria from contaminated Hg sites and 1-10% of the bacteria in non-contaminated sites (Barkay et al. 1989).

1.5 Hg(II) uptake in bacteria that do not contain the *mer* operon

The only identified mechanism of Hg(II) uptake is that mediated by proteins encoded by the *mer* operon. However, there are many bacteria involved in the cycling of Hg that do not contain the *mer* operon. Therefore, knowledge of how Hg enters a bacterial cell, and what environmental conditions favors some transformations of Hg over others, is crucial for our understanding and potential mitigation of Hg toxicity in aquatic ecosystems. One possibility is that Hg(II) diffuses passively into the cell in the absence of specific transport proteins for Hg(II).

The rates of uptake via passive diffusion can be estimated by the equation $V = P \times C \times A$, where P is the ability of a molecule to cross the membrane, C is the extracellular concentration of the molecule, and A is the area of the cell (Stein 1986). P depends on the molecule's ability to partition into and its diffusability within a lipid bilayer membrane, which can be estimated by measuring a molecule's octanol-water coefficient (K_{ow}). The K_{ow} can range from near 0 for hydrophilic molecules to 10^8 for

very hydrophobic (i.e. lipophilic) ones (Morel 1998) (for comparison of K_{ow} for various molecules see Table 1.1). Passive diffusion of Hg(II) across biological membranes has been implied from studies using artificial membranes (Bienvenue et al. 1984, Gutkneckt 1981) and diatoms (Mason et al. 1996) that demonstrated the passive diffusion of μM – mM concentrations of $HgCl_2$ (K_{ow} only 3.3). On this basis it was hypothesized that in the absence of the *mer* operon, Hg uptake is mediated by passive diffusion of lipophilic Hg species across bacterial membranes (e.g. Benoit et al. 1999, Morel et al. 1998). For example, in anoxic sediments the calculated formation of neutral Hg-sulfide species formed in the bulk solution was implicated as the likely species available for Hg(II) uptake in *Desulfobulbus propionicus* (Benoit et al. 1999). The rates of Hg(II) methylation in pure culture were later used as an indicator of Hg(II) bioavailability in this bacterium (Benoit et al. 2001) and *Desulfovibrio desulfuricans* ND132 (Jay et al. 2002), and correlations were found between the calculated neutral Hg(II)-sulfide species formed in the bulk solution and Hg(II)-methylation rates. However, passive diffusion of Hg(II) has never been directly demonstrated in bacteria.

1.5.1 Limitations for the passive diffusion of Hg in bacteria Studies demonstrating passive diffusion of Hg using artificial phospholipid membranes cannot take into account the complexity of gram-negative bacterial cells (Figure 1.7). This lipophilic species formed in the bulk solution would have to diffuse unaltered through the bacterial boundary layer, across the outer membrane, and through the periplasmic space before it even reaches the cytoplasmic membrane of a gram-

Table 1.1. Comparison of octanol/water partition coefficients (K_{ow}) for various molecules.

Molecule	K_{ow}	Reference
$HgCl_2$	3.3	Mason et al. (1996)
$Hg(OH)_2$	0.05	Mason et al. (1996)
HgOHCl	0.05	Mason et al. (1996)
CH_3HgCl	1.7	Mason et al. (1996)
$(CH_3)_2Hg$	182	Major et al. (1991)
HgS	25	Benoit et al. (1999a)
$CdCl_2$	0.21	Mason et al. (1996)
AgCl	0.09	Reinfelder and Chang (1999)
PCBs (polychlorinated biphenyls)	19,952 – 181,970,086	CCME (1992)
DDT (dichlorodiphenyltrichloroethane)	8,203,515	De Bruijn et al. (1989)
DDE (dichlorodiphenyldichloroethylene)	9,036,495	De Bruijn et al. (1989)

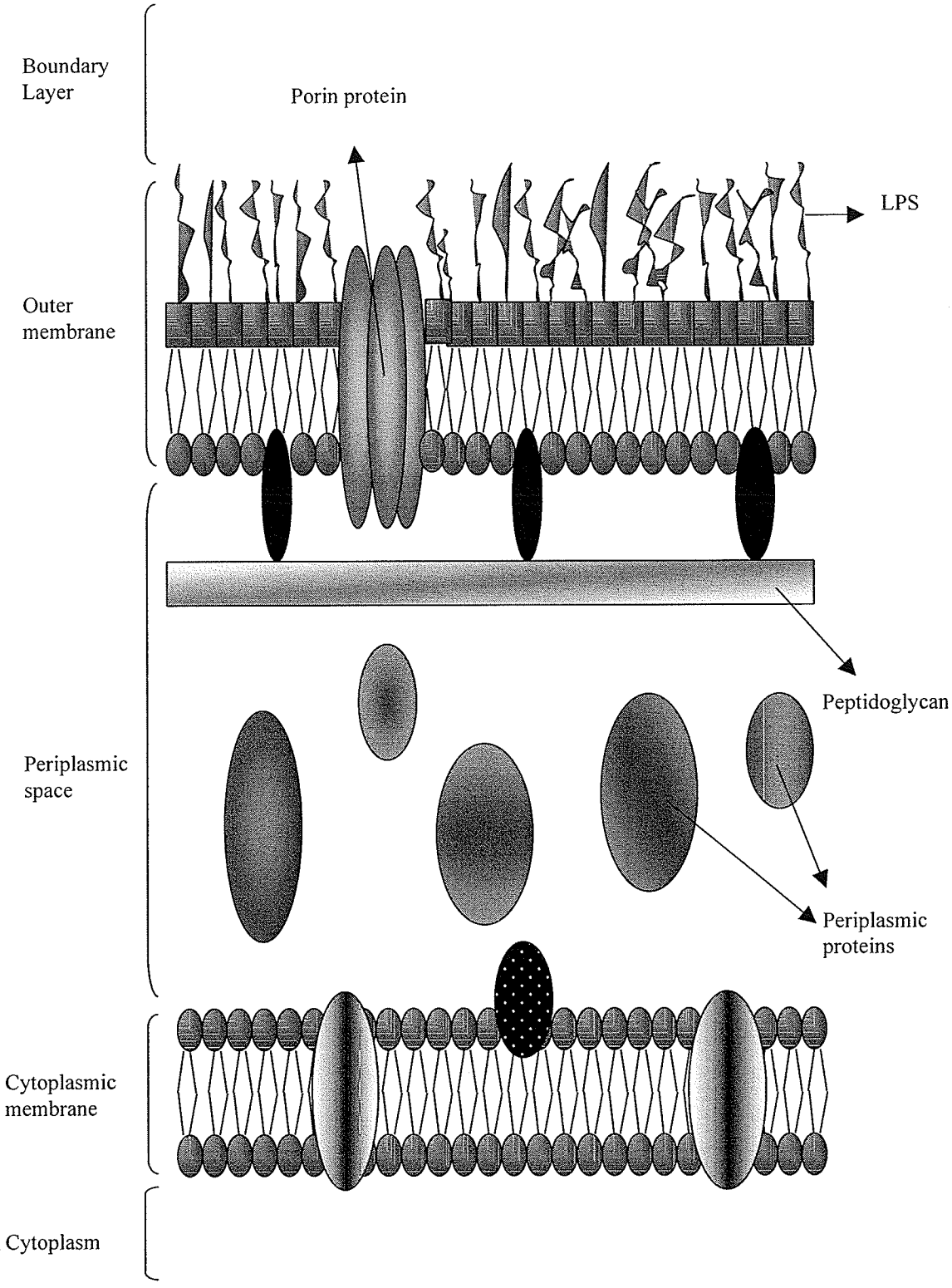

Figure 1.7. Overview of the structure of gram-negative bacterial cells.

negative bacterial cell. However, strong ligands for Hg exist outside of the cell from bacterial exudates, in the outer membrane, within the periplasmic space, and on the cytoplasmic membrane surface that will strongly influence the diffusion rates and chemical speciation of Hg(II) before reaching the cytoplasmic membrane. In addition to the potential sites of ligand exchange, unique structural characteristics of gram-negative bacterial cells provide further limitations for the passive diffusion of hydrophobic molecules.

The outer membrane (OM) of gram-negative bacteria is chemically distinct from other biological membranes. It consists of two types of lipids, lipopolysaccharides (LPS) and phospholipids, forming an asymmetric bilayer. The inner leaflet of the OM is composed of phospholipids, with a slight enrichment of phosphatidylethanolamine (Neidhardt et al. 1990). The outer leaflet contains lipopolysaccharides (LPS), which is composed of a hydrophilic O antigen polysaccharide, a core polysaccharide region, and a hydrophobic lipid A region. The LPS is in immediate contact with the surrounding environment and is responsible for the hydrophilic and negative charge of the bacterial surface. In addition, the LPS contains only saturated fatty acids and therefore, the hydrocarbon interior would be much less fluid than that of the usual phospholipid membrane, thereby making it much more difficult for hydrophobic molecules to penetrate into (Labischinski et al. 1985). In fact, the main function of the OM is to act as a permeability barrier that is highly effective in excluding hydrophobic compounds. The OM also possesses a variety of functional groups, which provide potential sites for Hg ligand exchange. Hg is a soft metal and tends to form complexes with soft ligands (Stumm and Morgan

1996). Therefore, the amino groups would provide the majority of Hg binding sites in LPS, followed by phosphoryl, and to a lesser extent the carboxyl sites. The structure of LPS varies among species of bacteria and so the extent of potential mercury-binding sites would be species dependent.

In addition to lipids, the OM contains a variety of proteins that would present additional ligands for Hg binding, including sulfhydryl groups. The majority of proteins in the OM form relatively nonspecific pores or channels that act as a molecular sieve allowing the passage of small hydrophilic compounds (< 600-700 daltons) across the outer membrane (Nikaido and Vaara 1987). Hydrophobic molecules would come into contact with the polar wall of the channel and be excluded or retarded (Nikaido and Vaara 1985). Therefore, the exclusion of hydrophobic compounds by the LPS and porin proteins, and the potential binding capacity of the outer membrane for Hg, would be the first barrier lipophilic Hg species would have to surmount intact in order to diffuse passively across the cytoplasmic membrane.

Separating the outer membrane and the cytoplasmic membrane is the periplasm. The periplasm constitutes 20-40% of the cell volume (Stock et al. 1977), and has been largely neglected in Hg speciation models. Within the periplasm exists a 2-15 nm thick peptidoglycan layer. Peptidoglycan is an enormous polymer composed of two sugar derivatives, N-acetylglucosamine and N-acetylmuramic acid, and several D- and L-amino acids (Park 1987). Generally, solutes are assumed to diffuse readily through the peptidoglycan layer, but peptidoglycan possesses functional groups that would be accessible for Hg binding. In gram-positive bacteria, the peptidoglycan

layer contributes significantly to the metal-binding capacity of the cell (Beveridge and Murray 1980, Doyle et al. 1980). However, in gram-negative bacteria the peptidoglycan layer is much thinner and so to what extent metals bind peptidoglycan in gram-negative cells is unknown (Langley and Beveridge 1999).

The periplasmic space is tightly compacted, forming a gel-like solution of proteins and molecules that can slow the diffusion of "non-reactive" solutes ~ 100-fold lower than those observed in the bulk solution (Neidhardt et al. 1990). However, these rates would likely be much slower for a "reactive" metal species such as Hg. The periplasm contains molecules such as glutathione and numerous sulfhydryl-containing proteins. Many of these proteins are essential for nutrient acquisition, but could also play a significant role in Hg sequestration, transport, and/or sites of toxicity. Therefore, within the periplasm Hg would likely no longer exist as the same species that was formed in the bulk solution, but would rather be controlled by thiol complexation reactions. To what extent these reactions influence the chemical speciation of Hg remain to be determined, but can be speculated to be quite significant.

The cytoplasmic membrane is a ~ 5-10 nm thick phospholipid bilayer (Cronan et al. 1987). The major function of the cytoplasmic membrane is to act as a hydrophobic barrier, which is dependent on the physical state of the membrane and lipid composition. The outer surface of the bilayer is hydrophilic, whereas the hydrophobic ends are buried in the interior away from the surrounding water. Therefore, one also has to consider possible interactions with the hydrophilic head groups on the surface of the membrane, which can vary in different organisms. For

example, the studies supporting passive diffusion used primarily eukaryotic-based lipids comprised of phosphatidylcholine, whereas the predominant lipid in bacterial membranes is phosphatidylethanolamine (Neidhardt et al. 1990). NMR studies could not detect Hg(II) complexes with egg phosphatidylcholine (Delnomdedieu et al. 1992). However, the polar head of phosphatidylethanolamine contains an accessible amine group, which was shown to be specific for $HgCl_2$ binding (Delnomdedieu et al. 1989). Hg(II) interactions with these bacterial phospholipids provide a potential site for toxicological effects at the membrane level.

Interactions of Hg with proteins in the cytoplasmic membrane also need to be taken into consideration. Upward of 200 different kinds of proteins, many of which are present as thousands of copies per cell, are embedded in the cytoplasmic membrane (Cronan et al. 1987). The exact extent of these proteins would be dependent on the species of bacteria under study and the metabolic status of the cell. Many of these proteins are involved in bioenergetic and biosynthetic reactions, whereas others are involved in the transport of solutes across the membrane. Non-specific binding of Hg via accessible sulfhydryl, amino, or carboxyl sites on these proteins can potentially disrupt catalytic function, ultimately resulting in toxicity to the cell. However, Hg might also mimic an essential element, thus the binding of Hg to a transport system might result in the non-specific uptake of Hg across the cytoplasmic membrane.

The notion of passive diffusion differentiates Hg from all other non-essential metals, which are taken up by transport systems specific for other essential nutrients (reviewed in Nies 1999). Such a mechanism of Hg uptake in bacterial cells would

imply that chemical speciation models, focusing only on the formation of lipophilic species in the bulk solution, would need to be reconsidered.

1.5.2 Non-specific uptake? A number of essential metabolic processes in bacteria require heavy metal ions. Therefore, the ability of bacterial species to accumulate metal ions, in often-metal limiting environments, is of uttermost importance. However, high intracellular concentrations of heavy metal ions can be deleterious to the cell. In addition, the non-specificity of many of these systems can result in the transport of toxic non-essential heavy metals. Heavy metal ions are structurally very similar, and therefore molecular homology is likely the reason for the non-specific uptake of other metals. For example, the ionic diameters of Mn(II), Fe(II), Co(II), Ni(II), Cu(II), and Zn(II) range from 138-160 pm, a difference of only 14 % (Nies 1999).

In bacteria, the fast and non-specific metal transporter CorA is ubiquitous in gram-negative and gram-positive bacteria (Smith et al. 1998a, Smith and Maguire 1995, Townsend et al. 1995). This protein is the main transporter of Mg(II) in bacteria, but is also capable of transporting a variety of different divalent metals. In *Salmonella typhimurium* CorA is involved in the uptake of Mn(II), Co(II), and Ni(II) in addition to Mg(II) (Snavely et al. 1989). The reported K_M for Mg(II) was 15 μM and the K_i (cation inhibition) for Co(II), Mn(II), and Ni(II) was 50, 30, and 500 μM respectively (Snavely et al. 1989). A mutant CorA protein resulted in a 30 % decrease of Fe(II) accumulation in *E. coli* and *S. typhimurium* (Hantke 1997). Strains containing this mutation were also resistant to Fe(II) concentrations up to 75 μM,

whereas cells containing a functional CorA had a survival rate of ~ 10 and < 1 % at 25 and 75 μM respectively. In addition, the toxicity of Fe(II) in $CorA^+$ cells was alleviated by the addition of 3mM Mg(II) (Hantke 1997), demonstrating that CorA is also likely involved in the transport of Fe(II). Kinetic parameters of metal uptake in *Alcaligenes eutrophus* proposed that Zn(II) and Cd(II), in addition to Mg(II), Co(II), Mn(II), and Ni(II), were also transported via CorA (Nies and Silver 1989). The average K_M for Zn(II), Cd(II), Co(II), Ni(II), and Mn(II) was 137, 136, 40, 40, and 58 μM respectively. Therefore, CorA is a constitutively expressed transport system that is capable of accumulating a broad spectrum of metal ions.

Non-specific uptake of metals has also been documented in inducible high affinity metal ion transport systems. For example, the P-type ATPases MgtA and MgtB in *S. typhimurium* are specific for Mg(II) uptake and are inducible in the presence low Mg(II) concentrations (Tao et al. 1995). Both of these transport systems are also capable of transporting Ni(II) and Co(II) (Snavely et al. 1989). In addition, Mg(II) uptake by MgtA was inhibited by Zn(II) and Ca(II), K_i = 7 and 300 μM respectively, suggesting that Zn(II) might also be taken up by this system. Whereas MgtB was not inhibited by Zn(II) and Ca(II), but was inhibited by Mn(II), K_i = 40 μM (Snavely et al. 1989), suggesting a role in Mn(II) uptake.

Another example is NRAMP (MntH), which is a selective Mn(II) transporter in bacteria and higher organisms. This transporter is stimulated by H^+ and regulated by multiple transcriptional control systems (Kehres et al. 2000). In *E. coli* and *S. typhimurium* the K_M for Mn(II) uptake is 1 μM and 100 nM respectively (Kehres et al. 2000). Cd(II) was a potent inhibitor of Mn(II) transport (K_i = 1 μM) and appears

to be an alternative non-physiological substrate for MntH (Kehres et al. 2000). In addition, the overexpression of MntH resulted in increased Cd sensitivity, further supporting the uptake of Cd(II) by this system. Additional substrates for MntH might also include Fe(II) (K_M = 25-50 μM) and Co(II) (K_i 15-30 μM). Inhibition of Mn(II) uptake was also noted for Pb(II), Fe(III), Ni(II), Cu(II), and Zn(II), but the concentrations required were not physiologically relevant (Kehres et al. 2000). One last example is CopA and CopB, which are P-type ATPase transport systems specific for Cu(I) transport, but is also capable of transporting Ag(I) (Solioz and Odermatt 1995, Odermatt et al. 1994).

Mechanisms of non-specific metal uptake not involving metal ion transport systems have also been reported. These include metal uptake through transport systems specific for phosphate, sulfate, and citrate. Experiments involving the uptake of phosphate, via the phosphate inorganic transport system (Pit), have revealed additional low-affinity mechanisms for metal accumulation in bacteria. One example is the formation of neutral metal-phosphate species, $MeHPO_4$, which is required for phosphate uptake via the Pit system. In the presence of EDTA, a strong metal chelator, the uptake of phosphate is inhibited (Van Veen et al. 1994). The metals Mg(II), Ca(II), Mn(II), and Co(II) all form soluble neutral complexes with phosphate. In *Acinetobacter johnsonii* and *E. coli* these neutral metal-phosphate conjugates are transported, via Pit, in symport with H^+ with a mechanistic stoichiometry of 1 (Van Veen et al. 1994, Van Veen et al. 1993). Insertional mutagenesis of the *pit*A gene in *E. coli* conferred increased resistance across Zn(II) gradient plates and a decrease in intracellular accumulation of Zn(II) (Beard et al. 2000). This suggests that in addition

to the ZnuABC high-affinity uptake system (Patzer and Hantke 1998), a second, low affinity uptake system for Zn(II) exists in *E. coli*. Increased resistance to Cd(II) was also observed on gradient plates, but the Cd(II)-resistant phenotype could not be confirmed in batch culture (Beard et al. 2000). This Pit system appears to represent a low affinity system that could be capable of transporting a wide range of divalent metal ions, assuming that the formation of these metal phosphate species are soluble.

Resistance to arsenate in *E. coli* also results from deletion mutants of the Pit system (Bennet and Malamy 1970). This arsenic form structurally resembles phosphate, PO_4^{3-}, and is transported via the Pit system. However, it is not known whether AsO_4^{3-} requires additional metal binding to form a neutral species, such as $MeHAsO_4$. This may present an alternative ligand for metal binding and uptake via the Pit system. The uptake of chromate in *Alcaligenes eutrophus* (Nies and Silver 1989), and many other microorganisms (Nies and Silver 1995), occurs via the sulfate-uptake system. Similar to the proposed uptake of AsO_4^{3-} by Pit, the uptake of chromate, CrO_4^{2-}, stems from molecular mimicry with sulfate, SO_4^{2-}.

Citrate is relatively abundant in nature and forms strong complexes with metal ions. These citrate-metal complexes are another example of non-specific metal accumulation in bacteria. In *Bacillus subtilis* two citrate transport systems have been identified, CitM and CitH. Krom et al. (2000) demonstrated that both transport systems take up citrate-metal complexes, but with complementary metal specificities. Metal specificities were determined by expressing the transporters in *E. coli*. Unlike most enterobacteria, *E. coli* does not express any citrate transporters under aerobic conditions (Kay et al. 1987, cited in Krom et al. 2000). CitM transports citrate when

complexed with Mg(II), Ni(II), Co(II), Mn(II), and Zn(II), but not Ca(II), Sr(II), and Ba(II). In contrast CitH only transports citrate when complexed with Ca(II), Sr(II), and Ba(II) (Krom et al. 2000). The discrimination between the two groups of metal-citrate complexes by CitM and CitH remains to be resolved, but may be related to the size of the metal ion (Krom et al. 2000).

Surprisingly, however Hg(II) was neither considered nor examined in any of the metal transport studies that demonstrated non-specific uptake of other metals. This could be a result of the high cost of Hg^{203} isotopes, the difficulty in working with Hg(II) because of its high affinity for cellular ligands, or the assumption that Hg uptake is via passive diffusion. It is interesting to note however, that silver (Ag) accumulation in *Thalassiosira weissflogii* has also been hypothesized to be the result of $AgCl^0$ diffusing passively into the cell, even though the K_{ow} of $AgCl^0$ is only 0.09 (Reinfelder and Chang 1999). However, recent data using *Chlamydomonas reinhardtii* found no evidence for the passive diffusion of $AgCl^0$, but rather suggested the involvement of active transport of Ag(I) via transport systems specific for Cu(I) (Fortin and Campbell 2000). An additional metal that was previously believed to diffuse passively into cells was As(III), but was recently shown to be facilitated by the aquaglyceroporin GlpF in *E. coli* (Meng et al. 2004).

1.6 Measurements of "bioavailable" Hg

Currently, chemical analytical techniques have been used to measure total Hg levels in environmental samples (Stratton and Lindberg 1995, Bloom and Fitzgerald

1988). The levels reported are then used for regulating Hg releases to aquatic systems. Although analytical techniques are very sensitive, they are incapable of distinguishing what fraction of the total Hg is available for uptake (i.e. bioavailable), and therefore transformations by the microorganisms involved in the cycling of Hg. For example, Hg methylation rates do not correlate well with total Hg measurements (Benoit et al. 1998, Kelly et al. 1995, Krabbenhoft et al. 1995). One factor that may disrupt this expected correlation is microorganisms containing *mer* operons, enabling the demethylation and reduction of MeHg and Hg(II) respectively (Schaefer et al. 2004). Therefore, the rate of Hg uptake and transformation by various bacteria containing the *mer* operon could potentially lower the total pool of MeHg (Hintelmann et al. 2000). In addition, Hg(II) uptake by the bacteria responsible for the methylation of Hg(II) would also affect levels of MeHg.

Essential for the uptake rates of Hg and MeHg is the bioavailability of the metal, especially for the bacteria that have no identified Hg transport proteins. Bioavailability can be defined as the degree to which a contaminant in a potential source is free for uptake into an organism (Newman and Jagoe 1994). Many previous methods have attempted to distinguish bioavailable from unavailable forms of Hg. One such method is the measurement of "easily reducible" Hg by $SnCl_2$ reduction of acidified samples (Bloom 1994). However, this method has the potential to overestimate bioavailable Hg by releasing Hg previously bound or absorbed, which would otherwise have been unavailable for uptake (Schuster 1991). Other methods have involved the use of computer programs, which models Hg speciation by utilizing known binding affinities of Hg for potential ligands identified in the water.

However, these theoretical methods cannot directly measure or quantitate the amount of Hg that is actually available for uptake.

1.6.1 Bioluminescent biosensors The development of biosensors has alleviated some of the limitations of previous methods attempting to predict bioavailability. Biosensors are genetically engineered bacteria that use a reporting system for the detection or toxicity of a compound of interest. One of the first and most commonly used reporting systems utilizes bioluminescent genes isolated from a variety of different organisms. Examples include bacteria, fungi, insects, and squid to name only a few (Nealson and Hastings 1979). Interestingly, the enzymes that catalyze the bioluminescent reaction from different organisms exhibit no evolutionary relationship. The only common feature amongst these organisms is the requirement of O_2 for light emission, which was demonstrated more than 300 years ago (Boyle 1668). The most abundant and distributed of the luminescent organisms are bacteria. Luminous bacteria have been isolated from both freshwater and terrestrial environments (Harvey 1952), but are most commonly found in marine environments, living as symbionts or saprophytes (Hastings 1986). In *Vibrio fischeri*, the enzymes responsible for the bioluminescent reaction are encoded in the *lux* operon (Engebrecht and Silverman 1984). The *lux* operon consists of seven genes in the structural order *lux*RICDABE. In other luminous bacteria a number of other additional genes have been identified (Meighen 1991).

i) Biochemistry of Light Production

*lux*R is transcribed divergently from the structural genes and is responsible for regulating transcription of the operon (Engebrecht and Silverman 1984). *lux*I encodes α-ketocaproyl homoserine lactone, which is the autoinducer of the operon (Engebrecht and Silverman 1986, Eberhard et al. 1981). The autoinducer is species specific and is produced at a low constitutive rate (Eberhard et al. 1986). In order to stimulate transcription of the *lux* operon the autoinducer must accumulate to a sufficient level. When this level is reached, the autoinducer interacts with LuxR and enhances transcription of *lux*ICDABE (Engebrecht and Silverman 1986).

*lux*A and B encode the heterodimeric enzyme luciferase (Engebrecht and Silverman 1984), which is the enzyme involved in catalyzing the light-emitting reaction. Both subunits of luciferase form an $(\alpha/\beta)_8$ barrel motif (Fisher et al. 1996), which catalyzes the oxidation of $FMNH_2$ and a long chain fatty aldehyde (Hastings and Nealson 1977) as outlined below:

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + hv$$

The reaction involves the formation of several enzyme-flavin intermediates. For the reaction to proceed, luciferase first interacts with $FMNH_2$, which subsequently reacts with O_2 forming an oxygenated enzyme-flavin complex (Becvar et al. 1978). This complex interacts with the aldehyde and the reaction goes to completion with light emission (Hastings and Gibson 1963), or the complex decomposes through a pathway known as a dark reaction (Becvar et al. 1978). In addition to *lux*AB, three other proteins are required for light emission and are encoded by the *lux*CDE genes.

LuxCDE form the fatty acid reductase complex, which is required for the synthesis and recycling of the aldehyde substrate (Engebrecht and Silverman 1984).

The natural aldehyde for the luminescence reaction is believed to be tetradecanal (Ulitzur and Hastings 1979), but could easily be replaced by a number of other saturated straight chain aldehydes. For example, aldehydes with varying chain lengths of C_7 to C_{16} have been shown to work with varying degrees of effectiveness (Strehler and Cormier 1954).

ii) Applications of the *lux* operon in non-luminous bacteria

Non-luminous bacteria lack the genes that encode the *lux* operon, but most are capable of supplying the metabolites required for bioluminescence. Therefore, all that is required for these bacterium to become bioluminescent is the transfer of the luciferase and fatty acid reductase genes, providing that these genes are transcribed, translated, and sufficiently stable in the new host. The first example involved the cloning and transfer of the *lux* operon from *V. fischeri* into *E. coli* (Engebrecht and Silverman 1983). In addition to *E. coli*, a number of other nonluminous bacteria have since been shown to be capable of bioluminescence upon the transformation of the *lux* operon (Meighen 1991). Initial applications of luciferase were to assay a variety enzymes, substrates, and cofactors using ATP as a biotic marker (Kricka 1988). Recent advances in biotechnology however, has unleashed numerous applications for bioluminescence and are in continuous progress. For example, the expression of luciferase in various bacterial species has provided a simple and sensitive system for the presence of specific bacteria (Stewart et al. 1992, Stewart et al. 1989), monitoring of growth and distribution of bacterial species in the environment (Janson 1995, Shaw and Kado 1986), susceptibility to various toxins (Stewart et al. 1989), and reporters of

gene expression (Billard and DuBow 1998, Meighen 1984). Luminescence based reporter assays are advantageous compared to other commonly used reporter systems in that they produce a physical signal. Assays that are chemically based may be toxic when accumulated and therefore prevent real-time processing. Luminescence on the other hand is non-invasive, nondestructive, 10^2 - 10^3 times more sensitive than chemically based assays, and can be measured in real-time (Stewart et al. 1992).

A routinely used quantifiable marker of toxicity in bacteria has been bioluminescence in specific bacterial strains (reviewed in Kong et al. 1995). Bioluminescence requires RNA and protein synthesis, ATP, $FMNH_2$, and NADPH for light production (reviewed in Meighen 1991). Interference with any of these basic cell processes and metabolites would alter a cell's ability to produce light. Thus, a correlation has been made between toxicity, as measured using bioluminescence, and effects of the metal on these metabolic processes. For example, the addition of toxic concentrations of Hg(II) resulted in the concomitant loss of light (e.g. Virta et al. 1995, Dodd et al. 1990).

The most useful application of luminescence-based assays is reporters of gene expression. The regulation of transcription from various promoters can readily be monitored using the *lux* genes as reporters of gene expression. In order to accomplish this a promoter of interest is fused to a promoterless *lux* operon, either *lux*CDABE or *lux*AB. In the latter, the aldehyde substrate is added exogenously for the measurement of light production. By excluding *lux*CDE the aldehyde substrate is neither synthesized nor recycled. Therefore, the *lux*AB reporter cannot be measured in real-time.

Biosensor assays using *lux*CDABE or *lux*AB gene fusions have been utilized to exploit the responses of bacteria to toxic environmental compounds. Of particular use is the fusion of operons whose expression is induced by a particular toxic compound. For example, a variety of heavy metals have been shown to activate a set of genes whose products protect the cells from metal toxicity (Nies 2003). A variety of these toxic metals including copper, cobalt, cadmium, zinc, nickel, thallium, chrome, arsenic, tin, and mercury have been examined using *lux*-fusion constructs, resulting in sensitive detection systems for these specific heavy metals (Briscoe et al. 1996, Diorio et al. 1995, Corbister et al. 1993, Guzzo and DuBow 1994, van der Lelie et al. 1994, Selifonova et al. 1993). The development of bacterial biosensors have provided a method that is fast and inexpensive, and is the only method capable of distinguishing available from non-available forms of metals (reviewed in Köhler et al. 2000, Billard and DuBow 1998) (overviewed in Figure 1.8).

iii) Hg(II)-bioreporters

The regulatory region of the *mer* operon has been fused to a number of different promoterless reporting systems for the purpose of studying Hg(II) bioavailability, e.g. *lac*Z (Hansen and Sorensen 2000), *dsred* (Hakkila et al. 2002), *bla*Z (Chu et al. 1992), *gfp* (Hakkila et al. 2002, Hansen and Sorensen 2000), *luc* (Hakkila et al. 2002, Tauriainen et al. 1999, Virta et al. 1995), and *lux* (Hakkila et al. 2002, Hansen and Sorensen 2000, Selifonova et al. 1993, Condee and Summers 1992, Moelders 1990). The bioluminescent reporting systems, *lux* and *luc*, appear to be the most sensitive.

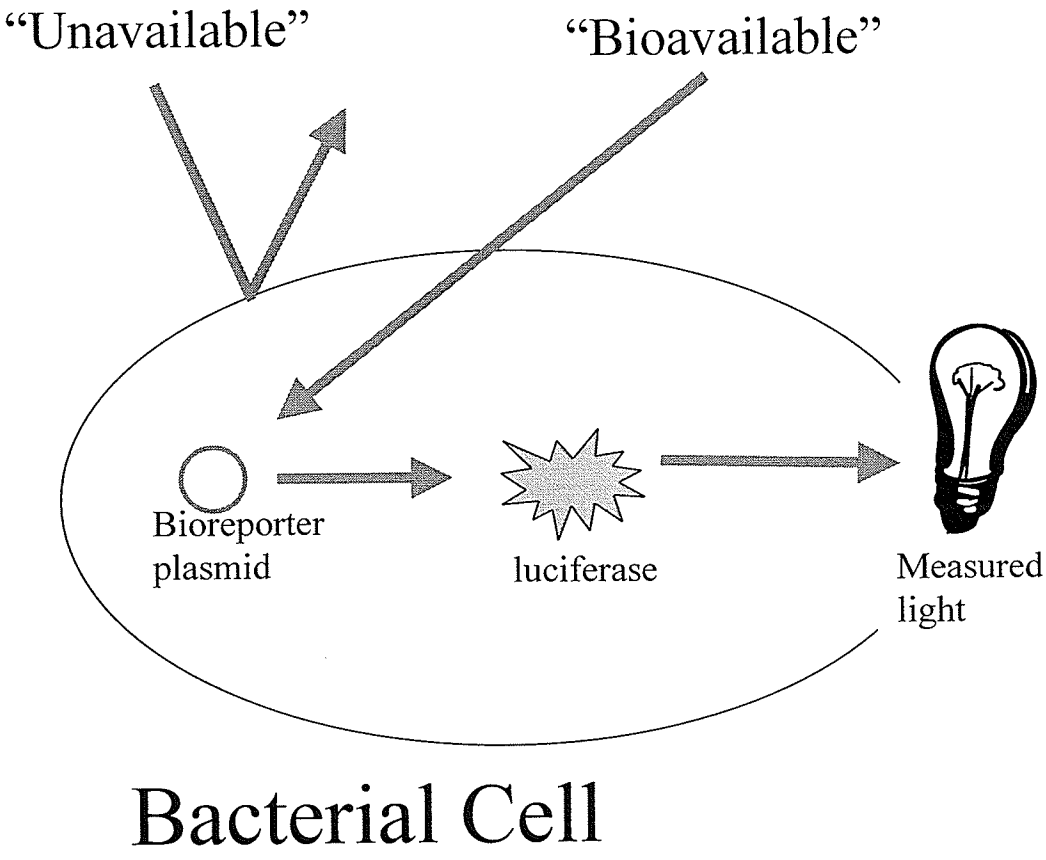

Figure 1.8. Schematic overview of bioluminescent bioreporters

However, in the *luc* system the substrate luciferin has to be added for the measurement of luminescence, which therefore prevents "real-time" measurement of gene expression. In addition, the luciferin substrate is quite expensive. Therefore, due to the sensitivity of *lux* and the high affinity of MerR for Hg(II), a *mer-lux* fusion is ideal for the measurement of low levels of Hg(II).

One of the first *mer-lux* bioreporter plasmids (pCC306) was employed to evaluate in vivo gene expression rates of the Tn*21 mer* structural gene promoter in *E. coli* K-12 (CB806) (Condee and Summers 1992). This construct consisted of a fusion of the regulatory region, *mer*Ro/p, from the Tn*21 mer* operon to a promoterless *lux*AB operon from *Vibrio harveyi*. The kinetics of Hg(II)-induced bioluminescence produced a sigmoidal curve with a $K_{0.5}$ for MerR induction of ~ 9.3 X 10^{-8} M Hg(II), which was in good agreement with the in vitro observations of 1 X 10^{-8} to 5 X 10^{-8} M (Ralston and O'Halloran 1990, O'Halloran et al. 1989). Induction of the *mer* operon also demonstrated an "ultimate threshold" effect in vivo and in vitro, where only a 4.2 fold and 5.1 fold respectively increased the activity from 10 to 90% of the maximum. It was therefore concluded that the luciferase assay was "capable of faithfully portraying gene expression kinetics in vivo" (Condee and Summers 1992).

In 1993, Selifonova et al. constructed the *mer-lux* bioreporter plasmid (pRB28) (Figure 1.9). This plasmid contains a fusion of the regulatory region, *mer*Ro/p, from the Tn*21 mer* operon to a promoterless *lux*CDABE operon from *Vibrio fischeri*. The inclusion of the aldehyde reductase, *lux*CDE, in this construct enabled Hg(II) uptake to be monitored in "real-time". The genes encoding Hg(II) transport, *mer*TPC, were deleted from the construct so that Hg(II) bioavailability

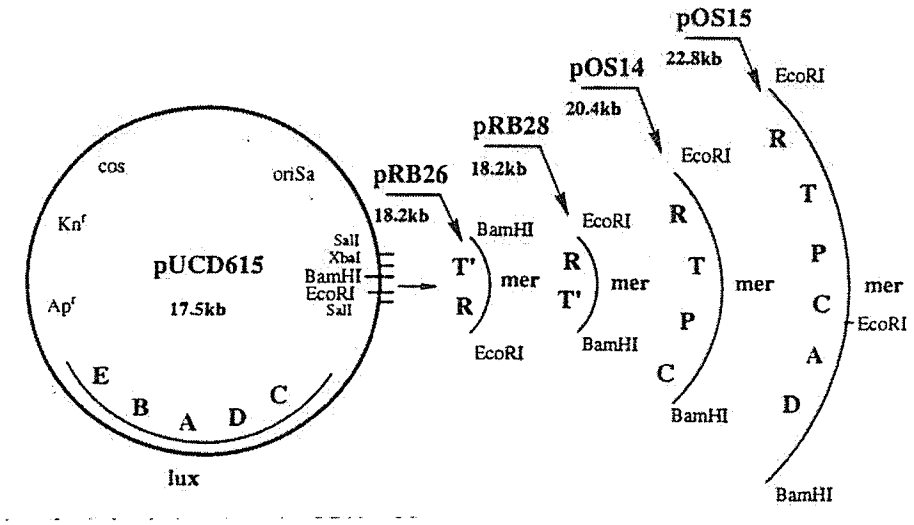

Figure 1.9. Construction of *mer-lux* fusion plasmids pRB28, pOS14, and pOS15 (Selifonova et al. 1993)

could be studied as it occurs in the majority of bacteria, which do not contain the *mer* operon, and hence Hg(II) specific transport systems. Initial investigations using (pRB28) in *E. coli* demonstrated a semi-quantitative reporting system for Hg(II) concentrations ranging from 1nM – to 1μM (Selifonova et al. 1993). This bioreporter was the first to demonstrate the potential of bioreporters to quantitatively measure bioavailable Hg(II) in samples isolated from a Hg(II)-polluted lake. However, the bioreporter required the addition of Hg(II) for a detectable response in natural water samples, which contains lower Hg(II) concentration (Selifonova et al. 1993). Therefore, the requirement of nM concentrations of Hg(II) to induce the *mer* operon initially limited the bioreporters for the study of Hg(II)-polluted aquatic ecosystems only.

From previous in vivo and in vitro kinetic studies involving Hg(II) induction of the *mer* operon it was assumed that bacteria would not play a significant role in the reduction of Hg(II) in non-contaminated ecosystems (e.g. Morel et al. 1998). However, *mer*A transcripts are commonly detected in sites containing concentrations of Hg(II) below what is believed to be required for induction of the *mer* operon (e.g. Nazaret et al. 1994). The capability of detecting pM concentrations of Hg(II), which is commonly encountered in pristine aquatic ecosystems (Fitzgerald 1979), was achieved by simply decreasing the cell concentration of the bioreporter assays from 10^7 to 10^5 cells ml^{-1} (Rasmussen et al. 1997). Therefore, this study indicated that the importance of bacteria containing the *mer* operon should not be limited to Hg(II)-contaminated sites, as proposed by Morel et al. (1998), but should also include pristine or less-contaminated locations. Further improvements to the bioreporter

assays have enabled Hg(II) concentrations as low as 0.25 pM using the bacterial luciferase (Kelly et al. 2003) and 0.1 fM using the firefly luciferase (Virta et al. 1995) to induce a measurable bioluminescent response. The increased sensitivity of the firefly luciferase over bacterial luciferase might be a result of the quantum yields of these enzymes, which is about 90% and 5 % respectively (Virta et al. 1995). This increased sensitivity has enabled the potential for bioreporters to study Hg(II) bioavailability in a wide range of non-contaminated natural waters.

iV) Potential limitations of bioluminescent bioreporters

The production of luminescence depends on the physiological state of the cell (Dorn et al. 2003) and therefore, any substance or environment that compromises cellular viability can affect the results and interpretations of the bioreporter assays. Therefore, the use of a constitutive light producing plasmid is important to ensure that any differences in light production from a bioreporter was dependent on metal bioavailability and not due to differences in bacterial physiology that could effect the light producing capability of the cell (Barkay et al. 1998). The constitutive plasmid (pRB27), which expresses *lux* independently of non-toxic concentrations of metals, has been used as a control to monitor light emission in varying samples (Kelly et al. 2003, Barkay et al. 1997). It is believed that the constitutive phenotype of (pRB27) resulted from a small deletion upstream from the *mer* insert in (pRB28) (Barkay et al. 1997, see Appendix 1 for DNA sequence analysis).

A bioreporter response is not only the concentration of metals that are transported into the cell, but is also dependent on the "intracellular" proportion of

metals that is available to the "sensor" protein. There are a number of different external and internal factors that can influence the intracellular availability of a metal (reviewed in Rensing and Maier 2003). For example, metals forming strong complexes with intracellular ligands might not be available to the "sensor" protein. In such a case these metals would not be detectable using the bioreporter system, despite being transported into the cell. In addition, metals might not be detectable by a bioreporter if it is quickly transported out of the cell by a detoxification efflux pump. This was demonstrated for an *E. coli* strain expressing ZntA, which is a P1-ATPase Zn(II) efflux pump that transports Zn(II) out of the cytoplasm. In the presence of *znt*A the bioreporter was not capable of detecting intracellular Zn(II) (Rensing et al. 1998). Whereas in a zntA$^-$ *E. coli* strain the same bioreporter construct was able to detect intracellular Zn(II) (Rensing et al. 1998). To date there have been no reports of Hg(II) detoxification via efflux transporters and therefore, this might not be a concern for the study of this metal.

Bioreporters are an indirect measurement of metal uptake, which should be used in conjunction with classical metal transport studies (e.g. radio labeled metals) when possible. However, previous attempts at studying Hg(II) uptake has not been successful. Therefore, despite some of the aforementioned limitations, the use of bioreporters is the only available method to study trace concentrations of Hg(II) "uptake" in bacteria.

1.7 Thesis Objectives

Of most environmental concern is the fate of Hg(II) in anaerobic sediments of aquatic ecosystems, where the bacterial mediated transformation of Hg(II) into MeHg predominantly occurs. In order for Hg(II) to be methylated it must be in a form that can be taken up by the cell. Therefore, knowledge of environmental parameters that could potentially affect Hg(II) bioavailability, and hence rates of Hg(II) methylation, is of outermost importance. However, the applicability of *mer-lux* bioreporters to study Hg(II) bioavailability under anaerobic conditions was unknown. Therefore, the first objective of my thesis was to develop an anaerobic *mer-lux* bioreporter assay that was capable of responding to environmentally relevant concentrations of Hg(II). However, there were many results in these assays that did not coincide with previous assumptions of Hg(II) uptake in bacteria, which was proposed to be via passive diffusion of lipophilic Hg(II) species formed in the bulk solution.

Taking into consideration results from the *mer-lux* bioreporter assays, the complexity of bacterial cells, the uptake of other essential and non-essential metals in bacteria, and the facilitated uptake of Hg(II) in higher organisms, we hypothesized that secondary transport systems for Hg(II) should also exist in bacteria. The focus of my thesis was then shifted to study the mechanism(s) of Hg(II) uptake in bacteria and how differences in physiology and/or environmental parameters affected these

processes. To attempt to identify the mechanism(s) of Hg(II) uptake my objectives were:

1) Investigate the effect that inorganic and organic Hg(II) speciation had on the uptake of Hg(II).

2) Investigate the effect of varying competing metal ions for their potential to mitigate Hg(II) uptake.

3) Investigate the effect of environmental parameters that appear to stimulate MeHg production on uptake.

4) Investigate the effect of mutagenesis via transposon mutagenesis for the creation of Hg-resistant and Hg-sensitive clones.

Chapter 2

General Methods

2.1 Bacterial strains

The two bacterial cultures used predominantly in this thesis were *Escherichia coli* HMS174 ATCC 47011 [F⁻ *recA1 rpoB331 hsdR19* λ⁻ IN(*rrnD-rrnE*)*1*] and *Vibrio anguillarum* ATCC 14181. *E. coli* HMS174 was the original bacterial host for the (pRB28) bioreporter plasmid (see Figure 1.9) (Selifonova et al. 1993) and the (pRB27) constitutive light producing plasmid (Barkay et al. 1997). The natural aquatic organism *V. anguillarum* was used in an effort to create a bioreporter more suitable for the study of Hg(II) uptake in natural waters. Both of these organisms are facultative anaerobes, which enabled the comparison of Hg(II) uptake under aerobic and anaerobic conditions in the same bacterial host.

2.2 Transformation Protocols

Transformation of the bioreporter plasmids (pRB28) and (pRB27) were carried out with modifications as described by Chung et al. (1989). Cultures were grown in 5 ml of LB (Luria-Bertani) medium (Table 2.1) containing 60 μg/ml kanamycin (Kan) and harvested in early mid-log phase by centrifugation at 7000 rpm for five minutes. The pellet was resuspended in 0.5 ml of 0.1 M $CaCl_2$, centrifuged for an additional five minutes, resuspended in 0.1 M $CaCl_2$, and maintained on ice for 30 minutes. In a 1.5 ml eppendorf tube, plasmid DNA was added to 0.1 ml of the suspension and maintained on ice for an additional 30 minutes. The cells were then heat shocked for 90 seconds at 42^0C, placed on ice for 3 minutes, and incubated at 37^0C in 1 ml of LB medium for 1 hour. Following the incubation, the cells were transferred into 2.5 ml of pre-melted R- Top agar (Table 2.2) and immediately

Table 2.1. Components of LB medium.

Component	
Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g
H_2O	up to 1 L
For LB agar plates:	
Bacto-agar	15 g

poured onto LB agar plates containing the appropriate selective marker to grow overnight.

2.3 Plasmid isolation

The isolation of (pRB28) and (pRB27) was conducted according to Sambrook et al. (1989) with slight modifications. Overnight grown cultures in 5 mL of LB broth were centrifuged for 3 minutes at 13,000 g in eppendorf tubes. The pellet was resuspended in 200 µL glucose-EDTA buffer (25 mM Tris-HCl, pH 8.0, 1 % glucose, 10 mM EDTA). The cells were lysed for 5 minutes on ice by adding 400 µL of a 0.2 M NaOH and 1 % SDS solution. 300 µL of 6.2 M ammonium acetate (pH=5.9) was then added to neutralize the solution. Following 10 minutes on ice, the tubes were centrifuged for 15 minutes. The supernatant was transferred to a new tube and centrifuged for an additional 10 minutes. The supernatant was transferred to a new tube. 550 µL of isopropanol was added to precipitate the DNA and remained at room temperature for 15 minutes. The tubes were then centrifuged for 10 minutes and the pellet was washed twice with 70% (v/v) ice-cold ethanol. The pellet was then dried for 30 minutes in a dessicator and resuspended in 50 µL of TE buffer (10 mM Tris, pH=8, 1 mM EDTA). Plasmids were stored at $-20^{O}C$.

2.4 Storage of cultures

For long-term storage of the bioreporter cultures, 1 ml of a mid-log culture grown in LB-Kan broth and 0.5 ml of 24 % (v/v) DMSO were mixed in a 2 ml

Table 2.2. Components of R-Top agar.

Component	
Bacto- yeast extract	1 g
Bacto-tryptone	10 g
NaCl	8 g
Bacto-agar	8 g
H_2O	up to 1 L
Autoclave then add:	
1 M $CaCl_2$	2.0 ml
1.67 M Glucose	3.36 ml

nominal capacity cryogenic vial and stored at -60^{0}C. Streaked cultures on LB-Kan agar plates were stored at 4^{0}C for short-term storage (~2-3 weeks).

2.5 Bioreporter assays

2.5.1 Reagents and Hg_{Total} analysis

Culture flasks, Teflon centrifuge tubes, and reagent bottles were washed in 30 % H_2S0_4 and rinsed thoroughly with Milli-Q water. Reagents commonly used in the *mer-lux* bioreporter assays are listed in Table 2.3. Although glucose is a reducing sugar, it did not appear to have an affect on Hg(II) in the time frame of our assays. For precautionary measures future bioreporter studies could try using a non-reducing sugar (e.g. sucrose) in the assay medium. These reagents were periodically analyzed for total Hg(II) by Flett Research Ltd. using a cold vapor atomic fluorescence spectrometer (CVAFS) (Brooks Rand, Ltd. Model 2) and EPA Method 1631, as adapted from Bloom and Creselius (1983). Samples were preserved for analyses by adding 0.25 ml concentrated hydrochloric acid per 125 ml sample. Milli-Q water from the Department of Fisheries and Oceans Canada was used in the growth and assay medium, as it contained very low Hg(II) concentrations (1.5-2.5 pM).

2.5.2 Preparation of anaerobic reagents

Heat sensitive components, such as antibiotics, were made anaerobic via the Balch-Wolfe method (Balch and Wolfe 1976). For this method, filter sterilized reagents were added to either sterile Balch-Wolfe tubes, or 125 ml nominal capacity serum vials. The tubes/vials were then sealed with a rubber butyl stopper and

Table 2.3. Common components used in the defined assay medium for the bioreporter assays.

Component	Brand	Grade	Stock Concentration
$(NH_4)_2SO_4$	Fisher	A.C.S certified	0.91 M
NaH_2PO_4	BDH	Analytical	} 1 M
K_2HPO_4	BDH	Analytical	
D-glucose	BDH	Analytical	1.67 M
NaCl	Fisher	A.C.S certified	1 M

crimped with an aluminum cap. To reduce O_2 levels, the tubes were placed on a gas/vacuum manifold and evacuated for 7 minutes and gassed for 1 minute with N_2, for a total of four cycles. Serum vials were gassed for 3 minutes and evacuated for 10 minutes for four cycles. TheHungate method (Hungate 1969) was used to eliminate O_2 for the reagents used in the anaerobic assays (e.g. water, growth medium, and stock solutions of reagents). The reagents were boiled for ~ 5-10 minutes and the head space was flushed with N_2 pre-purified gas, which was passed through a reduced heated copper column, until the solution cools to room temperature. A ChemMets O_2 Kit was used to ensure the reagents and media were O_2 free (O_2 < 1 ppb).

2.5.3 Growth of cultures for mer-lux assays

For maintenance of the plasmid Kan was added to a final concentration of 60 µg/ml in all the growth media. 5 ml of aerobic LB-Kan broth was inoculated with a single isolated colony from a LB-Kan agar plate (preferably < 2 weeks old) and incubated at 28^0C with shaking until late-log phase. The culture was brought into an anaerobic Coy® glove bag, maintained under a 93% N_2:7% H_2 atmosphere, and 100 µl was transferred to a serum vial containing 5.90 ml of anaerobic glucose minimal medium-Kan broth (GMM) (Table 2.4). The vials were sealed with a rubber butyl stopper and crimped with an aluminum cap. The culture was then removed from the glove bag and reincubated at 28^0C with shaking at 150 rpm until late/mid-log phase. The culture was brought back into the anaerobic glove bag and 20 ml of anaerobic GMM-Kan broth was added to the 5 ml anaerobic culture. The cultures were re-incubated at 28^0C with shaking at 150 rpm until mid-log phase.

Table 2.4. Components glucose minimal medium (GMM).

Component	
0.5 M Na_2HPO_4	8.0 ml
1.0 M KH_2PO_4	2.0 ml
2.0 M NH_4Cl	1.0 ml
0.86 M NaCl	0.2 ml
Milli-Q water	up to 100 ml
Autoclave and add:	
1.67 M Glucose	1 ml
**Trace elements	0.1 ml
1 M $MgSO_4$	0.1 ml
1 mg/ml Vitamin B_1	0.1 ml
** Trace elements stock solution	
$ZnSO_4$ $7H_2O$	100 mg
$MnCl_2$ $4H_2O$	30 mg
H_3BO_3	300 mg
$CoCl_2$ $6H_2O$	10 mg
$NiCl_2$ $6H_2O$	20 mg
Na_2MoO_4 $2H_2O$	30 mg
Milli-Q water	up to 1 L

The initial inoculum and growth of cultures for the aerobic assay is as described for the anaerobic *mer-lux* assays with the exception that it was all carried out on the bench top. 50 µl of the mid-log culture was transferred to 4.95 ml of aerobic GMM-Kanbroth and incubated at 28^0C with shaking at 150 rpm until mid-log phase. The entire 5 ml mid-log culture was then transferred to 20 ml of aerobic GMM-Kan broth in a 125 ml nominal capacity serum vial and grown until mid-log phase. The mean generation times (g) of *V. anguillarum* and *E. coli* in LB and GMM are shown in Table 2.5.

The growth was monitored over time by measuring the optical density at 600 nm (OD_{600} nm) in a Biochrom Novaspec II spectrophotometer. The mean growth rate constant (k) was calculated using the formula k= (Log A2-Log A1)/(.301t), where A2 and A1 represent OD_{600} readings measured in exponential phase and t is the time. The mean generation time (g) was calculated by taking the reciprocal of the mean growth rate constant (g = 1/k).

2.5.4 Cell preparation for mer-lux assays

The final 25 ml mid-log culture was decanted into 50 ml nominal capacity O-ring Teflon centrifuge tubes in the glove bag. The tubes were then removed from the glove bag and centrifuged at 12,000 g for 10 minutes at 4^0C. The supernatant was discarded in the glove bag and the pellet was resuspended in 20 ml of anaerobic 67 mM phosphate buffer (pH=7). The centrifugation and resuspension in anaerobic phosphate buffer was then repeated. The supernatant was discarded in the glove bag and the pellet was resuspended in 3-5 ml of anaerobic phosphate buffer. In the glove

Table 2.5. Mean generation times (g) of *V. anguillarum* and *E. coli* in the growth media described for the preparation of cells for the *mer-lux* bioreporter assays. Averages of quadruplicate samples are shown.

Organism	**Medium**	**Aerobic (g) (hours/generation)**	**Anaerobic (g) (hours/generation)**
V. anguillarum	LB	0.88	ND*
V. anguillarum	GMM	2.1	2.8
E. coli	LB	1.1	ND
E. coli	GMM	2.4	2.9

* ND= not determined

bag the resuspended pellet was aliquoted into a Balch-Wolfe tube containing 5ml of anaerobic phosphate buffer. The tubes were then sealed using a rubber butyl stopper and an aluminum crimp and removed from the glove bag. The optical density (OD) of the final cell suspension was diluted to an OD_{600} of 0.4 using anaerobic phosphate buffer via syringe. The cultures were brought back into the glove bag and diluted another 100-fold in anaerobic phosphate buffer to a final cell concentration of approximately 2 $X10^6$ cells/ml.

Cell preparation for aerobic *mer-lux* assays are those of the anaerobic method described above, with the exception that it was carried out on the bench top with aerobic components.

2.5.5 mer-lux assays

In the glove bag the anaerobic components of the defined assay medium (Table 2.3) are mixed directly into the scintillation vials, to a final volume of 19 ml. The primary mercury standard was a 1 μg/ml Hg(II) solution, containing 1 % BrCl, provided by Flett Research Ltd. For anaerobic assays, 0.5 ml of the primary standard is transferred to a Teflon vial and brought into the glove bag. The primary Hg(II) standard was diluted twice in anaerobic Milli-Q water, in Teflon vials left overnight in the Coy anaerobic glove bag. Assays were initiated by adding 1 ml of the diluted cell suspension at timed intervals of 45 seconds, which coincides with the time it takes for the scintillation counter to measure each vial. The vials were incubated at room temperature in the glove bag until the desired time of measurement. At this time the samples were removed from the glove bag. For light measurement the vials

were quickly opened and shaken three times, to ensure full aeration, and then immediately placed in the scintillation counter for counting (Beckman LS 6500, set in non-coincidence mode for 30s count intervals).

Aerobic *mer-lux* assays are carried out as described for the anaerobic method described above, with the exception that the assays were prepared on the bench top with aerobic components, the diluted cell suspension was added at 38s intervals, and the samples were immediately placed in the scintillation counter and continuously monitored over time.

The growth and cell preparation for the aerobic and anaerobic assays involving constitutive light production using (pRB27), for either cellular energetics or toxicity, were carried out as described above for the *mer-lux* assays.

For the growth measurements in defined assay media under aerobic and anaerobic conditions, the cells were initially grown and prepared as described for the *mer-lux* bioreporter assays. Balch-Wolfe tubes containing 10 ml of the assay medium was inoculated with the bacterium for an initial cell concentration of ~ 1 X 10^5 cells/ml. To mimic the bioreporter assays the tubes were maintained at room temp without shaking and the growth was monitored over time by measuring the OD_{600} nm in a Biochrom Novaspec II spectrophotometer.

2.6 Hg(II)-speciation calculations

The thermodynamic speciation model program MINEQL+ version 4.5 was used to calculate the speciation of Hg(II) in the bulk solution. Stability constants used in the calculations were obtained from the NIST database (Table 2.6) unless

otherwise stated. It should be noted that the thermodynamic speciation database for Hg(II) was limited and therefore, the stoichiometries and stability constants of many complexes might not be accounted for or reliable. To limit the carry over of any potential bacterial exudates that might have been present in the initial growth medium the cells were washed in phosphate buffer prior to the bioreporter assays. Under aerobic and anaerobic conditions the mean generation times for *V. anguillarum* and *E. coli* ranged from 4-7 hours/generation in the defined assay media. Therefore, the effect of growth was not considered in the speciation calculations since the bioreporter assays were typically only 1-2 hours. Since no reducing agents were used under anaerobic conditions the effect of E_h was also not considered in the speciation calculations. The addition of glucose had no significant effect on the speciation of Hg(II) and was also not considered in the calculations.

Table 2.6. Stability constants used in the calculation of Hg(II) speciation. Constants were measured at 25^{O}C and ionic strength =0 or corrected to 0. Exceptions were stability constants for ligands $(NH_3)^{2+}$ (I=1) and HPO_4^{2-} and PO_4^{3-} (I=3).

Species	LogK [A]
$HgCl^+$	7.2
$HgCl_2$	14
$HgCl_3^-$	15.1
$HgCl_4^{2-}$	15.4
HgOHCl	4.1
$HgOH^+$	-3.4
$Hg(OH)_2$	-6.2
$Hg(OH)_3^-$	-21.1
$HgNH_3^{2+}$	8.8
$Hg(NH_3)_2^{2+}$	17.4
$Hg(NH_3)_3^{2+}$	18.4
$Hg(NH_3)_4^{2+}$	19.1
$HgPO_4^-$	14.5
$HgHPO_4$	20.3
$HgHis^+$	7.7
$HgHHis^{2+}$	28.2
$Hg(His)_2$	22
$HgH_2His_2^{2+}$	33.6

[A] Constants obtained from Martel and Smith (2001) with the exception of the Hg-histidine complexes, which were obtained from Van der Linden and Beers (1973).

Chapter 3

The development and characterization of a *mer-lux* bioreporter for the detection of trace concentration of Hg(II) under anaerobic conditions in *Vibrio anguillarum* and *Escherichia coli*.

3.1 Introduction

The only method that can distinguish bioavailable from non-available forms of Hg(II) are bioreporters. The sensitivity of these assays has enabled studies of Hg(II) bioavailability in a number of different environmental samples; e.g. soil (Rasmussen et al. 2000), snow (Scott et al. 2001), precipitation (St. Louis et al. 2001), and natural lake water (Hintelmann et al. 2002). To date *mer-lux* bioreporters have only been used to study Hg(II) bioavailability in aerobic environmental samples. However, of most environmental concern is the fate of Hg(II) in anaerobic sediments of aquatic ecosystems, where the bacterial mediated transformation of Hg(II) into MeHg predominantly occurs (King et al. 2001, Macalady et al. 2000, Benoit et al. 1999, Pak and Bartha 1998, Compeau and Bartha 1984).

In order for Hg(II) to be methylated it must be in a form that can be taken up by the cell. Therefore, knowledge of environmental parameters that could potentially affect Hg(II) bioavailability, and hence rates of Hg(II) methylation, is of outermost importance. The development of a bioreporter assay that would distinguish available from non-available forms of Hg(II) under anaerobic conditions would be an invaluable tool that would increase our knowledge of how different environmental conditions affect the bioavailability of Hg(II) under anaerobic conditions. This in turn would enhance our understanding of the biogeochemical cycling of Hg, increase our predictive abilities of the fate of Hg in the environment, and potentially provide new alternatives for the bioremediation of MeHg contaminated ecosystems.

The applicability of bioreporters to study Hg(II) bioavailability under anaerobic conditions was unknown at the start of this thesis. However, in 1993 the *lux* system was investigated as a possible reporter system under anaerobic conditions (Phillips-Jones 1993). In this study a shuttle vector was constructed that contained the α-toxin gene promoter region fused to *lux*AB. This plasmid was then transformed into an obligatory anaerobe, *Clostridium perfringens*, and the facultative anaerobe, *E. coli*. Since bioluminescence requires O_2 (Meighen 1991), the samples were incubated under anaerobic conditions for a set duration of time, following which the samples were aerated briefly and measured in a scintillation counter. *lux* expression following aeration in both bacterial species demonstrated that bioluminescence could be used as a quantitative reporter of gene expression under anaerobic conditions (Phillips-Jones 1993).

On the basis of the expression of the *lux* genes under anaerobic conditions (Phillips-Jones 1993), my first objective was to develop a *mer-lux* bioreporter assay for the determination of Hg(II) bioavailability under anaerobic conditions. To accomplish this we utilized the *mer-lux* bioreporter (pRB28) and the constitutive derivative (pRB27), previously described by Selifonova et al. (1993) and Barkay et al. (1997) respectively.

3.2 Additional Methods

E. coli HMS174 (pRB28) and (pRB27) were kindly provided by Tamar Barkay. *V. anguillarum* was transformed with the bioreporter plasmid (pRB28), by Fiona Punter, and (pRB27) as described in Chapter 2 (section 2.2). The aerobic and

anaerobic *mer-lux* bioreporter assays in *V. anguillarum* and *E. coli* HMS174 were carried out as described in Chapter 2 (section 2.5). One exception was the growth of the bacteria for the bioreporter assays involving the effect of reducing agents, which was supplemented with Ti-citrate, Ti-NTA, or dithionite in the corresponding reducing agent experiments.

Batch to batch variations in total light emission from the bioreporter assays can vary orders of magnitude, which could result from a number of different factors that were not stringently controlled e.g. differences in temperature (Dorn et al. 2003). While total light emission at times was quite variable from assay to assay, the trends observed were all reproducible in the data presented. Therefore, most of the data presented are illustrative examples of experiments that were repeated several times.

Sediment Sampling An Ekman dredge was used to collect sediment samples from Lake 114 at the Experimental Lakes Area, Ontario, Canada. Sediments were of high porosity (~98% water) and high organic content (Rudd et al. 1986). 500 ml screw capped jars were thoroughly rinsed with surface lake water and were used to store the sediment collected by the dredge. Sediment was transferred to over-flowing in the jars, tightly screwed closed, and opened inside an anaerobic Coy glove bag. Disposable sterile surfactant-free cellulose acetate 0.45 μM filters (Nalgene), left overnight in the glove bag, were pre-rinsed with approximately 75 ml of low Hg Milli-Q anaerobic water prior to filtering the sediment samples. The concentration of O_2 in the porewater measured immediately after filtration was below the detection limit of the ChemMets O_2 Kit (< 1 ppb). The collected porewater was supplemented with 5 mM glucose, 2 mM K_2HPO_4, 1 mM NaH_2PO_4, 9 mM $(NH_4)_2SO_4$, and +/- 1

mM fumarate or 1 mM histidine, and was used the same day for the bioreporter assays.

3.3 Results

3.3.1 Kinetics of Hg(II)-induction under aerobic conditions The *mer-lux* bioreporter plasmid (pRB28) has successfully been used to study factors that affect the bioavailability of Hg(II) under aerobic conditions in *E. coli* HMS174 (Barkay et al. 1997, Selifonova et al. 1993) and *V. anguillarum* (Kelly 2003, Scott PhD dissertation, University of Manitoba). Typical responses of *V. anguillarum* (pRB28) and *E. coli* HMS174 (pRB28) under aerobic conditions in samples spiked with Hg(II) are shown in Figure 3.1A and 3.1B. Comparison of the Hg(II)-induced light emission in these two assays demonstrates that the bioreporter assay is somewhat comparable between the two bacterial species. The kinetics of light production in the samples spiked with trace concentrations of Hg(II) begins with an initial lag phase. As the concentration of Hg(II) in the samples increase, from 3.75 to 15 pM, the initial lag phase is shortened. The general length of the lag phase for the aerobic assay in these two bacteria is approximately 30-60 minutes, depending on the concentration of Hg(II) added and possibly the physiological state of the bacteria. This lag phase is likely the result of 1) the rates of uptake of Hg(II) into the cell, 2) the rates of transcription and translation of the aldehyde reductase and luciferase enzymes, which is required for light emission, and 3) amplification of luciferase to levels high enough to be detected by the photometer (Barkay et al. 1998). In a previous study using a *mer-lux*AB construct, which does not require the synthesis of the aldehyde reductase,

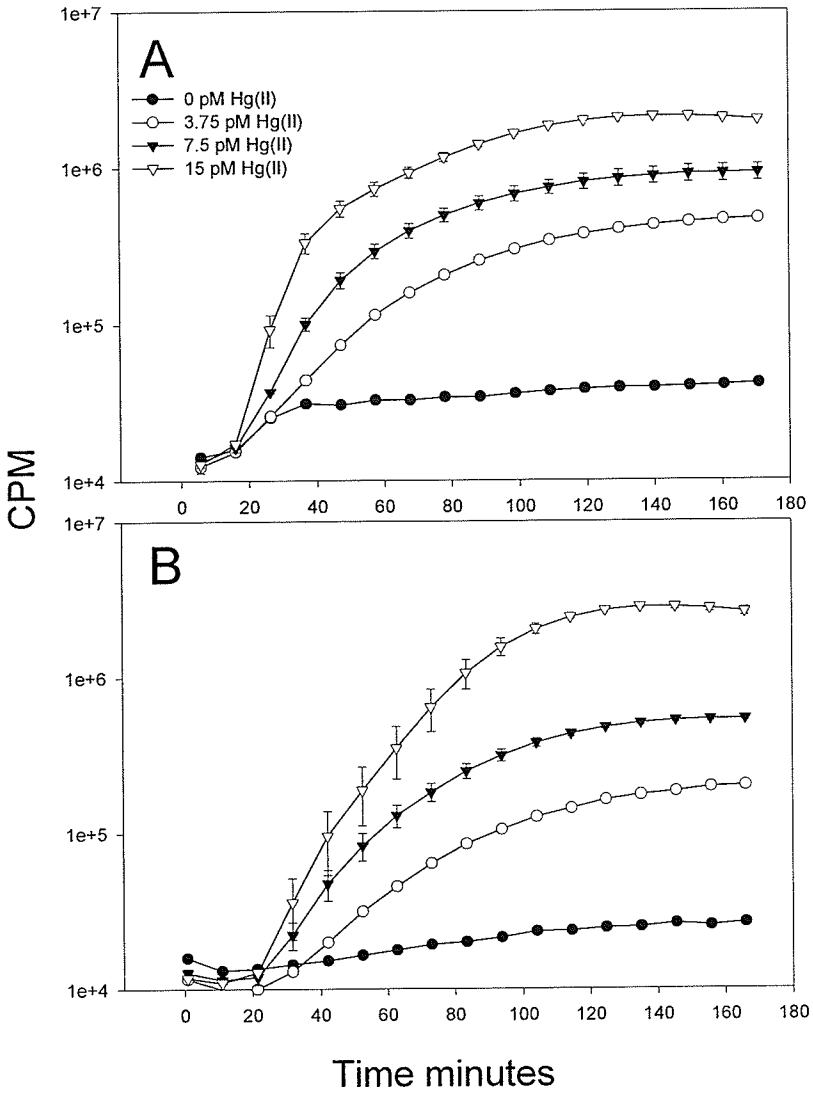

Figure 3.1. Hg(II)-induction of *mer-lux* in (A) *V. anguillarum* (pRB28) and (B) *E. coli* HMS174 (pRB28) under aerobic conditions. The assay medium consisted of 5 mM Glucose, 67 mM Phosphate buffer, 9 mM $(NH_4)_2SO_4$, and 2 mM NaCl. Error bars represent the standard deviation of duplicate samples.

and μM concentrations of Hg(II) induction could be detected after only 2-3 minutes (Condee and Summers 1992). Following the lag phase, induction of light emission becomes evident. As the concentration of Hg(II) increases, from 3.75 to 15 pM, the initial slopes increase in a linear fashion (Figure 3.2). Finally, light production plateaus and decreases slightly over time, yielding a sigmoidal shaped curve.

The amount of Hg(II) added to the samples was proportional to, and could be estimated from the length of the lag period, the plateau of light emission, or the slopes of the increase in light production. From previous experiments it has been determined that the initial slopes of induction are the most accurate measurements for quantitatively measuring bioavailable Hg(II) (Barkay et al. 1998).

3.3.2 Lack of Hg(II)-induction under anaerobic conditions Aerobic *mer-lux* bioreporter assays are advantageous over anaerobic assays in that they can be measured in "real time" (i.e. the same sample can be measured repeatedly over a time course). Since light emission requires O_2, time points based on the kinetics of the aerobic assays were chosen for the anaerobic *mer-lux* assays. Anaerobic assays where nitrate was added to replace O_2 as the terminal electron acceptor for respiration, using *V. anguillarum* (pRB28) (Figure 3.3A) and *E. coli* HMS174 (pRB28) (Figure 3.3B) resulted in no response following the addition of Hg(II). This lack of induction was even evident when the time period of the assay was extended up to 24 hours and the concentrations of Hg(II) were increased more than 8-fold in comparison to the highest Hg(II) concentration used for the aerobic assays (Figure 3.1A and B).

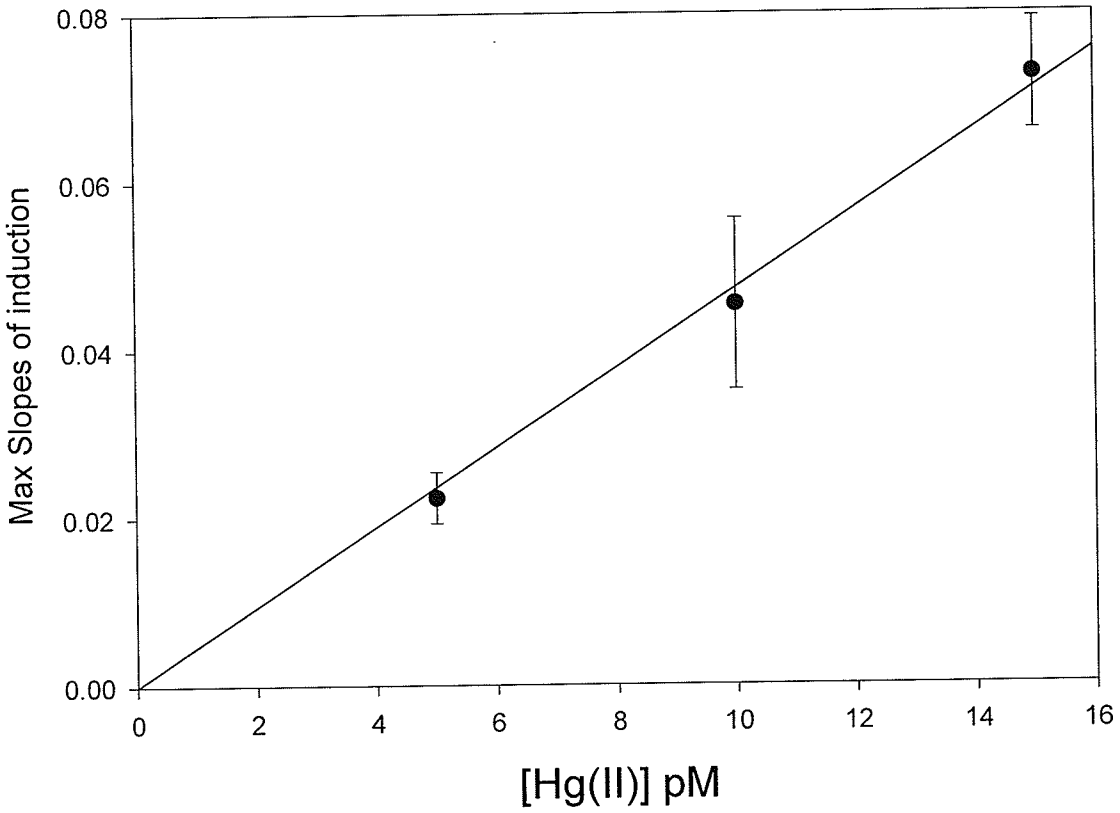

Figure 3.2. Correlation of Hg(II) concentration in the assay medium with max slopes of induction measured using *V. anguillarum* (pRB28). The error bars represent the standard deviation of three independent assays.

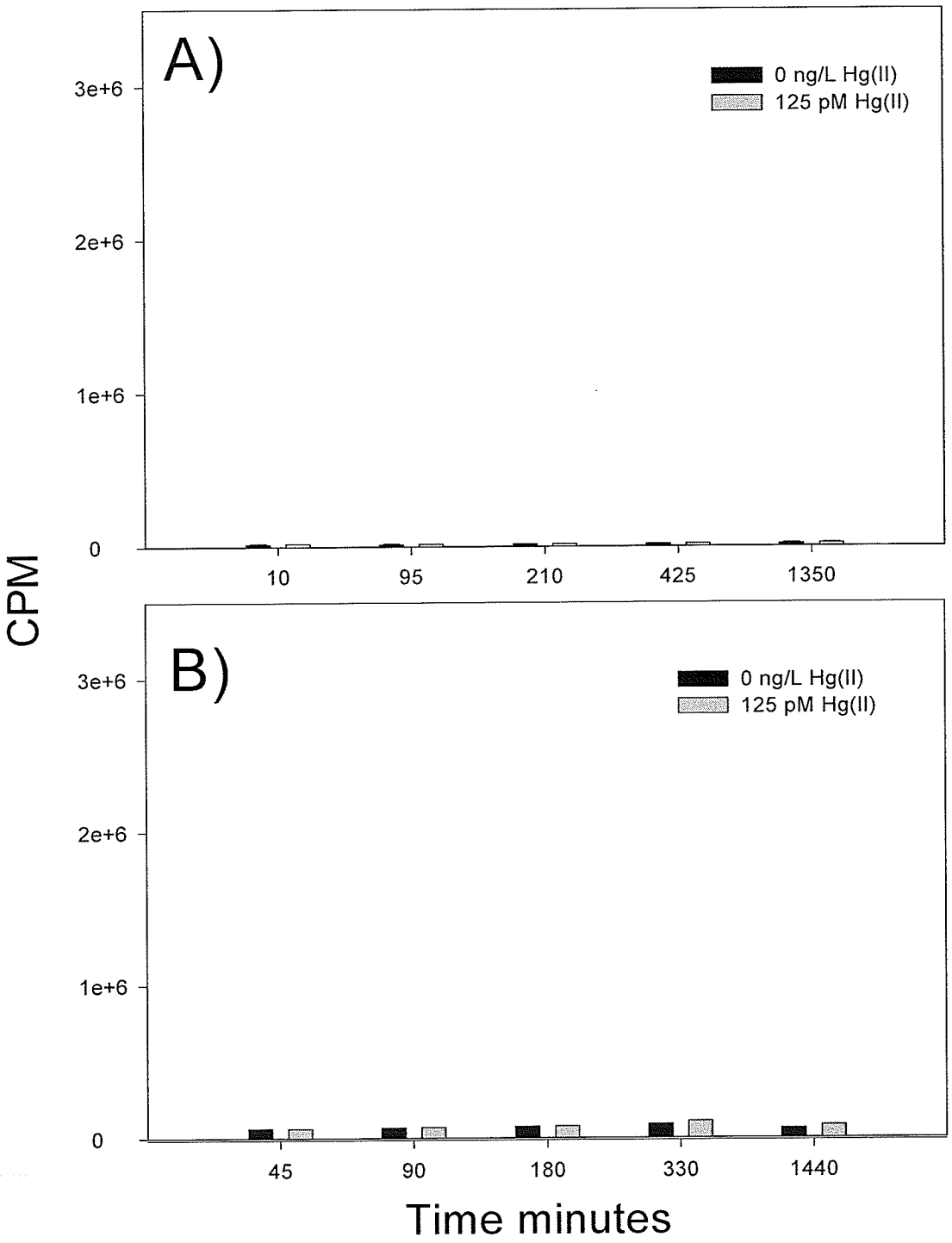

Figure 3.3. Lack of Hg(II)-induction of *mer-lux* in A) *V. anguillarum* (pRB28) and B) *E. coli* HMS174 (pRB28) under anaerobic conditions in defined assay media. The medium consisted of 5 mM Glucose, 67 mM Phosphate buffer, 9 mM $(NH_4)_2SO_4$, 2 mM NaCl, and 10 mM KNO_3. Error bars represent the standard deviation of duplicate samples.

If nitrate had a deleterious effect on the bioavailability of Hg(II) we would expect a lessened response from the bioreporter in aerobic samples containing nitrate. Therefore, an aerobic *mer-lux* assay was performed +/- nitrate in *V. anguillarum* (pRB28) as a control. Under aerobic conditions the uptake of Hg(II) is virtually identical in the presence or absence of nitrate (Figure 3.4A). Therefore, the addition of nitrate to the assay medium did not affect the bioavailability of the added Hg(II). In addition, anaerobic assays using assay medium not supplemented with nitrate, where the organisms fermented glucose, failed to induce upon the addition of Hg(II) in *V. anguillarum* (pRB28) (Figure 3.4B). Nitrate was then eliminated as a possible explanation as to why the biosensors were not responding to Hg(II) anaerobically and was no longer supplemented in the assay medium.

3.3.3 Constitutive light production under aerobic and anaerobic conditions

Physiological differences arise when facultative bacteria are grown under anaerobic or aerobic conditions and it is also known that light production is affected by altering the state of the cell (Dorn et al. 2003). Therefore, the plasmid which is Hg(II)-independent and constitutively produces light, (pRB27), was assayed to determine if the lack of light emission in the anaerobic *mer-lux* assays (Figure 3.3A and B) was a result of a reduction in cellular energetics or metabolites required for light emission. For example, insufficient levels of ATP, $FMNH_2$, NADPH, or the inability to produce or recycle the aldehyde substrate, would result in very little to no light production. Under aerobic conditions light production in *V. anguillarum* (pRB27) remained relatively constant throughout the period of the assay (Figure 3.5). As with the

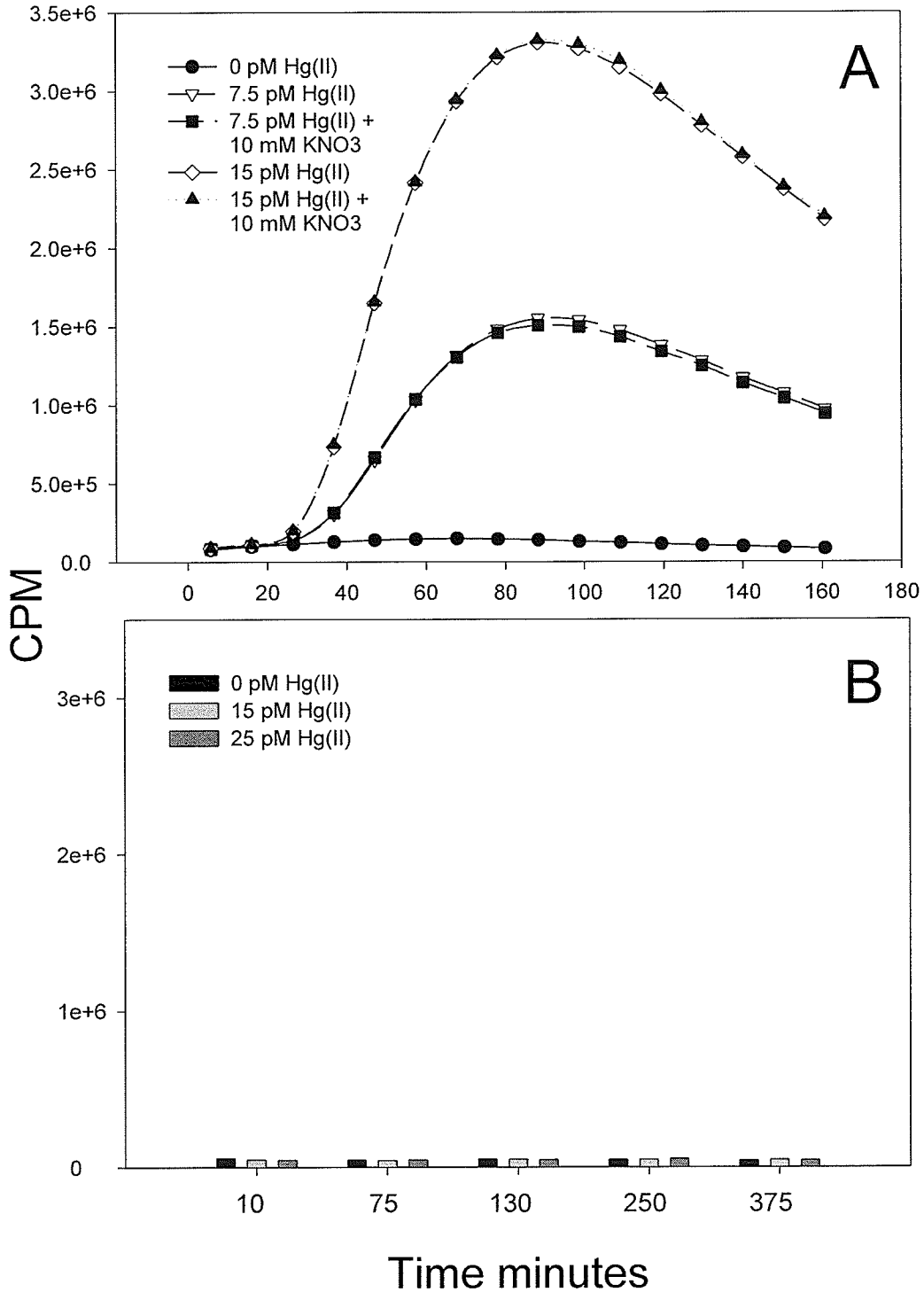

Figure 3.4. Induction of *mer-lux* in *V. anguillarum* (pRB28) under A) aerobic conditions in the presence of KNO_3 and B) the effect of not supplementing the assay medium with KNO_3 under anaerobic conditions. The assay medium consisted of 5 mM Glucose, 67 mM Phosphate buffer, 9 mM $(NH_4)_2SO_4$, and 2 mM NaCl. (Aerobic assays contained +/- 10 mM KNO_3).

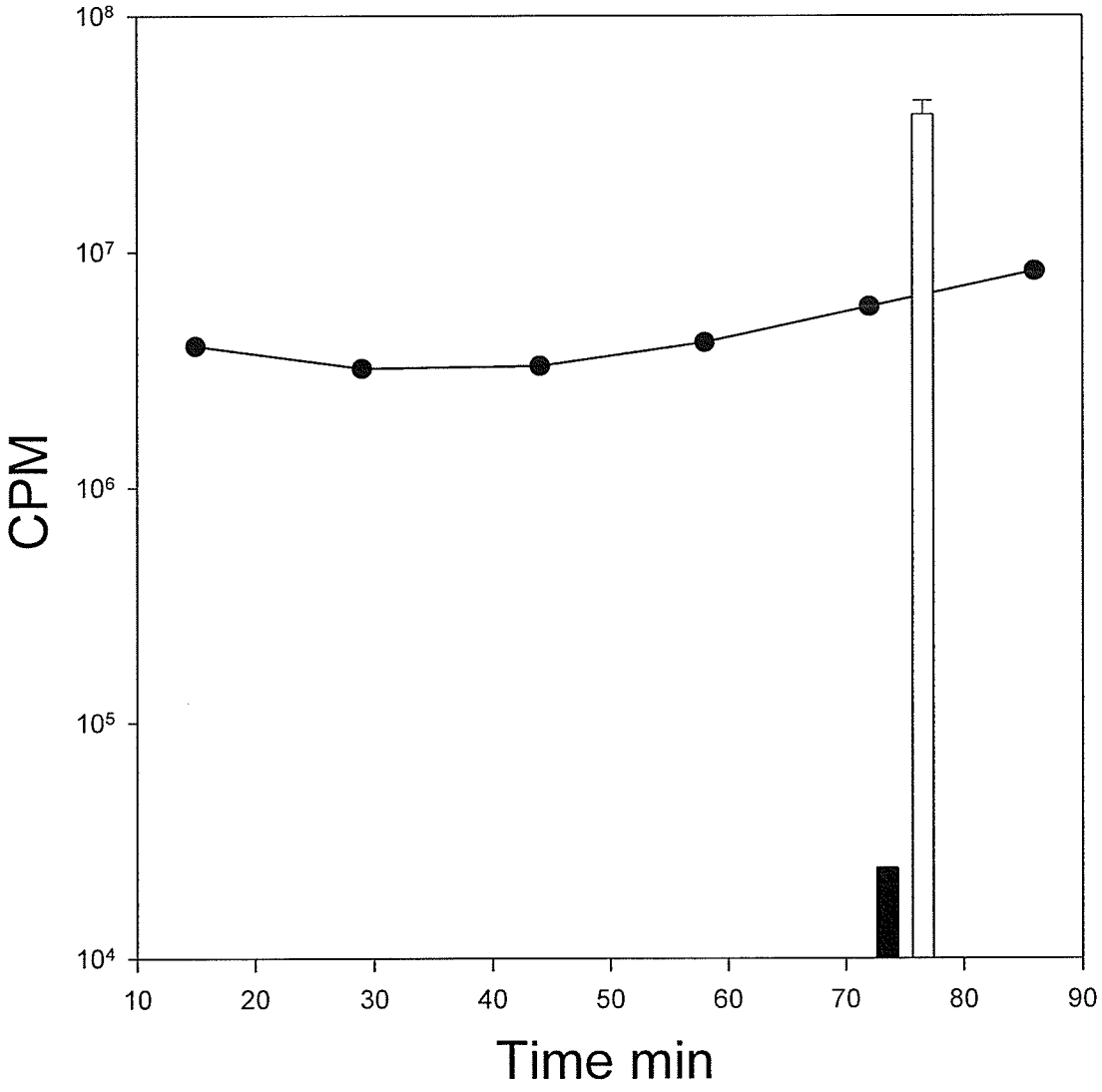

Figure 3.5. Light production in *V. anguillarum* (pRB27) under aerobic and anaerobic conditions in defined assay media. The assay medium consisted of 5 mM glucose, 67 mM phosphate buffer, 9 mM $(NH_4)_2SO_4$, and 2 mM NaCl. Closed circles represent an aerobic "real time" assay; closed bar graph represents anaerobic assay prior to aeration; open bar graph represents anaerobic assay following aeration. Error bars represent the standard deviation of triplicate samples.

anaerobic *mer-lux* assays, time points were chosen to measure light emission in the anaerobic constitutive *lux* assays. Results from the anaerobic constitutive *lux* assay indicate that the cellular physiology of *V. anguillarum* (Figure 3.5) is not limiting for light production. In fact, light emission, once exposed to O_2, is comparable to levels of light production in the aerobic constitutive *lux* assay. These results were also routinely obtained using *E. coli* HMS174 (pRB27).

3.3.4 Hg(II)-induction in glucose minimal medium Despite the ability to grow, it was not clear whether or not luciferase is transcribed and translated in the anaerobic assay medium, since the light observed in the anaerobic constitutive *lux* assay (Figure 3.5) might be from the carry over of luciferase produced anaerobically in GMM. Therefore, anaerobic assays were performed using *V. anguillarum* (pRB28) and *E. coli* HMS174 (pRB28) in GMM. Neither *V. anguillarum* (pRB28) (Figure 3.6A) or *E. coli* HMS174 (pRB28) (Figure 3.6B) induced upon the addition of elevated levels of Hg(II) over an extended time period in GMM. An aerobic *mer-lux* assay was performed in GMM to determine if the added Hg(II) was hindered by the additional components present in GMM (e.g. trace elements) (Table 2.4). For this experiment a range of Hg(II) concentrations, 5-150 pM, was used in the assay (Figure 3.7). The addition of 15 pM Hg(II) did not result in the induction of *mer-lux* under aerobic conditions in *V. anguillarum* (pRB28). However, induction was observed in GMM containing Hg(II) concentrations lower than that used for the previous anaerobic assays (Figures 3.6A and B). Therefore, an undetermined fraction of the added Hg(II) to GMM remained bioavailable and was capable of inducing light

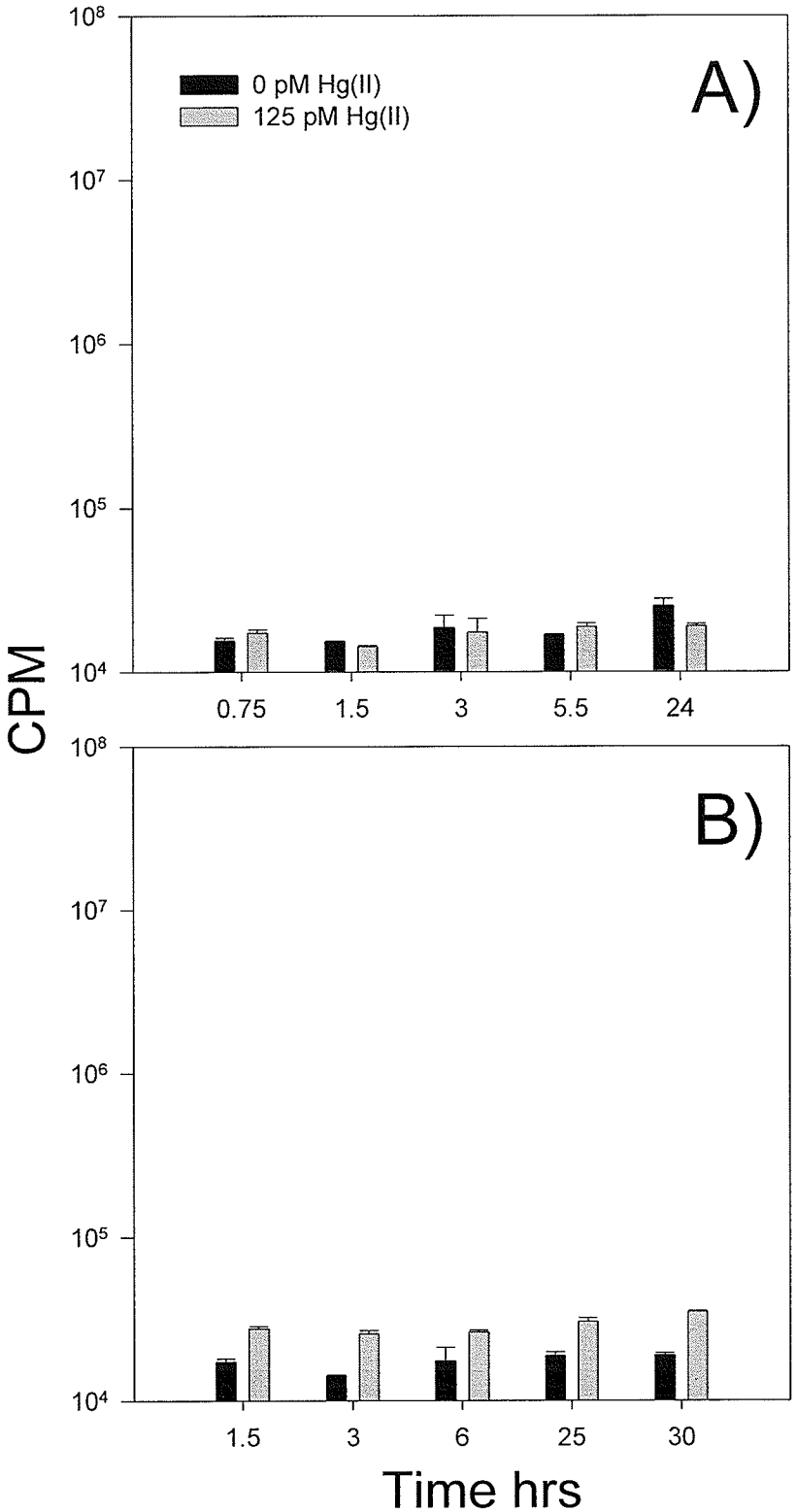

Figure 3.6. Lack of Hg(II)-induction in (A) *V. anguillarum* (pRB28) and (B) *E. coli* HMS174 (pRB28) under anaerobic conditions in GMM. Error bars represent the standard deviation of two independent assays.

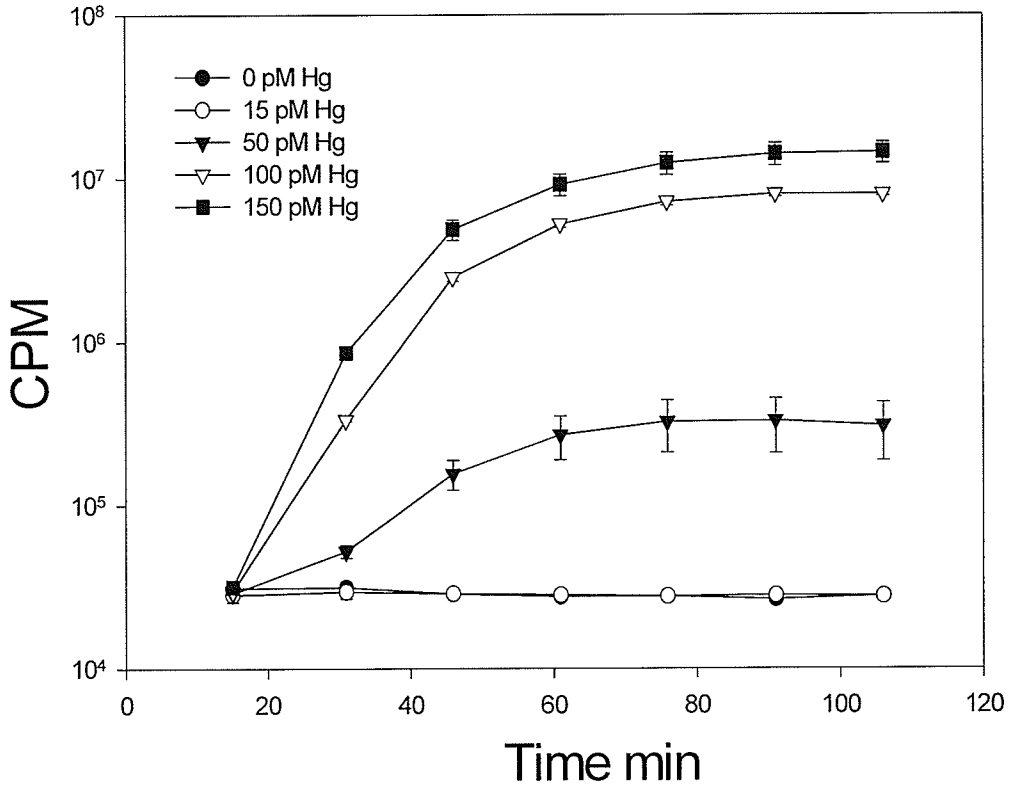

Figure 3.7. Hg(II)-induction of *mer-lux* in *V. anguillarum* (pRB28) under aerobic conditions in GMM. Error bars represent the standard deviation of duplicate samples.

production. This same fraction of bioavailable Hg(II) should also be present in the anaerobic assays. From these results, and the results obtained using the constitutive assays, it is unlikely that the lack of induction under anaerobic conditions is a result of the inability to synthesize protein.

3.3.5. Effect of organics on Hg(II) uptake Since the assay medium is quite limiting in nutrients, yeast extract was added to the samples. Anaerobic assays for *V. anguillarum* (pRB28) (Figure 3.8A) and *E. coli* HMS174 (pRB28) (Figure 3.8B) supplemented with 0.1% yeast extract resulted in induction. As with the aerobic assays performed in the defined medium (Figure 3.1A and B), light production was shown to increase with increasing Hg(II) concentration. These assays were the first to demonstrate the detection of a heavy metal under anaerobic conditions using a bioreporter system. However, the components in yeast extract are complex, which might disrupt the speciation of Hg(II), and hence bioavailability of Hg(II), in environmental samples. Therefore, a reagent was needed that was not as complex as yeast extract, but would still enable an anaerobic response to trace concentrations of Hg(II).

An aerobic assay was performed with varying concentrations of casamino acids and 50 pM Hg(II) in *V. anguillarum* (pRB28) (Figure 3.9A). The addition of 0.0005 % casamino acids did not result in a substantial difference in light production. However, the addition of 0.001 to 0.01 % casamino acids did inhibit the Hg(II)-response significantly, and the addition of 0.1% casamino acids resulted in no light production aerobically. From this experiment it appears that casamino acids decrease

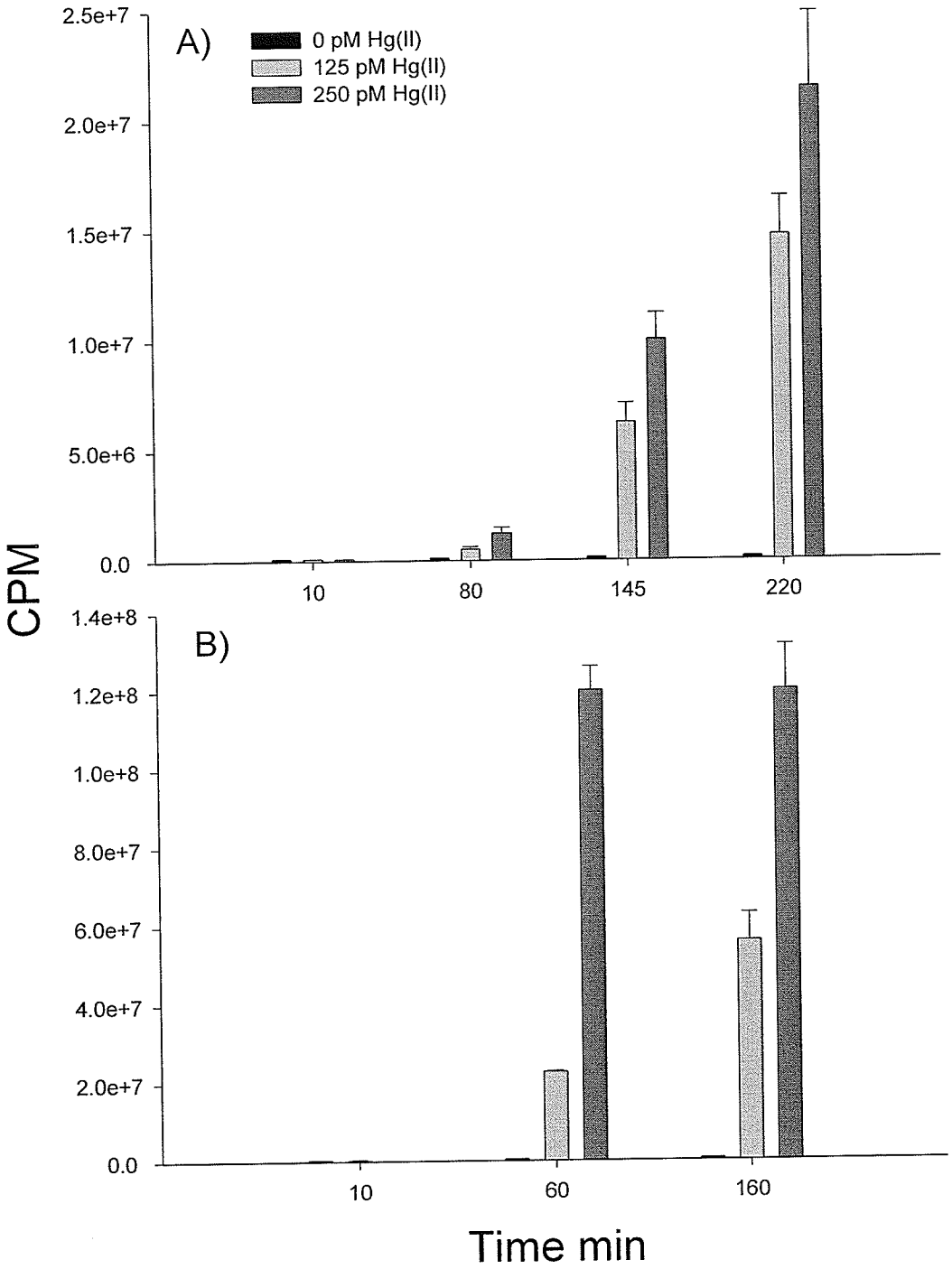

Figure 3.8. Hg(II)-induction of *mer-lux* in (A) *V. anguillarum* (pRB28) and (B) *E. coli* HMS174 (pRB28) under anaerobic conditions in defined assay media supplemented with yeast extract. The assay medium consisted of 5 mM Glucose, 67 mM Phosphate buffer, 9 mM $(NH_4)_2SO_4$, 2 mM NaCl, and 0.1% yeast extract. Error bars represent the standard deviation of duplicate samples.

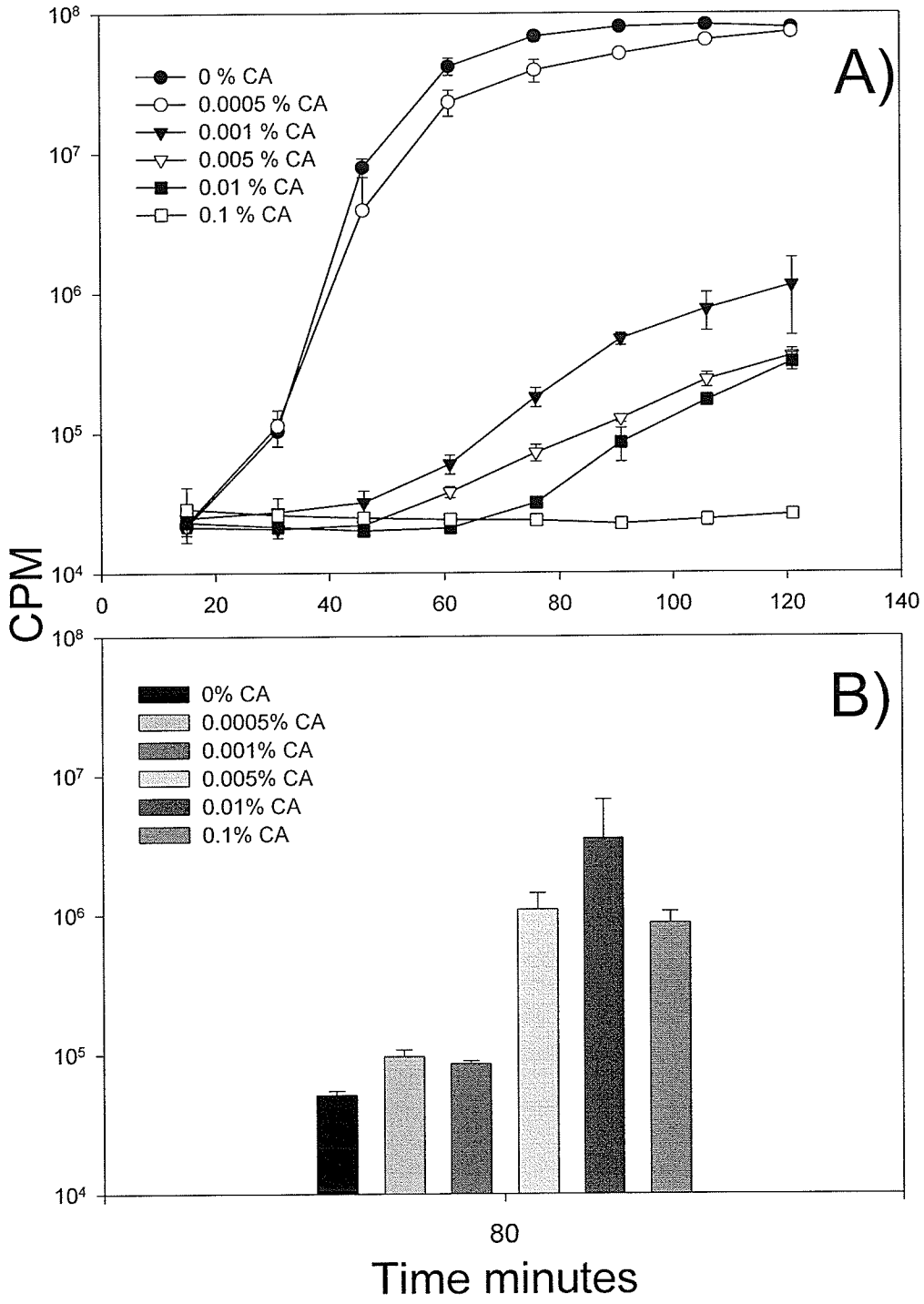

Figure 3.9. Effect of varying concentrations of casamino acids (CA) on Hg(II)-induction of *mer-lux* in *V. anguillarum* (pRB28) under (A) aerobic and (B) anaerobic conditions. The assay medium consisted of 5 mM Glucose, 67 mM Phosphate buffer, 9 mM $(NH_4)_2SO_4$, 2 mM NaCl, and varying concentration of casamino acids. For both aerobic and anaerobic assays the samples contained 50 pM Hg(II). Error bars represent the standard deviation of two independent assays.

the uptake of Hg(II). Under anaerobic conditions however, an opposite trend was observed (Figure 3.9B). The addition of 0 to 0.001% casamino acids and 50 pM Hg(II) did not induce *mer-lux*. Induction of *mer-lux* became evident when 0.005 and 0.01% of casamino acids were added to the samples. Light production was also shown to increase as the concentration of casamino acids increased from 0.005 to 0.01%. The addition of 0.1 % casamino acids, unlike the aerobic assay (Figure 3.9A), did result in light production, but lower than what was obtained for 0.005 and 0.01 %. Therefore, it appears that under anaerobic conditions casamino acids enhanced Hg(II) uptake. However, the addition of casamino acids appears to sequester Hg(II) aerobically, rendering it unavailable for uptake or for MerR. Under anaerobic conditions at a set concentration of casamino acids (0.005%) the bioreporter was responsive to varying concentration of Hg(II) (Figure 3.10). Casamino acids were then simplified by assaying *V. anguillarum* (pRB28) under anaerobic conditions supplemented with various amino acids.

Ten different amino acids were initially chosen and supplemented in the anaerobic defined assay medium (Figure 3.11). Of these ten, the addition of Hg(II) to samples containing glycine did not result in any increase in light production over background in *V. anguillarum* (pRB28). However, the other nine amino acids tested enhanced the Hg(II)-dependent light response in assay medium containing 125 pM Hg(II). The amount of light was dependent on which amino acid was supplemented, but due to the variability of the assays from day to day a direct comparison among the nine amino acids could not be accurately made.

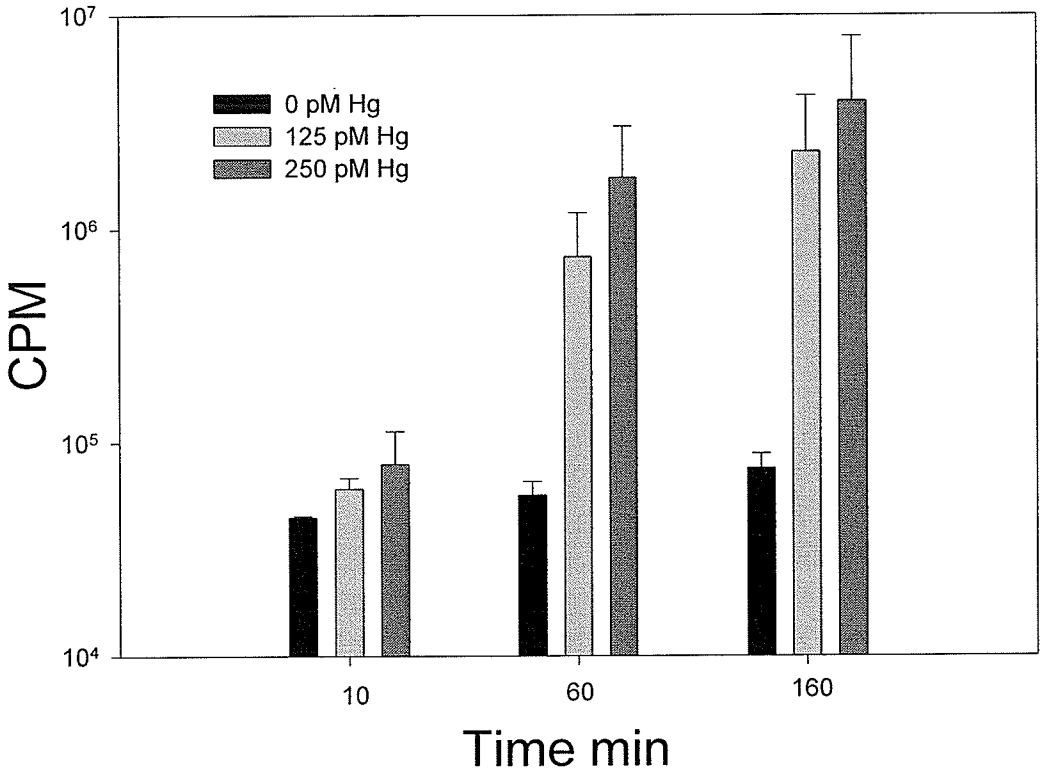

Figure 3.10. Hg(II)-induction of *mer-lux* in *V. anguillarum* (pRB28) under anaerobic conditions in defined assay media supplemented with varying concentrations of Hg(II) and 0.05% casamino acids. The assay medium consisted of 5 mM Glucose, 67 mM Phosphate buffer, 9 mM $(NH_4)_2SO_4$, 2 mM NaCl, and 0.05% casamino acids. Error bars represent the standard deviation of two independent assays.

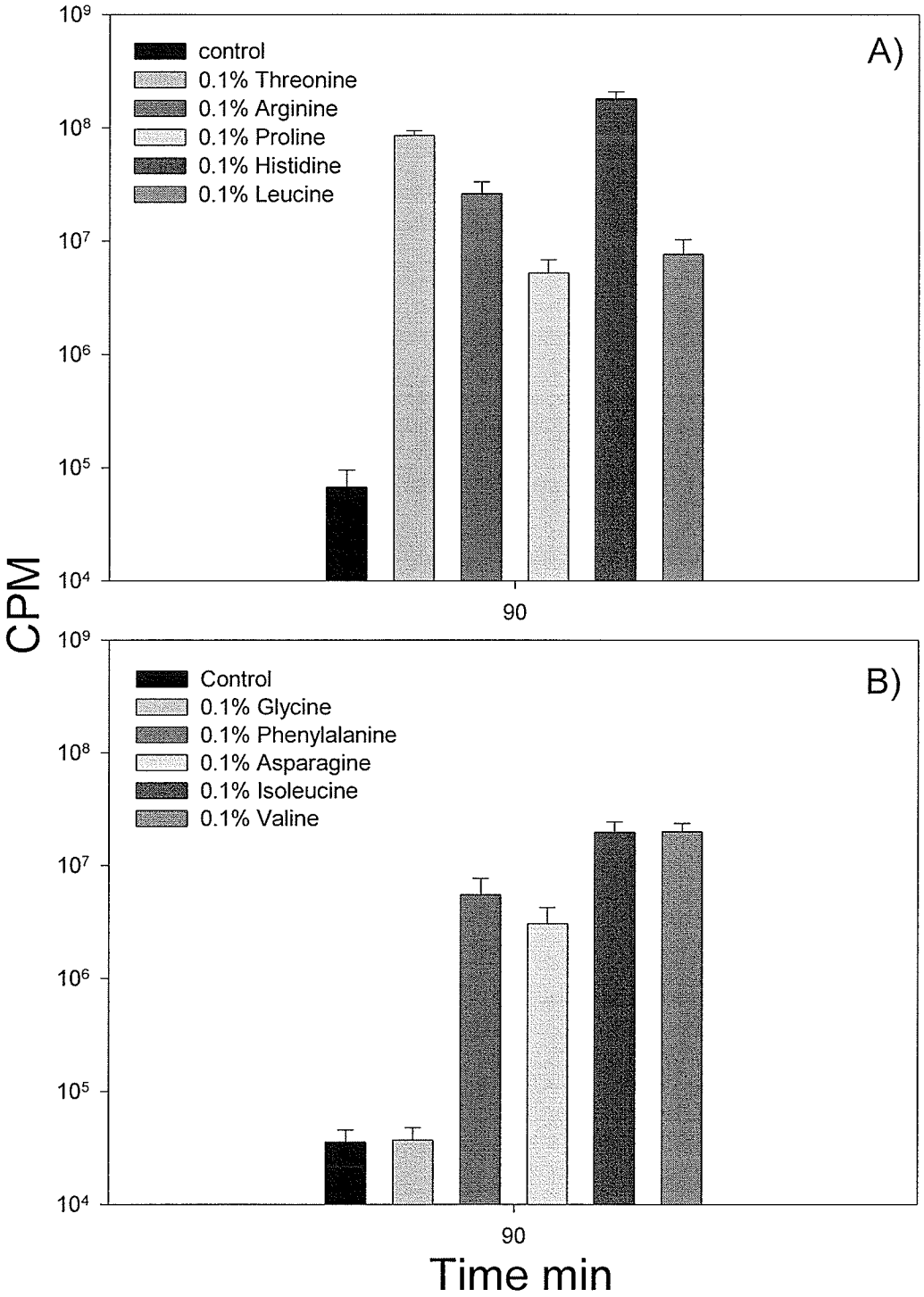

Figure 3.11. Effect of the addition of 0.1 % of various amino acids on the induction of *mer-lux* in *V. anguillarum* (pRB28) under anaerobic conditions. The assay medium consisted of 5 mM Glucose, 67 mM Phosphate buffer, 9 mM $(NH_4)_2SO_4$, and 2 mM NaCl. Concentration of Hg(II) in the spiked samples was 125 pM. Error bars represent the standard deviation of 2 independent assays (n=4).

Further experiments were performed using histidine, since it appeared to enhance Hg(II) uptake most significantly. An aerobic assay was performed for *V. anguillarum* using varying concentrations of histidine to determine if the addition of histidine to the assay medium affected the bioavailability of 50 pM Hg(II) (Figure 3.12A). With the exception of 5 mM of histidine, it appears that there is slight increase in the bioavailability of Hg(II) as a result of increasing histidine concentration. Anaerobically, increasing concentration of histidine largely enhanced the bioavailability of the Hg(II) (Figure 3.12B). Histidine concentrations > 100 μM was required to induce *mer-lux* in samples containing 50 pM of Hg(II). Increasing histidine concentrations, from 500 μM to 5 mM, resulted in a significant increase in light production.

Light production from *V. anguillarum* (pRB27) was measured in varying histidine concentration to ensure that the differences in light production from the *mer-lux* assays (Figure 3.12) were Hg(II) induced and not physiologically related. The addition of histidine, from 0 to 1 mM, under aerobic conditions did not appear to effect light production (Figure 3.13A). However, the addition of 5 mM of histidine did decrease light production, most likely due to some toxic effect at this concentration. Therefore, the lowered response to Hg(II) in the sample containing 5 mM of histidine (Figure 3.12A) might be the result of a toxic decrease in light production as opposed to the bioavailability of the Hg(II). Under anaerobic conditions there were no significant differences in light production in *V. anguillarum* (pRB27) in the samples containing 0 to 5 mM of histidine (Figure 3.13B). Therefore,

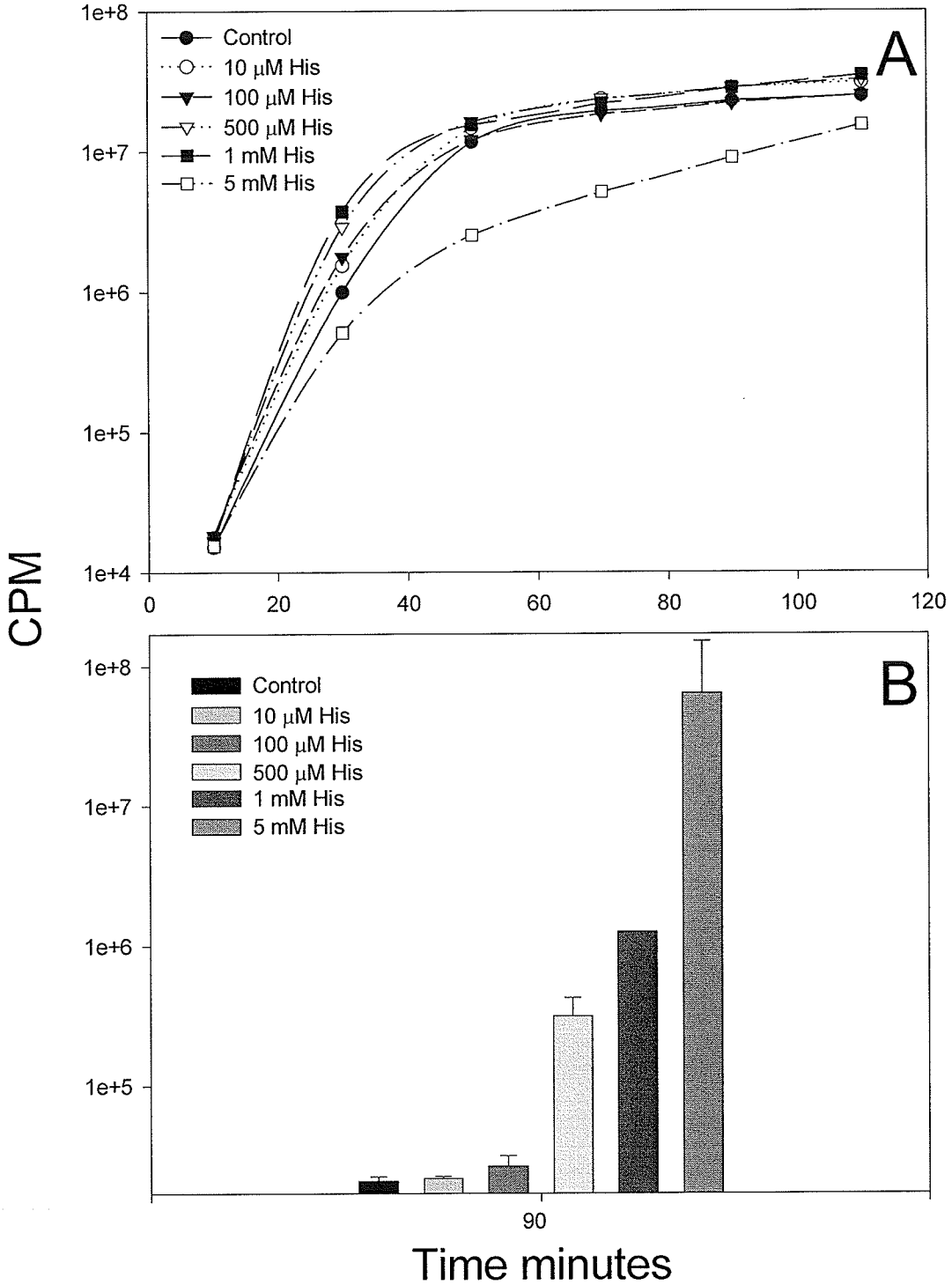

Figure 3.12. Effect of varying concentrations of histidine on the induction of *mer-lux* in *V. anguillarum* (pRB28) under (A) aerobic and (B) anaerobic conditions. The assay medium consisted of 5 mM Glucose, 67 mM Phosphate buffer, 9 mM $(NH_4)_2SO_4$, and 2 mM NaCl. All samples contained 50 pM Hg(II). Error bars represent the standard deviation of duplicate samples.

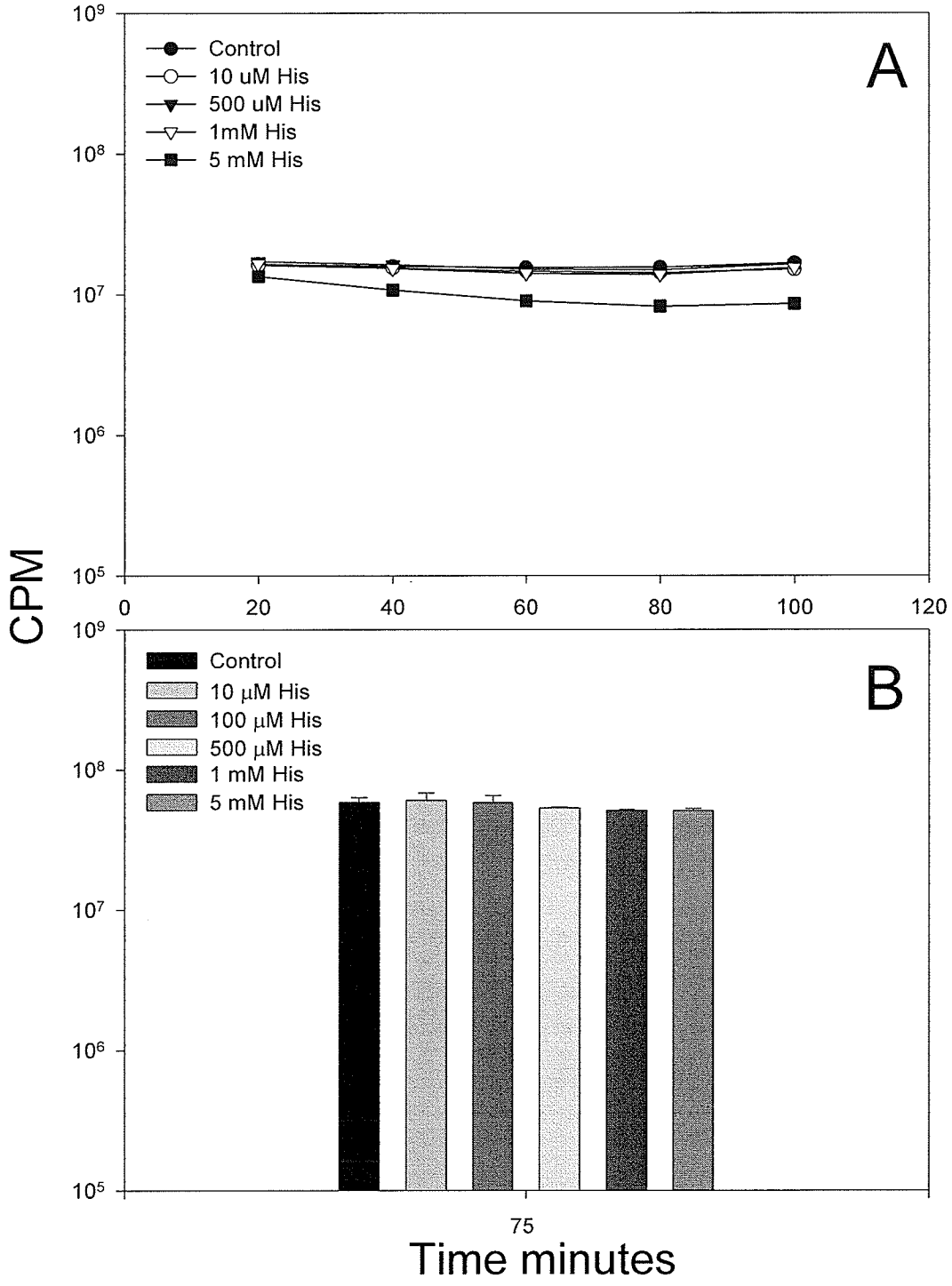

Figure 3.13. Effect of varying concentrations of histidine on light production in *V. anguillarum* (pRB27) under (A) aerobic and (B) anaerobic conditions. The assay medium consisted of 5 mM Glucose, 67 mM Phosphate buffer, 9 mM $(NH_4)_2SO_4$, and 2 mM NaCl. Error bars represent the standard deviation of duplicate samples.

the increase in light production with increasing histidine concentrations in the anaerobic *mer-lux* assays appears to be Hg(II)-induced and not related to increased light capability of the cells.

Under anaerobic conditions, in assay media supplemented with 1 mM histidine, the addition of increasing Hg(II) concentrations resulted in an increase in light emission in *V. anguillarum* (pRB28) (Figure 3.14A) and *E. coli* HMS174 (pRB28) (Figure 3.14B). As with the aerobic assays, light production was proportional to increasing trace concentrations of Hg(II) up to 25 pM and was capable of detecting concentrations of Hg(II) as low as 2.5 pM (Figure 3.15).

An anaerobic assay for *V. anguillarum* (pRB28) was performed to determine if the addition of 1 mM of D-histidine or 1 mM of imidazole would also affect the bioavailability of Hg(II) (Figure 3.16A). The addition of either D- or L- histidine enhanced Hg(II)-induction of *mer-lux*. Although the response to 50 pM of Hg(II) was similar, it was L-histidine that resulted in the larger response. The addition of Hg(II) to samples containing imidazole however, did not induce *mer-lux*. The addition of D-histidine, imidazole, or L-histidine did not significantly affect light production in the anaerobic constitutive *lux* assay (Figure 3.16B), suggesting that enhanced light production in the *mer-lux* assays (Figure 3.16A) was a Hg(II) induced response.

The effect that cysteine had on Hg(II) uptake in *E. coli* HMS174 was also investigated. *E. coli* HMS174 (pRB27) was first assayed as a control to determine if varying concentrations of cysteine affected the light producing capability of the cell. Under aerobic conditions cysteine concentrations greater than 0.01 mM appeared somewhat toxic, as indicated by the decrease in light production in *E. coli* HMS174

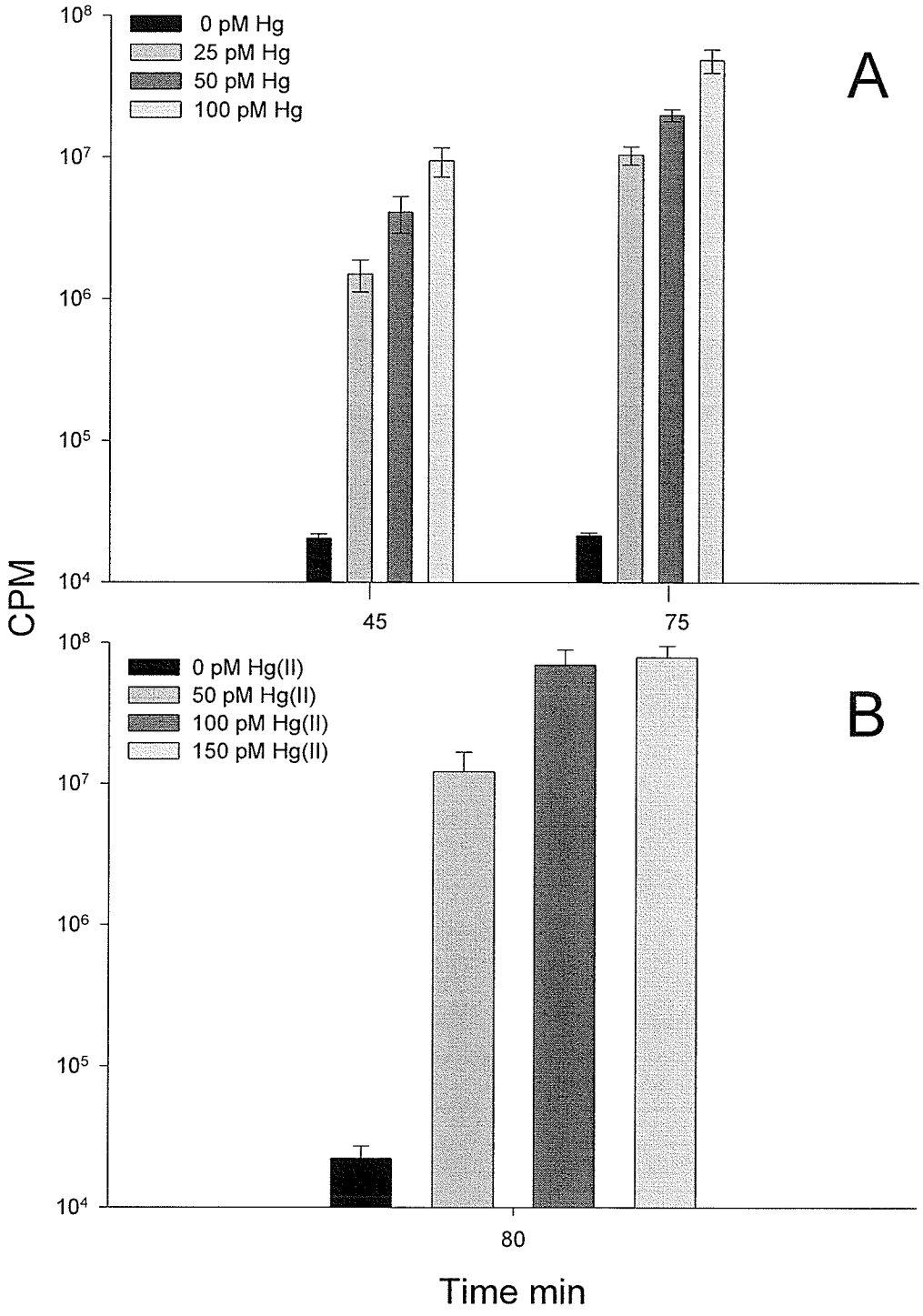

Figure 3.14. Anaerobic induction of *mer-lux* with varying concentrations of Hg(II) in defined assay media + 1 mM histidine in (A) *V. anguillarum* (pRB28) and (B) *E. coli* HMS174. (A) The assay medium consisted of 5 mM glucose, 67 mM phosphate buffer, 9 mM $(NH_4)_2SO_4$, 2 mM NaCl, and 1mM histidine. Error bars represent the standard deviation of two independent assays.

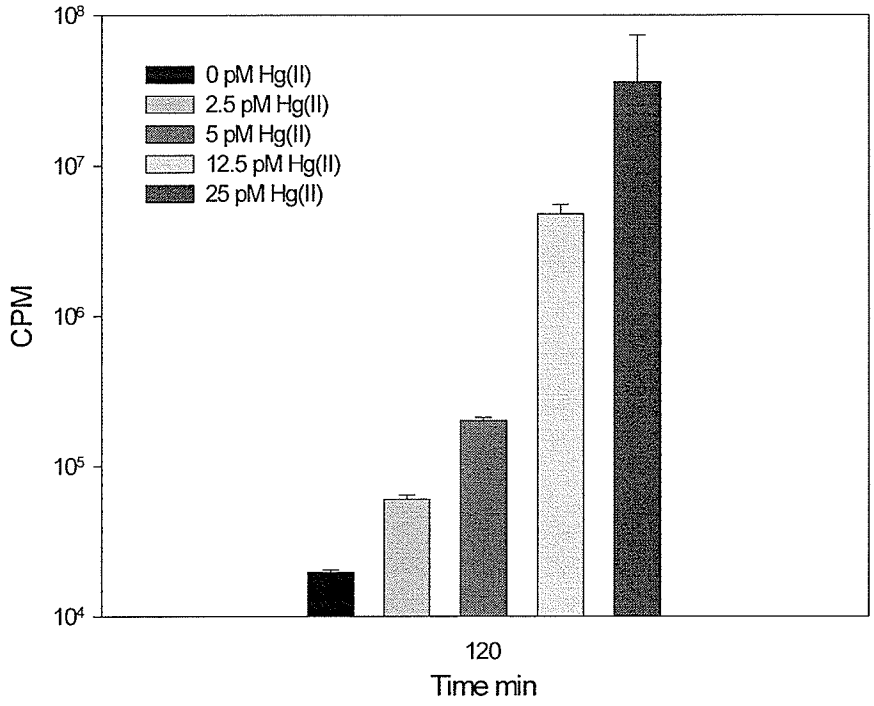

Figure 3.15. Induction of *mer-lux* with low levels of Hg supplemented with 1 mM histidine in defined assay medium for *V. anguillarum* (pRB28) under anaerobic conditions. The assay medium consisted of 5 mM Glucose, 3 mM phosphate buffer, and 9 mM $(NH_4)_2SO_4$. Error bars represent the standard deviation of duplicate samples.

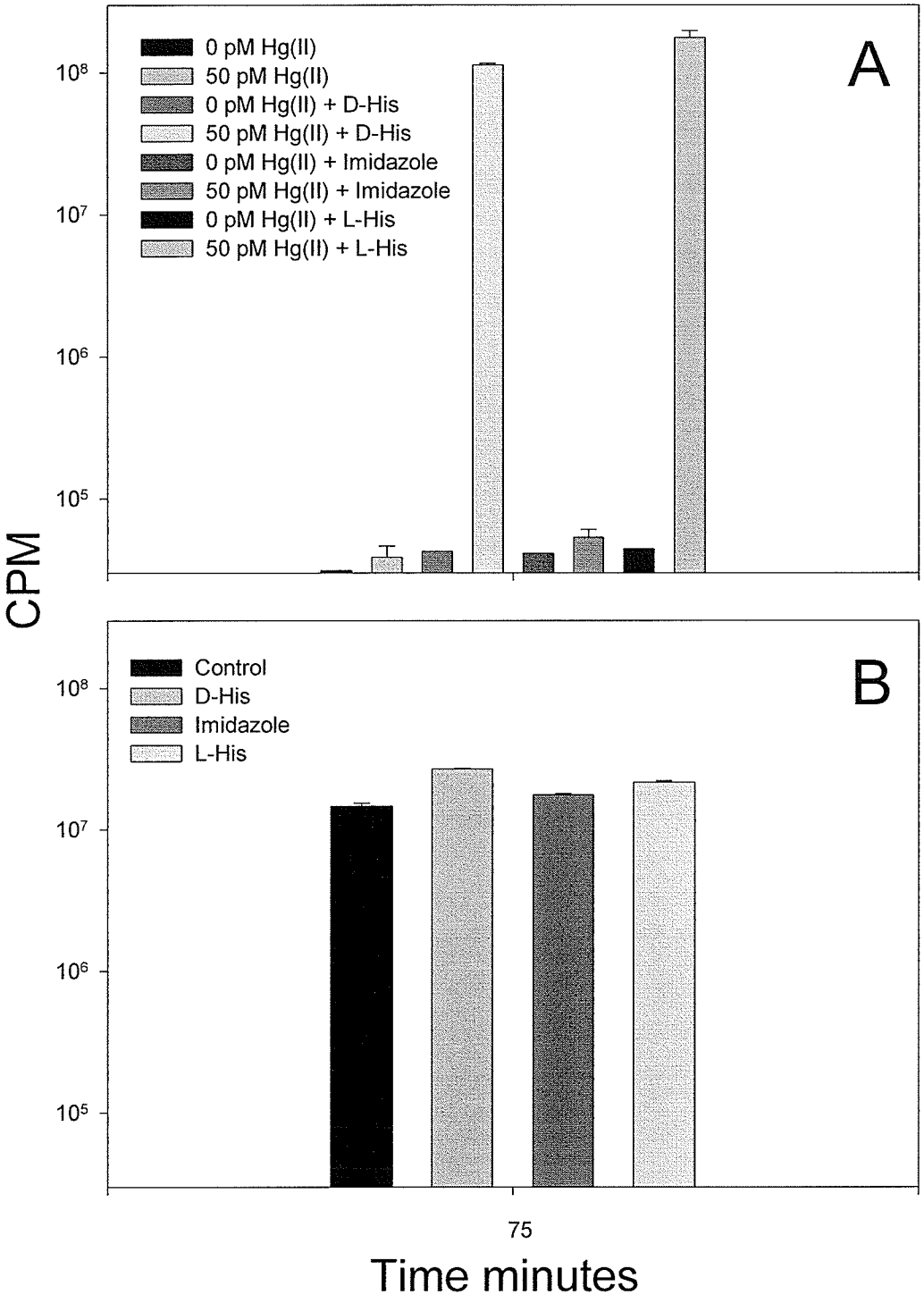

Figure 3.16. Effect of supplementing the assay medium with either 1mM of D-histidine, imidazole, or L-histidine on (A) the induction of *mer-lux* and (B) constitutive light production under anaerobic conditions in *V. anguillarum*. The assay medium consisted of 5 mM Glucose, 3 mM Phosphate buffer, and 9 mM $(NH_4)_2SO_4$. Error bars represent the standard deviation of duplicate samples.

(pRB27) (Figure 3.17A). Under anaerobic conditions, similar levels of light emission was observed in samples containing cysteine concentrations up to 0.01 mM, which decreased slightly at higher concentrations (Figure 3.17B).

Under aerobic conditions, there was a slight reduction in the Hg(II)-dependent light response from *E. coli* HMS174 (pRB28) in samples containing 0.001 mM cysteine, which was completely eliminated in samples containing higher concentrations (Figure 3.18A). However, these higher concentrations also affected the light producing capability of the cell and are therefore uninterruptible. Lower concentrations of cysteine, ranging from 1×10^{-5} - 1×10^{-9} mM, did not significantly affect the bioavailability of 25 pM Hg(II) under aerobic conditions, as light production from *E. coli* HMS174 (pRB28) was similar in comparison to samples not containing cysteine (Figure 3.18B). Under anaerobic conditions, cysteine concentrations up to 1 mM did not enable a Hg(II)-dependent light response in samples containing 25 pM Hg(II) (Figure 3.19A and B).

Six carboxylic acids were initially supplemented into the assay medium for the *mer-lux* anaerobic assays in *V. anguillarum* (pRB28) (Figure 3.20A). The addition of 1 mM malate and fumarate enhanced the bioreporter response in samples containing 50 pM of Hg(II) most significantly. The addition of acetate resulted in < 3-fold increase in light production and the addition of succinate did not enhance the response above background. The additions of organic compounds did not cause substantial changes in light emission in *V. anguillarum* (pRB27) (Figure 3.20B). Therefore, the enhanced light production from the *mer-lux* assays in the presence of malate and fumarate (Figure 3.20A) appear to be Hg(II)-induced, and not related to

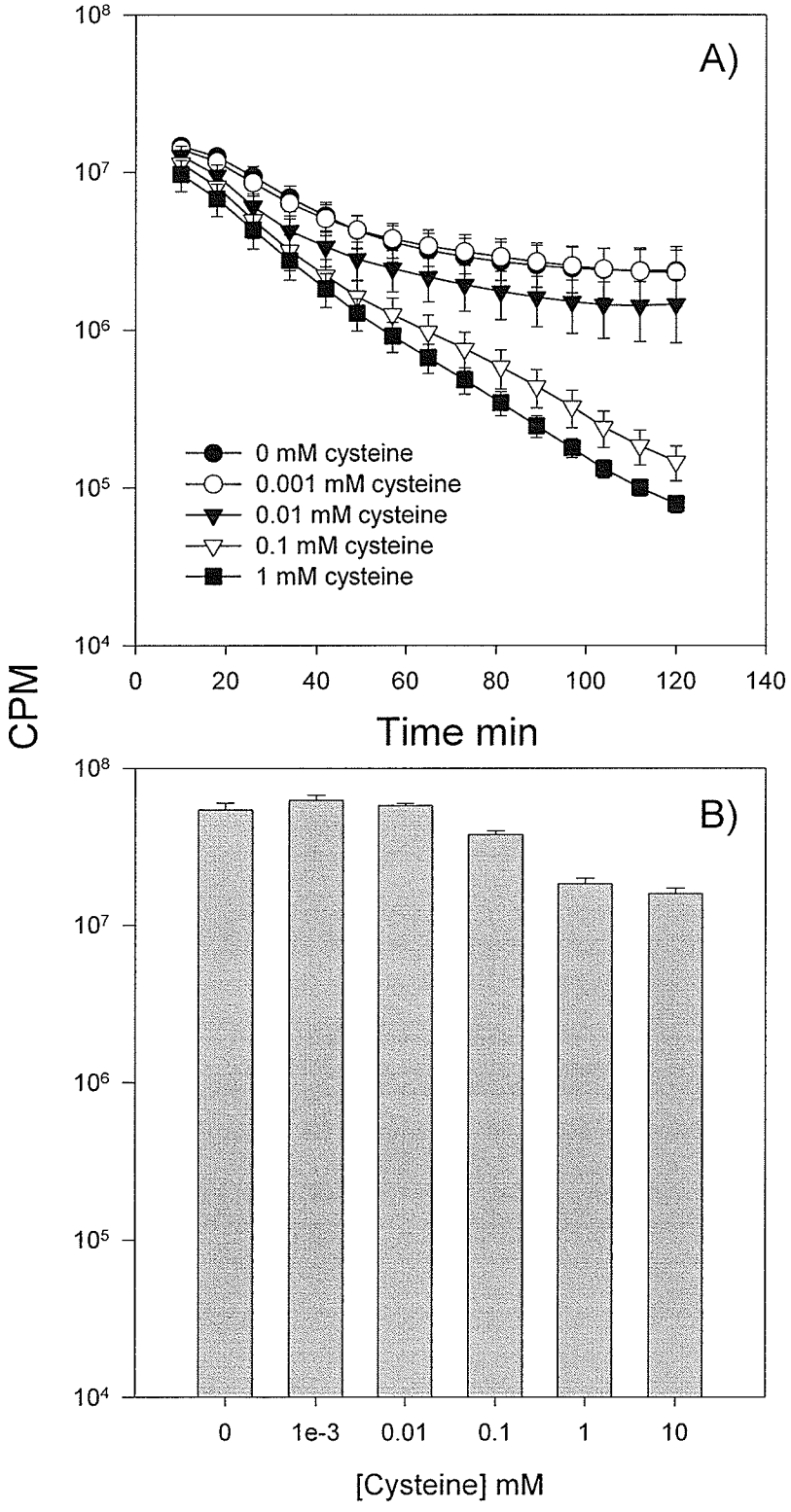

Figure 3.17. Effect of varying the concentrations of cysteine on constitutive light production in *E. coli* HMS174 (pRB27) under A) aerobic and B) anaerobic conditions. Error bars represent the standard deviation of two independent assays (n=4).

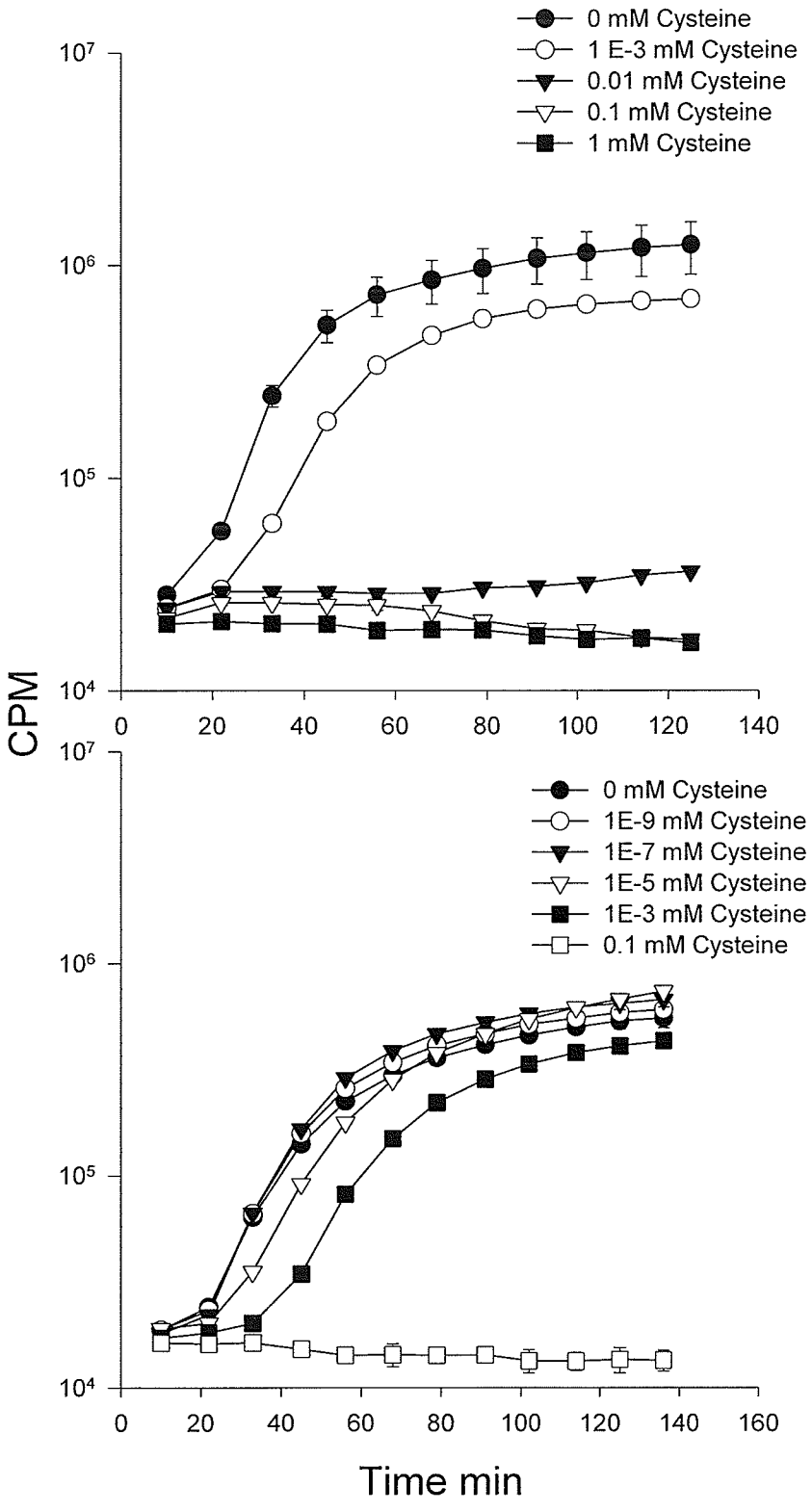

Figure 3.18. Effect of varying the concentrations of cysteine on Hg(II)-induction in *E. coli* HMS174 (pRB28) under aerobic conditions in assay media containing 25 pM Hg(II). Error bars represent the standard deviation of two independent assays (n=4).

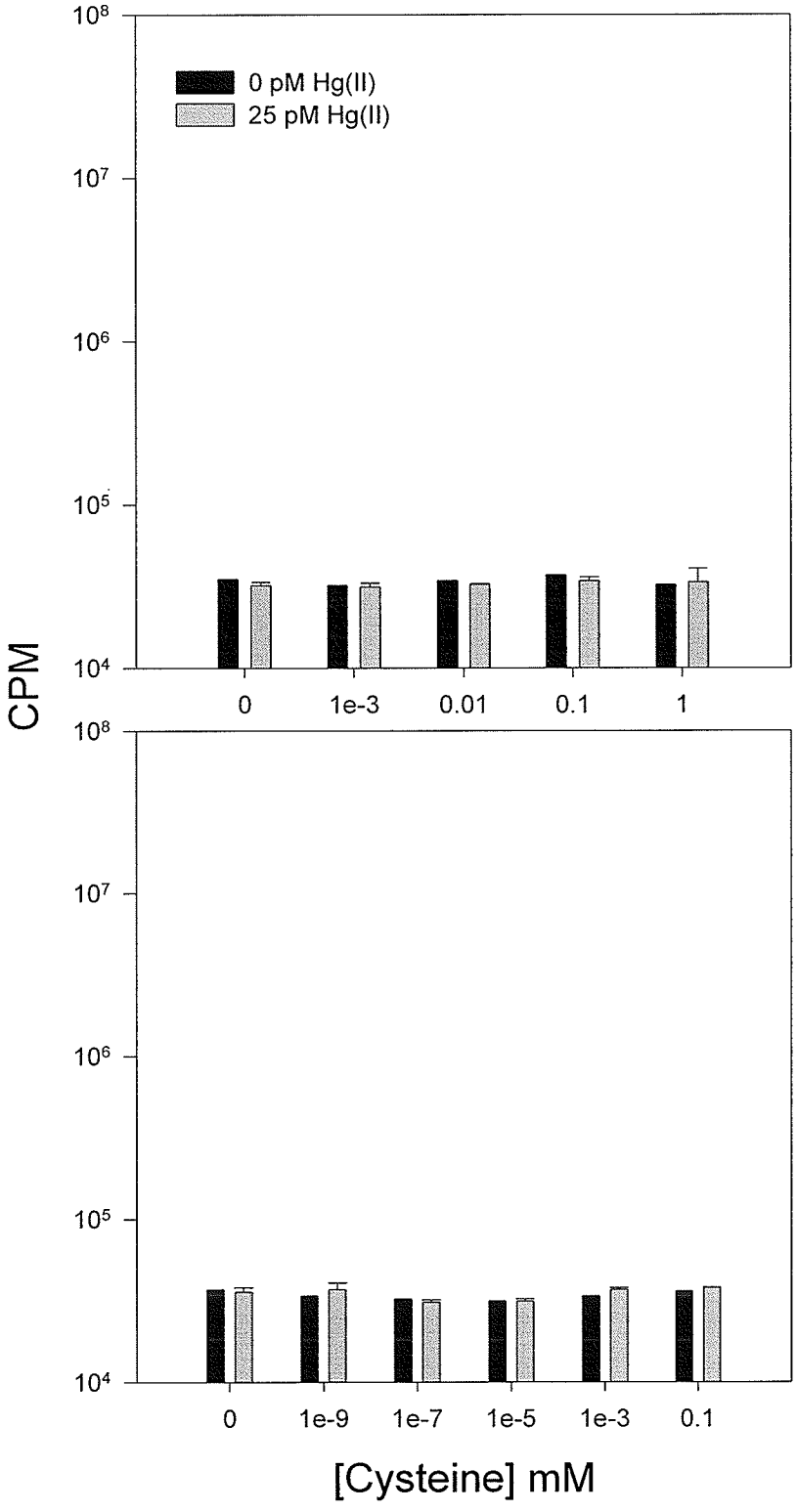

Figure 3.19. Lack of Hg(II)-induction in *E. coli* HMS174 (pRB28) under anaerobic conditions in assay media containing varying concentrations of cysteine and 25 pM Hg(II). Error bars represent the standard deviation of two independent assays (n=4).

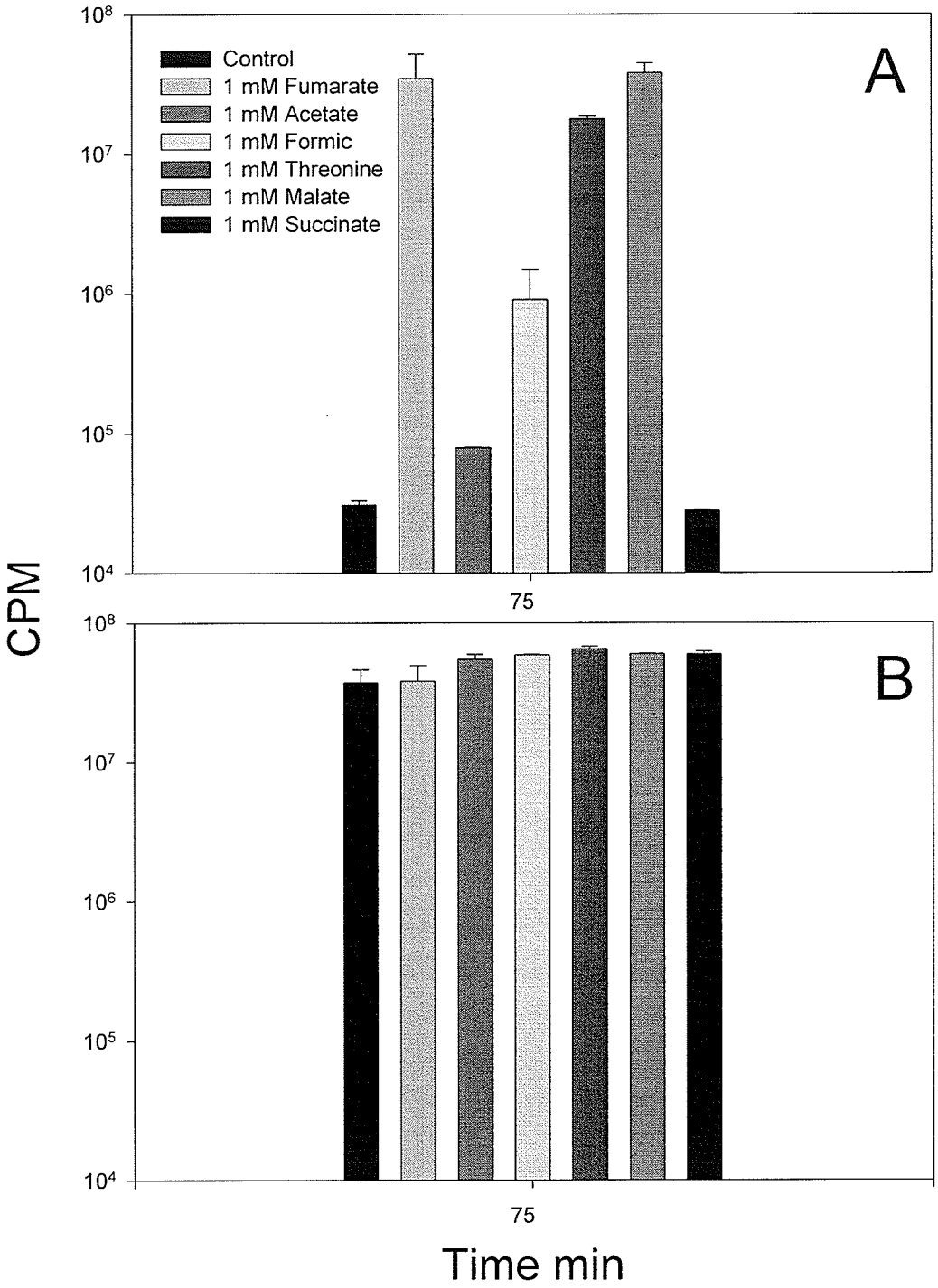

Figure 3.20. Effect of the addition of various organic acids on the (A) induction of *mer-lux* in *V. anguillarum* (pRB28) and (B) light production in *V. anguillarum* (pRB27) under anaerobic conditions. The assay medium consisted of 5 mM Glucose, 3 mM Phosphate buffer, and 9 mM $(NH_4)_2SO_4$. For the Hg(II) inducible assays the concentration of Hg was 50 pM Hg(II) in all samples. Error bars represent the standard deviation of two independent assays (n=4).

increased light capability of the cell.

Malate was supplemented at various concentrations into aerobic (Figure 3.21A) and anaerobic (Figure 3.21B) assay media to determine its effect on the bioavailability of 50 pM of Hg(II) in *V. anguillarum* (pRB28). The addition of 0.1 mM malate to the aerobic assay did not appear to affect the bioavailability of Hg(II) as the kinetics of light production remained similar with respect to samples containing no supplement and 50 pM of Hg(II) (Figure 3.21A). In the anaerobic assay, the addition of Hg(II) to samples containing 0.1 mM malate resulted in no induction of *mer-lux* (Figure 3.21B). However, the addition of Hg(II) to samples containing 1mM malate in the aerobic assay (Figure 3.21A) resulted in an increased response and significantly enhanced the induction of *mer-lux* under anaerobic conditions (Figure 3.21B). In both the aerobic and the anaerobic assay the addition of Hg(II) to samples containing 2.5 mM of malate resulted in no induction of *mer-lux*. It was not determined however, if this higher concentration of malate was toxic to *V. anguillarum*.

Three additional organic acids were supplemented into defined assay medium to determine its effect on the bioavailability of 50 pM of Hg(II) in *V. anguillarum* (pRB28) (Figure 3.22A). Of the three organic acids tested under anaerobic conditions, only the addition of Hg(II) to samples containing α-ketoglutarate enhanced the induction of *mer-lux*. The addition of Hg(II) to samples containing either 1 mM of citrate or 1 mM of oxaloacetate did not result in the induction of *mer-lux*. The effects on light production, following the addition of these organic acids,

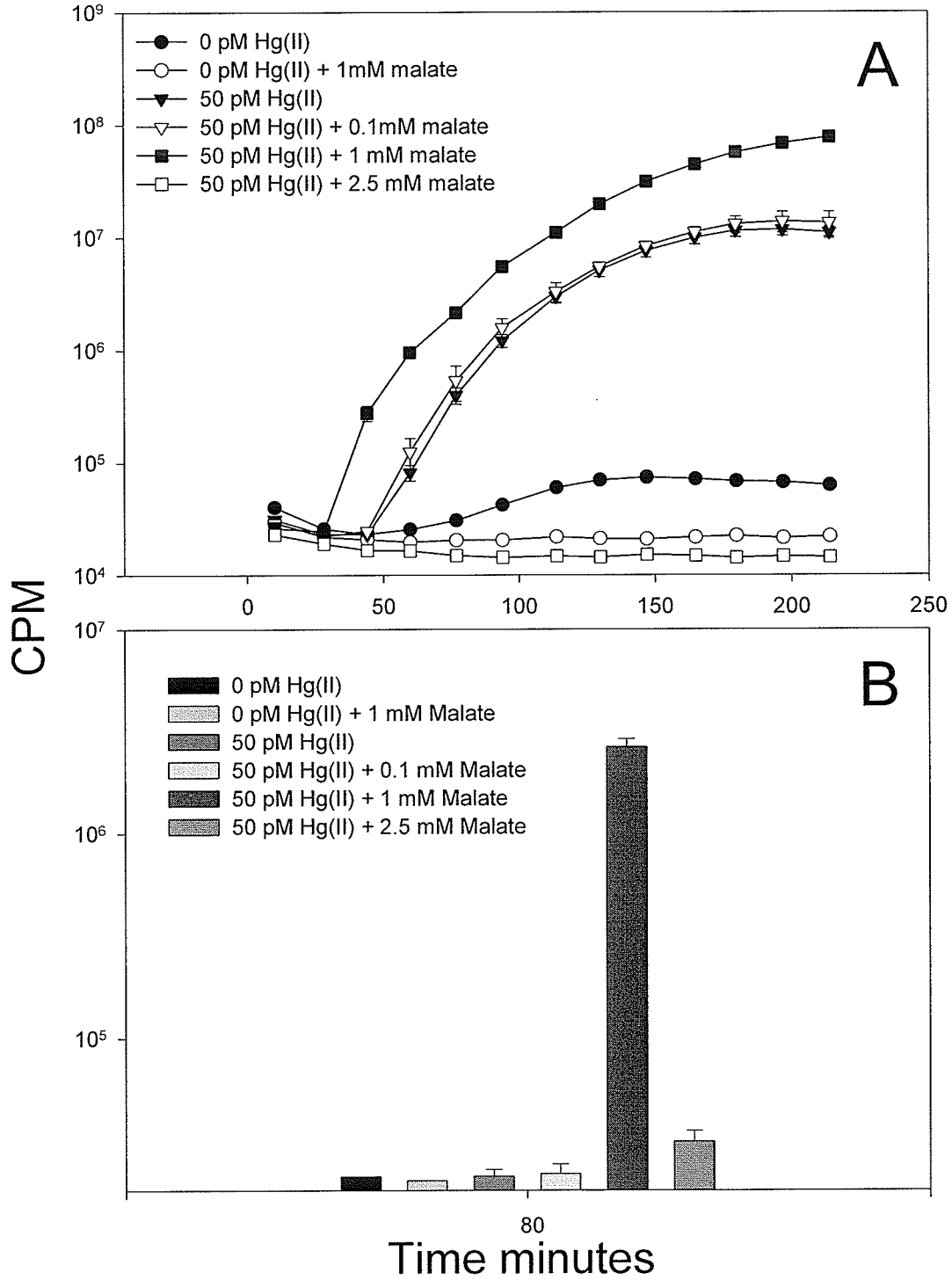

Figure 3.21. Effect of varying concentrations of malate in defined assay media on the induction of *mer-lux* in *V. anguillarum* (pRB28) under (A) aerobic and (B) anaerobic conditions. The assay medium consisted of 5 mM glucose, 3 mM phosphate buffer, and 9 mM $(NH_4)_2SO_4$. Error bars represent the standard deviation of duplicate samples.

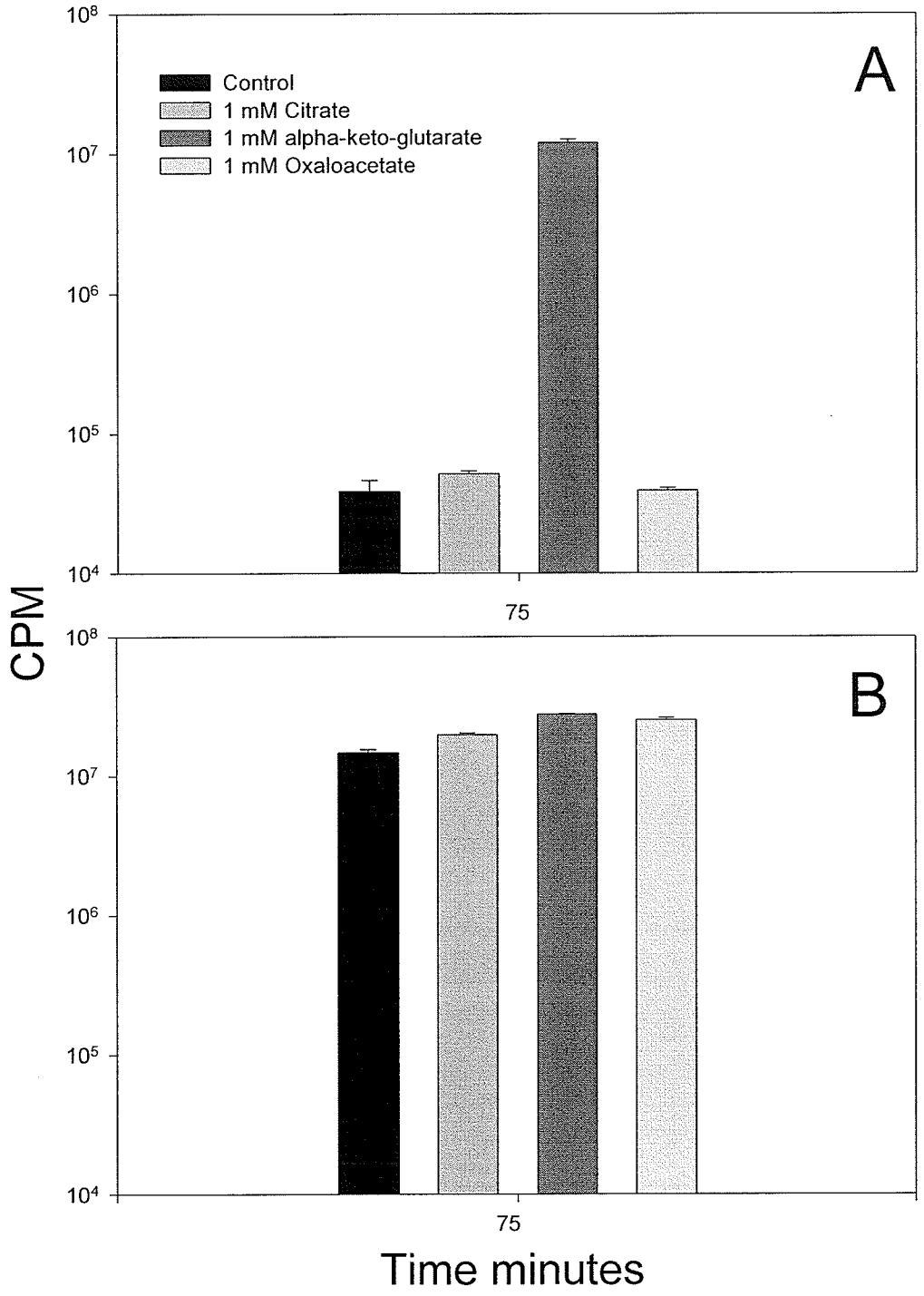

Figure 3.22. Effect of the addition of various organic acids on (A) the induction of *mer-lux* in *V. anguillarum* (pRB28) and (B) light production in *V. anguillarum* (pRB27) under anaerobic conditions. The assay medium consisted of 5 mM Glucose, 3 mM Phosphate buffer, 9 mM $(NH_4)_2SO_4$, and 50 pM Hg(II). Error bars represent the standard deviation of duplicate samples.

demonstrated that light production remains relatively the same in the presence or absence of the organic supplements (Figure 3.22B). Therefore, the enhanced light production in the *mer-lux* assays (Figure 3.22A) in the samples supplemented with α-ketoglutarate appears to be Hg(II) induced.

It should be noted that the addition of the carboxylic acids that enhanced the bioreporter response the most significantly also decreased the pH of the assay medium (Table 3.1). The role of pH in enhancing the bioavailability of Hg(II) is the main focus of chapter 6 and will be explored further in that chapter.

3.3.6. Effect of reducing agents on Hg(II) uptake At this point we had an anaerobic bioreporter that was capable of detecting trace concentrations of Hg(II) under non-reducing conditions. Therefore, in order to use this bioreporter to measure Hg(II) in anoxic environmental samples, at an E_h similar to sulfate reducing conditions, we needed to determine the effect that reducing conditions would have on the bioreporter. Ti-Citrate is a commonly used reducing agent, but citrate is also a known metal chelator and therefore, an aerobic assay in *V. anguillarum* was performed to determine the effect of citrate on the bioavailability of Hg(II) (Figure 3.23A). For this experiment citrate was added at varying concentrations, from 0 to 10 mM, in assay medium containing 50 pM of Hg(II). The addition of increasing concentrations of citrate only slightly decreases the bioavailability of Hg(II). However, light production from *V. anguillarum* (pRB27) was also slightly affected by the addition of increasing citrate concentrations (Figure 3.23B). Therefore, citrate did not appear to affect the bioavailability of Hg(II) under aerobic conditions.

Table 3.1. Hg(II)-induction in *V. anguillarum* (pRB28) in anaerobic defined assay media supplemented with varying carboxylic acids and 50 pM Hg(II).

Carboxylic acid (1mM)	Hg(II) induction [A]	pH of the assay medium
Fumarate	+	5.7
Acetate	-	6.6
Formic acid	-	6.5
Malate	+	5.8
Succinate	-	6.0
Citrate	-	7.0
α-Ketoglutarate	+	5.8
Oxaloacetate	-	6.5

[A] (+) indicates induction of greater than 100-fold over background

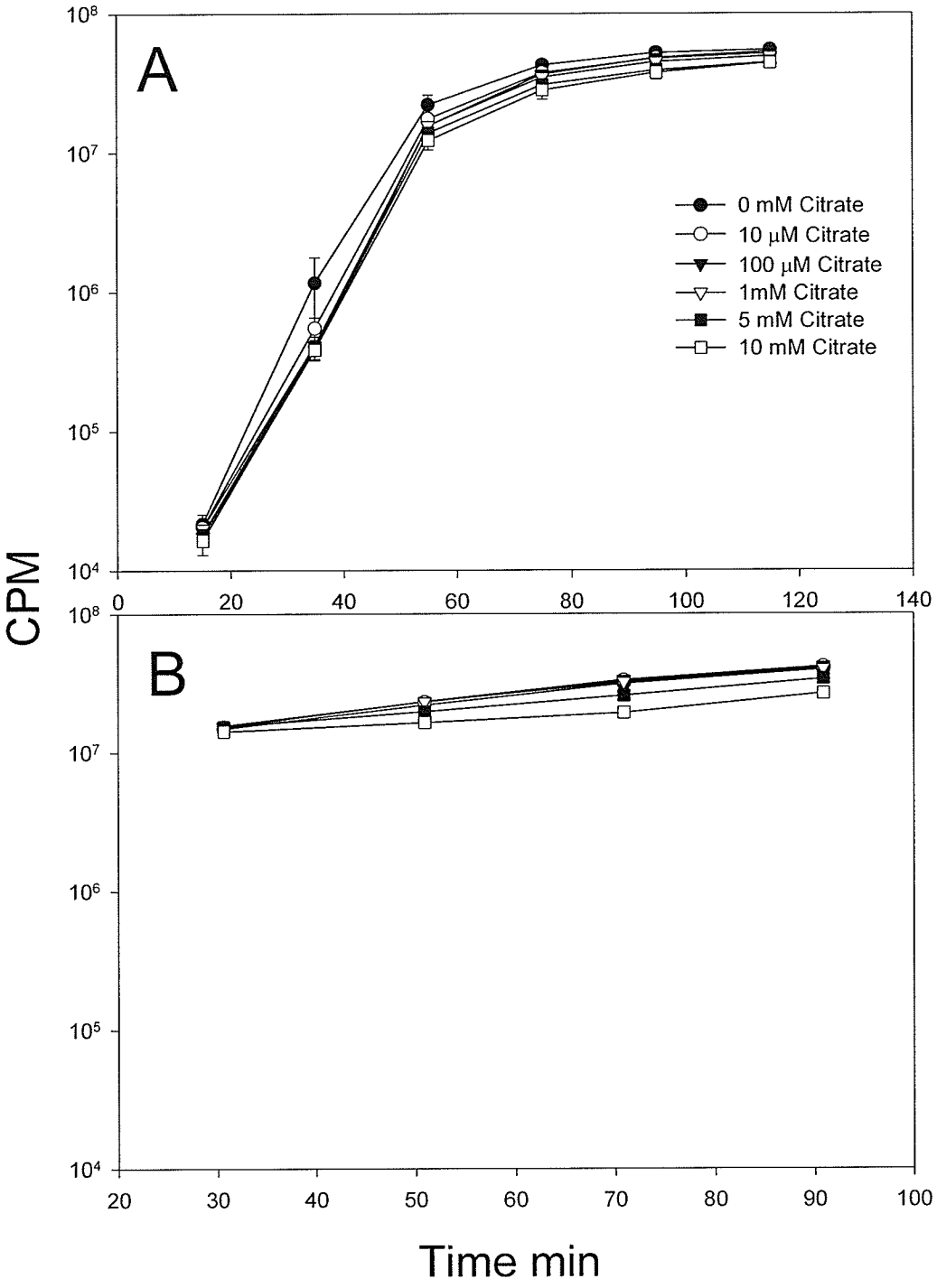

Figure 3.23. Effect of the addition of varying concentrations of citrate on (A) the induction of *mer-lux* in *V. anguillarum* (pRB28) and (B) light production in *V. anguillarum* (pRB27) under aerobic conditions. The assay medium consisted of 5 mM glucose, 3 mM phosphate buffer, and 9 mM $(NH_4)_2SO_4$. All samples in the Hg inducible assay contained 50 pM Hg(II). Error bars represent the standard deviation of duplicate samples.

Since citrate did not hinder the bioavailability of Hg(II), Ti-citrate was chosen as a reducing agent to determine if the bioreporter assay in *V. anguillarum* was applicable at low E_h. Ti-citrate was added to the anaerobic samples at varying concentrations (Figure 3.24A). Samples containing 75 pM of Hg(II) induced the bioreporter for all of the Ti-citrate concentrations added. However, as the concentration of Ti-citrate increased the Hg(II) induced response decreased. In contrast, light production appeared to be unaffected in *V. anguillarum* (pRB27) as the concentration of Ti-citrate was increased (Figure 3.24B). Therefore, the decreased response in the samples containing elevated concentrations of Ti-citrate appears to be due to a decrease in the uptake of Hg(II). It is unlikely that citrate is responsible since it was shown that citrate did not affect the bioavailability of Hg(II) (Figure 3.23A). However, the effects of lowering the E_h or the presence of any free titanium in the medium could not be ruled out as possible explanations for the apparent decrease in Hg(II) bioavailability.

An aerobic assay in *V. anguillarum* was performed to determine the effect of nitrilotriacetic acid (NTA) on the bioavailability of Hg(II) (Figure 3.25A). For this experiment Hg(II) was added to samples containing varying concentrations of NTA, from 0 to 1 mM. As the concentration of NTA increased there was a significant decrease in light production from *V. anguillarum* (pRB28). However, light production from *V. anguillarum* (pRB27) did not appear to be significantly affected from the addition of NTA (Figure 3.25B). Therefore, it appears that NTA affected the bioavailability of Hg(II) under aerobic conditions.

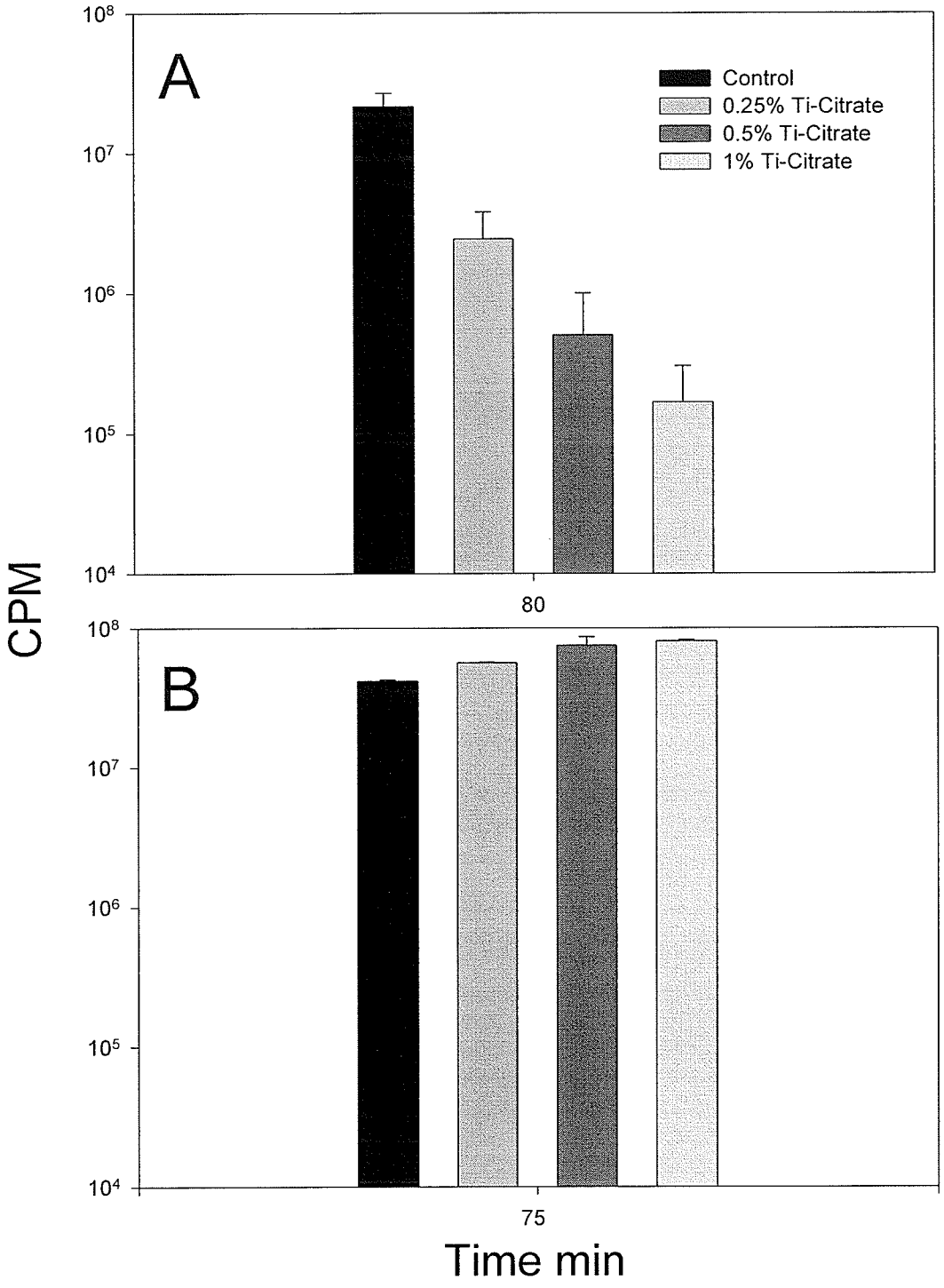

Figure 3.24. Effect of the addition of varying concentrations of Ti-citrate on (A) the induction of *mer-lux* in *V. anguillarum* (pRB28) and (B) light production in *V. anguillarum* (pRB27) under anaerobic conditions. The assay medium consisted of 125 pM Hg(II), 5 mM Glucose, 67 mM Phosphate buffer, 9 mM $(NH_4)_2SO_4$, 2 mM NaCl, and 1 mM histidine. Error bars represent the standard deviation of duplicate samples.

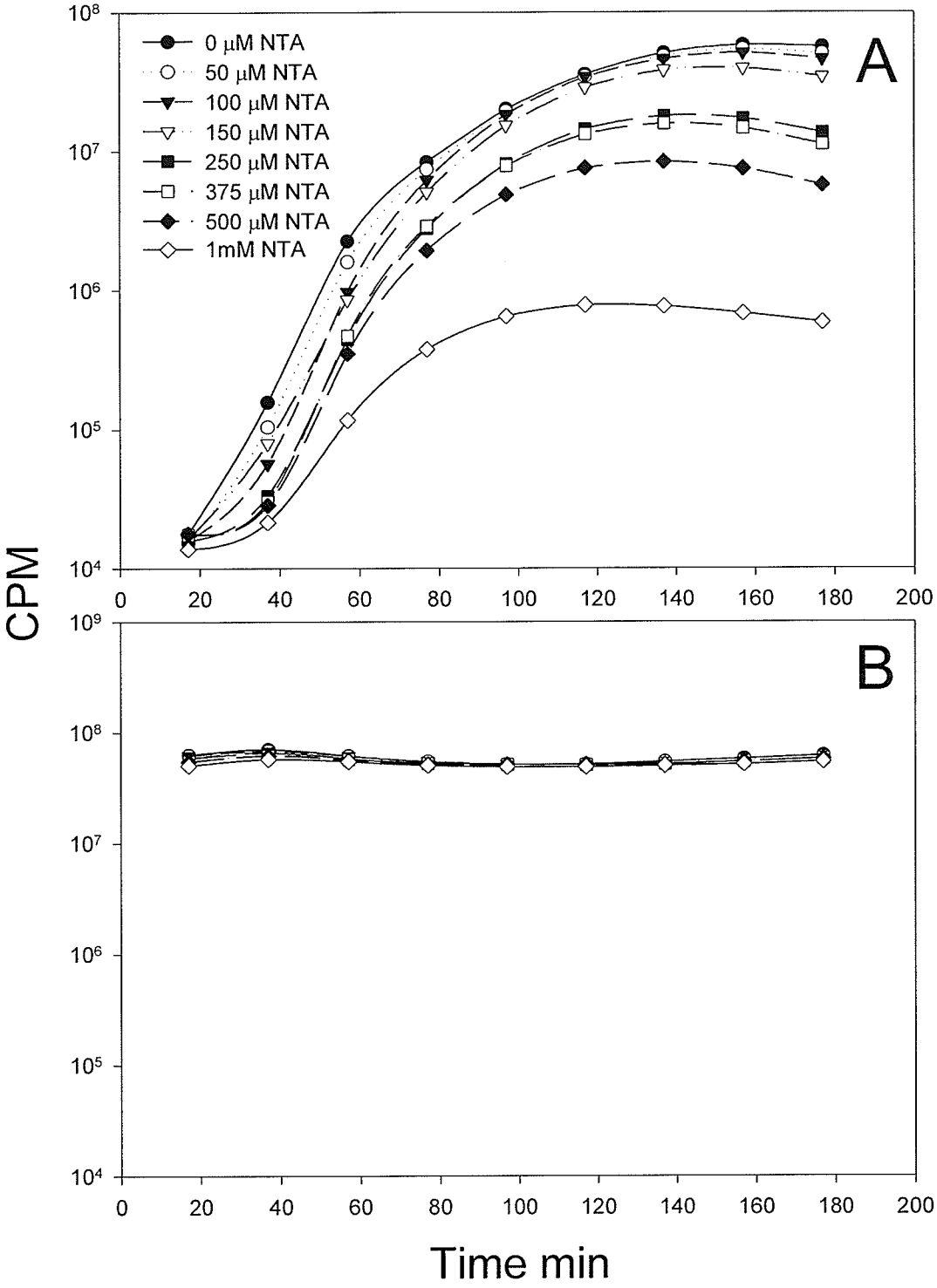

Figure 3.25. Effect of the addition of varying concentrations of NTA on A) the induction of *mer-lux* in *V. anguillarum* (pRB28) and B) light production in *V. anguillarum* (pRB27) under aerobic conditions. The assay medium consisted of 125 pM Hg(II), 5 mM Glucose, 67 mM phosphate buffer, 9 mM $(NH_4)_2SO_4$, and 2 mM NaCl.

Ti-NTA was added to the bioreporter assays in *V. anguillarum* to determine its effect on the bioavailability of Hg(II) under anaerobic conditions. Varying concentrations of Ti-NTA under anaerobic conditions resulted in a decrease in light production in *V. anguillarum* (pRB28) as the concentration of Ti-NTA was increased (Figure 3.26A). However, the addition of increasing concentrations of Ti-NTA did not appear to affect light production in *V. anguillarum* (pRB27) (Figure 3.26B). It could not be determined from this experiment if NTA, free titanium, or a lowering in E_h was responsible for the decrease in light production in the *mer-lux* assays (Figure 3.26A). However, *V. anguillarum* (pRB28) was capable of distinguishing varying concentrations of Hg(II) in the presence of Ti-NTA (Figure 3.27). As samples increased in Hg(II) concentration, from 0 to 150 pM, the induction of *mer-lux* increased. Therefore, as with Ti-citrate, *V. anguillarum* (pRB8) was capable of responding to Hg(II) in the presence of Ti-NTA.

One additional reducing agent was added to the *V. anguillarum* bioreporter assays. The addition of dithionite to the assay medium containing varying concentrations of Hg(II) resulted in the Hg(II) dependent induction of *mer-lux* (Figure 3.28). However, the response was relatively low and the bacteria took substantially longer to grow in the presence of this reducing agent in GMM. It is also possible that dithionite, which contains sulfhydryl groups, formed strong complexes with Hg(II) and should therefore be avoided in future studies.

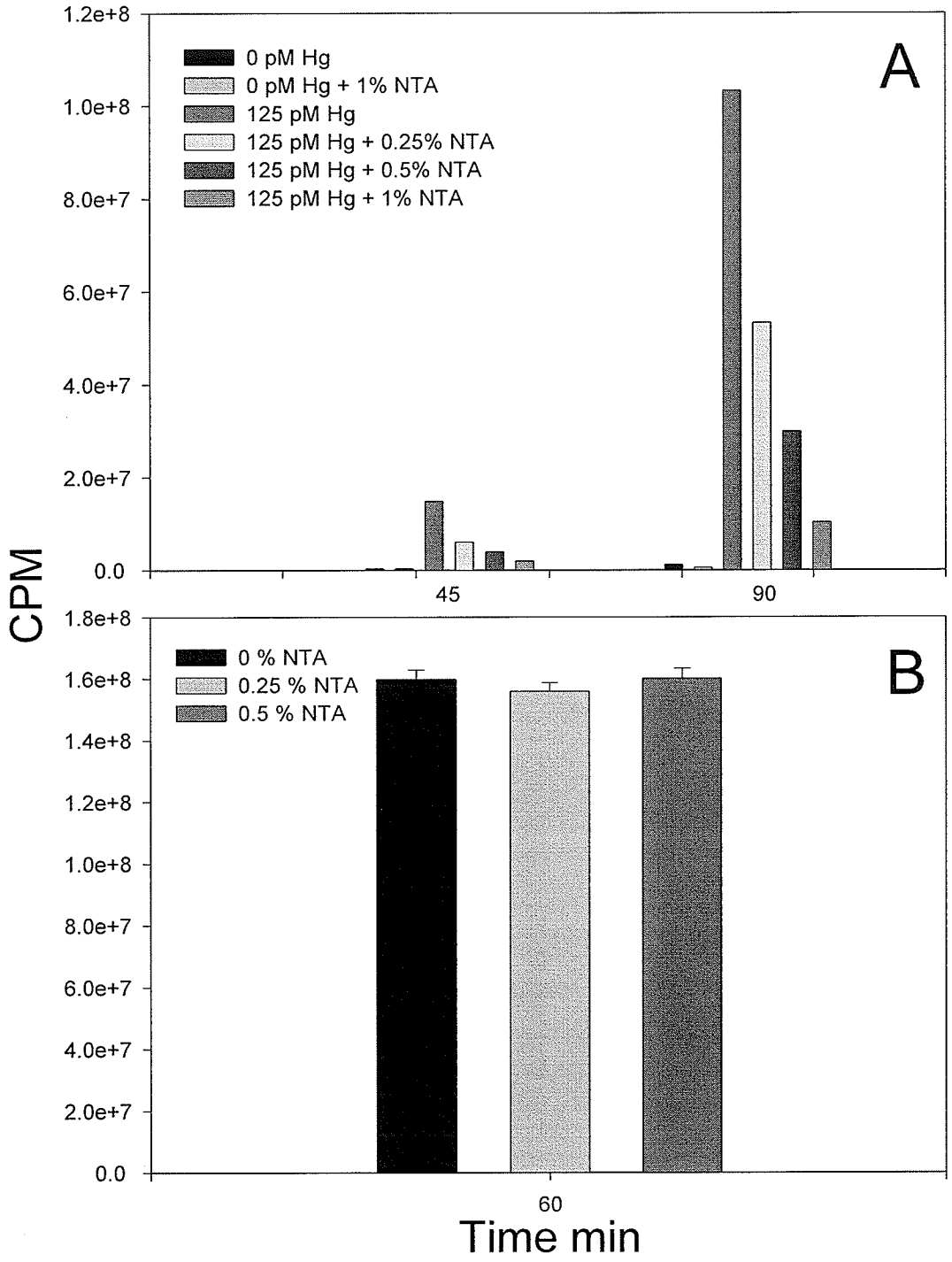

Figure 3.26. Effect of the addition of varying concentrations of Ti-NTA on A) the histidine enhanced induction of *mer-lux* in *V. anguillarum* (pRB28) and B) light production in *V. anguillarum* (pRB27) under anaerobic conditions. The assay medium consisted of 5 mM glucose, 67 mM phosphate buffer, 9 mM $(NH_4)_2SO_4$, 2 mM NaCl, and 1 mM histidine. Error bars represent the standard deviation of duplicate samples.

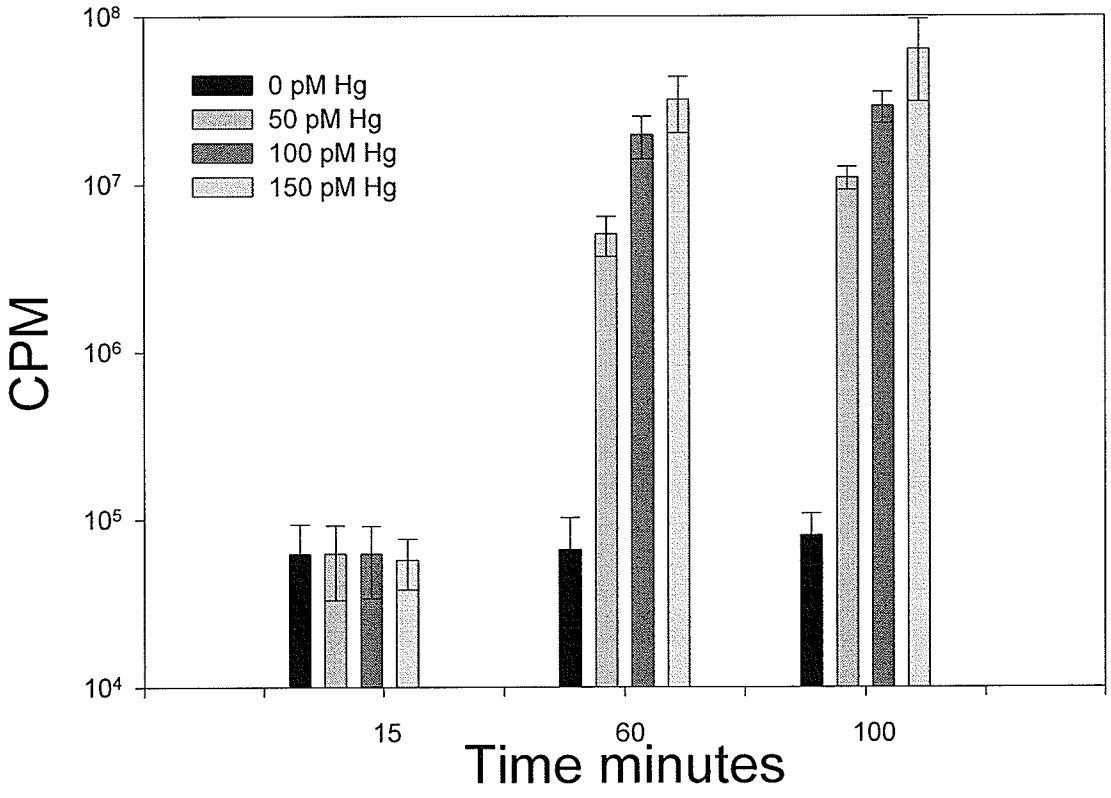

Figure 3.27. Induction of *mer-lux* in *V. anguillarum* (pRB28) under anaerobic conditions in samples containing Ti-NTA. The assay medium consisted of 5 mM Glucose, 67 mM phosphate buffer, 9 mM $(NH_4)_2SO_4$, 2 mM NaCl, 0.5 % Ti-NTA, and 1 mM histidine. Error bars represent the standard deviation of two independent assays.

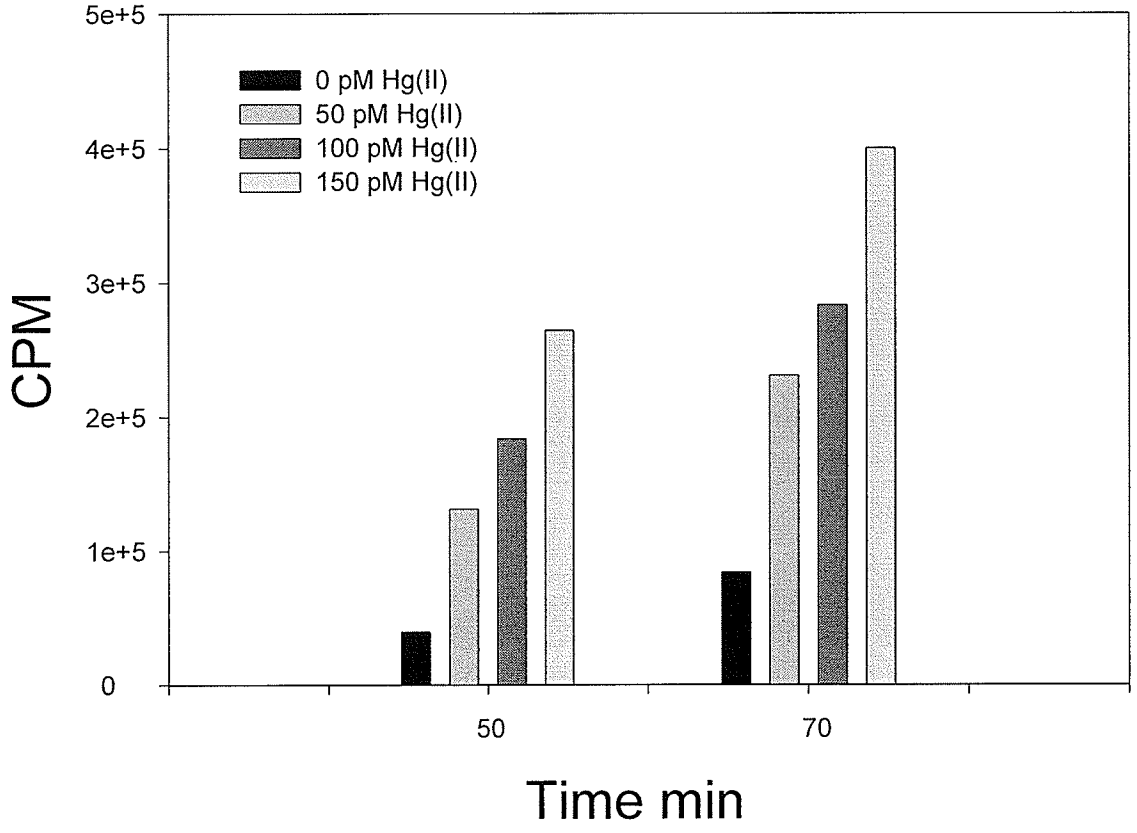

Figure 3.28. Effect of the addition of 0.1% dithionite on the histidine enhanced induction of *mer-lux* in *V. anguillarum* (pRB28) under anaerobic conditions in defined assay media. The assay medium consisted of 5 mM Glucose, 67 mM phosphate buffer, 9 mM $(NH_4)_2SO_4$, 2 mM NaCl, and 1 mM histidine.

3.3.7. Effect of organics on Hg(II) bioavailability in pore water At this point we were ready to use the bioreporter assay to measure trace concentrations of bioavailable Hg(II) in sediment pore water samples, however to standardize this assay we would have to add an amino or carboxlic acid. Due to the affinity of Hg(II) for e.g. histidine (Van der Linden and Beers 1973) it may not be applicable to use in pore water samples. For example, the addition of histidine to the pore water might result in an overestimation of bioavailable Hg(II) by exchanging with previous Hg(II) ligand complexes in the pore water, which may have otherwise been non-bioavailable. To test the effect of histidine in natural anaerobic water, we collected sediment pore water from Lake 114 at the Experimental Lakes Area, Ontario, Canada. The addition of 50 pM Hg(II) to pore water samples +/- histidine resulted in similar levels of induction (Figure 3.29A). Therefore, it appears that histidine was not required nor did it affect the bioavailability of Hg(II) in the pore water samples. For the pore water samples that Hg(II) was not added there was no response, suggesting that the endogenous Hg(II) was not bioavailable or too low a concentration to induce *mer-lux* (Figure 3.29A). The addition of histidine to the pore water samples did not appear to affect light production in *V. anguillarum* (pRB27) (Figure 3.29B). However, sediment samples obtained at a later date did not display the same results. The addition of 10 ng/L of Hg(II) to these pore water samples only induced *mer-lux* in samples that contained histidine (Figure 3.29C). For these experiments, the addition of histidine enhanced the bioavailability of Hg(II). These contradicting results are most likely due to the differences in the water chemistry of

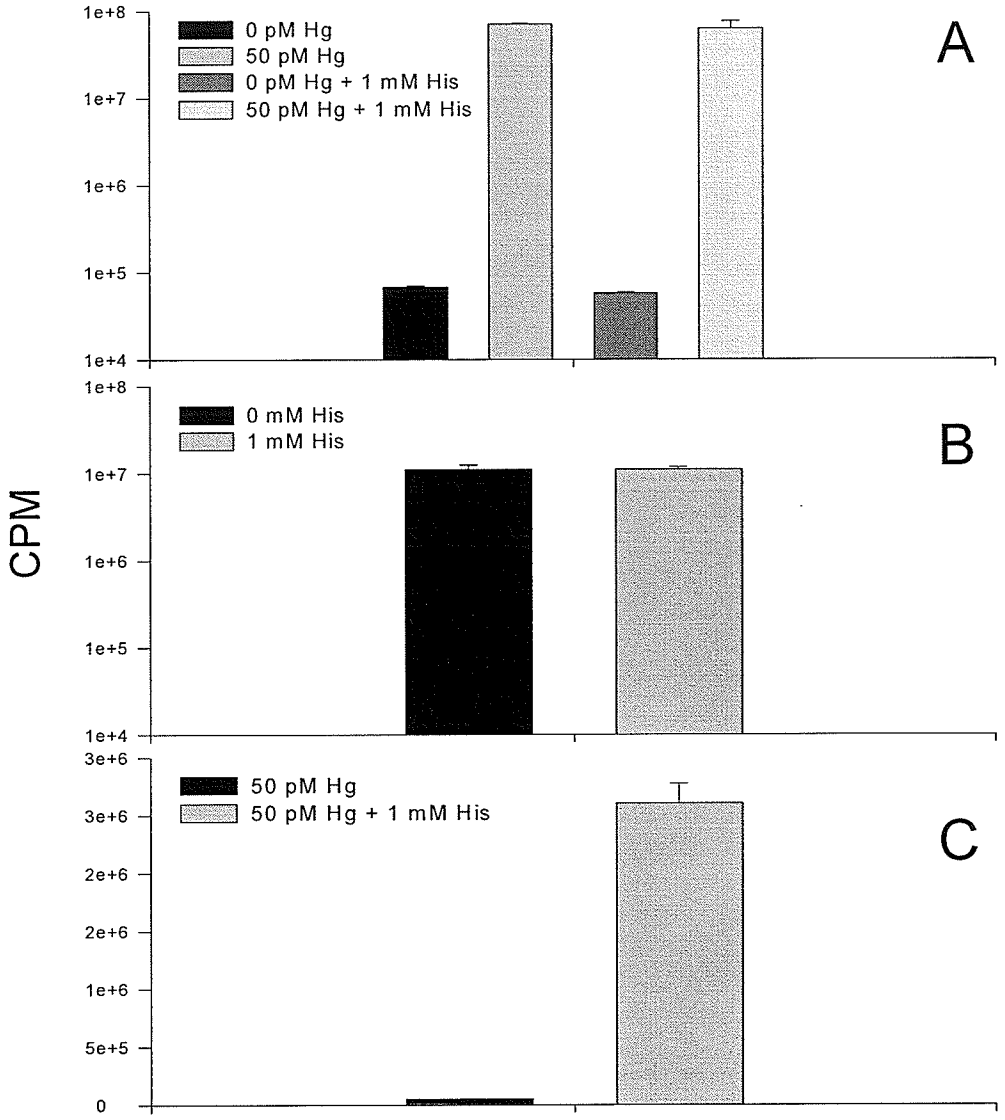

Figure 3.29. Effect of histidine on A) the induction of *mer-lux* in *V. anguillarum* (pRB28) and B) light production in *V. anguillarum* (pRB27) in filtered sediment pore water collected from ELA. Sediment pore water collected from ELA July 27/00. C) Induction of *mer-lux* in pore water collected from ELA Sept 12/00. The pore water was supplemented with 5 mM Glucose, 3 mM phosphate buffer, 9 mM $(NH_4)_2SO_4$, and +/- 1mM histidine. Samples were incubated 80 minutes prior to measurement. Error bars represent the standard deviation of duplicate samples.

different pore water samples, which might have contained varying ligands that competed with histidine for Hg(II)-binding. Therefore, additional organic acids with a lower affinity to Hg(II), which were capable of inducing *mer-lux* when supplemented into samples containing Hg(II), were attempted.

To test the effect of fumarate in natural anaerobic water, sediment pore water was collected from Lake 114 at the Experimental Lakes Area, Ontario, Canada. The addition of fumarate to the filtered pore water samples did not appear to affect light production for *V. anguillarum* (pRB27) (Figure 3.30A). The addition of 50 pM Hg(II) to pore water samples supplemented with fumarate resulted in only a slight increase in the induction of *mer-lux* in *V. anguillarum* (pRB28) (Figure 3.30B). For the pore water samples to which Hg(II) was not added, there was no significant response, indicating that the endogenous Hg(II) was not bioavailable or of a concentration too low to induce *mer-lux*. As with the histidine experiments (Figure 3.29), sediment samples obtained at a later date did not display the same results. The addition of 50 pM of Hg(II) to these pore water samples induced *mer-lux* in samples that contained fumarate to a much larger extent then samples not containing fumarate (Figure 3.30C). For these latter experiments, the addition of fumarate enhanced the bioavailability of the Hg(II). These contradicting results are once again most likely due to the variance in water chemistry between the pore water samples, which was not measured.

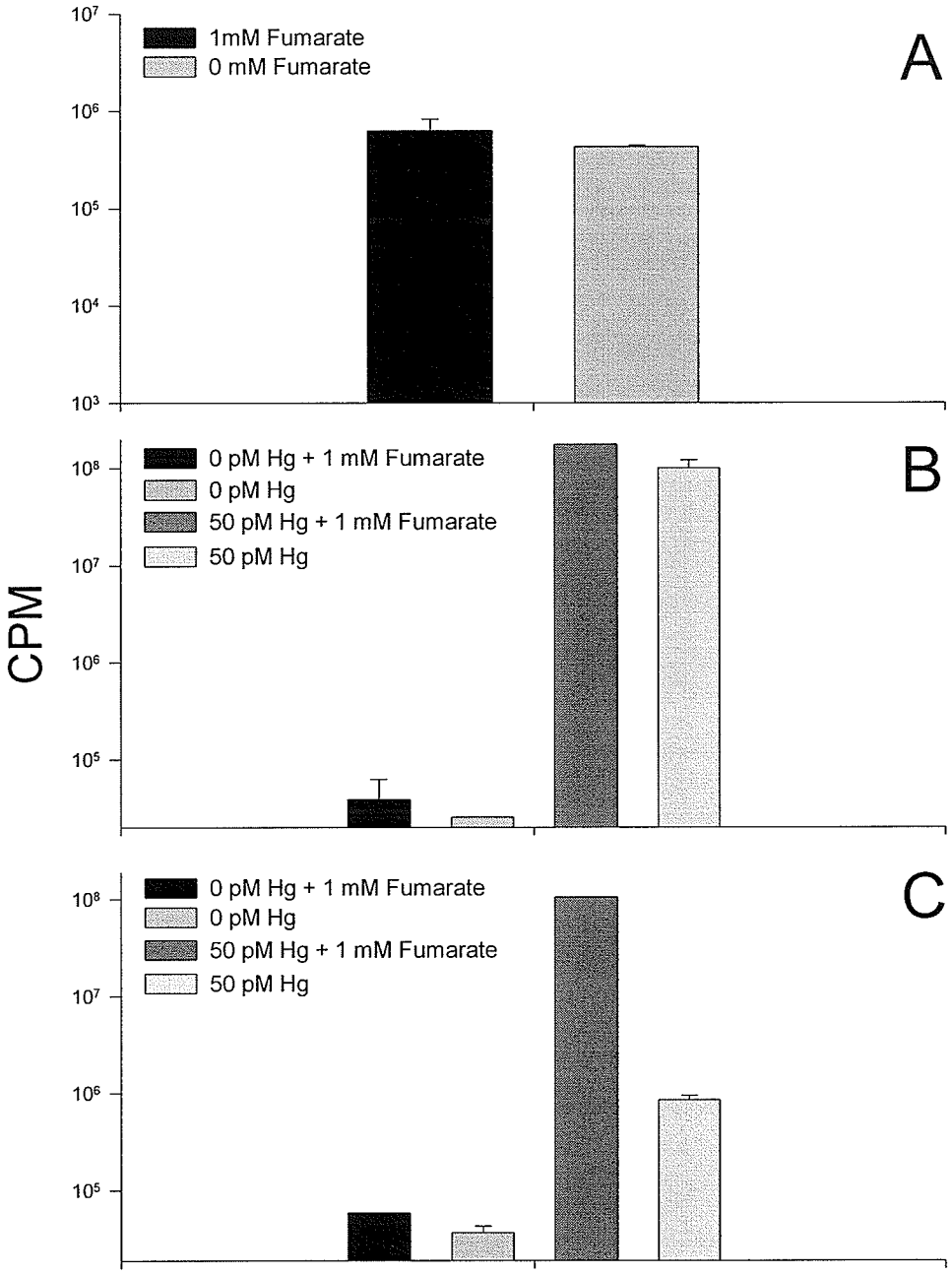

Figure 3.30. Effect of fumarate on A) light production in *V. anguillarum* (pRB27) and B) induction of *mer-lux* in *V. anguillarum* (pRB28) under anaerobic conditions in filtered sediment pore water collected from ELA Sept. 12/00. C) induction of *mer-lux* in filtered sediment pore water collected from ELA Sept 13/00. The pore water was supplemented with 5 mM glucose, 3 mM phosphate buffer, 9 mM $(NH_4)_2SO_4$, and +/- 1mM fumarate. Error bars represent the standard deviation of duplicate samples.

3.4 Discussion

3.4.1. Physiological role in Hg(II) uptake? In the development of a bioreporter assay to study Hg(II) uptake under anaerobic conditions many questions were raised regarding the mechanism of Hg(II) uptake in *V. anguillarum* and *E. coli* HMS174. It is often stated that passive diffusion of uncharged lipophilic complexes is responsible for the accumulation of Hg(II) in microbiota (e.g. Benoit et al. 1999, Morel et al. 1998, Mason et al. 1996). However, the importance of lipophilicity did not correlate with many aspects of Hg(II) transport and accumulation observed using the *mer-lux* bioreporter. For example, if passive diffusion were the major route for Hg(II) accumulation then why would there be a difference between aerobic and anaerobic Hg(II) bioavailability in the same bacterial species? The demonstration that major differences exist in both *V. anguillarum* and *E. coli* HMS174 suggests that Hg(II) uptake is also dependent on the physiological state of the cell.

Facultative bacteria exhibit differences between aerobic and anaerobic metabolism, which is mainly effected by transcriptional regulation. It has been estimated in *E. coli* that the expression of more than 200 genes is affected by the availability of O_2 (Unden et al. 1995). Therefore, some genes that are expressed aerobically will be repressed under anaerobic conditions, and vice versa. The differences in Hg(II) uptake in *E. coli* and *V. anguillarum* under aerobic and anaerobic conditions might then be due to the presence or absence of a protein(s), which may subsequently be involved in either sequestering or the accidental uptake of

Hg(II). The finding that Hg(II) uptake is limited under anaerobic conditions is in concordance with results obtained using a *merR-lacZ* and ^{203}Hg(II) volatilization as indicators of Hg(II) uptake in *Pseudomonas stutzeri* (Schaefer et al. 2002).

3.4.2. Potential role of organics in transport? The addition of a variety of amino acids (Figure 3.11) enhanced the bioavailability of Hg(II) under anaerobic conditions. The Hg(II)-induced light response was dependent on which amino acid was in the sample (Figure 3.11). Passive diffusion models would predict that Hg(II)-complexes with organic matter, forming hydrophilic charged complexes, would significantly reduce the uptake of Hg(II). However, the uptake of Hg(II)-amino acid complexes that enhanced the bioreporter response (Figure 3.11) did not correlate with the hydrophobicity of the amino acids (see Table 3.2). Hg(II) has a high affinity for amino acids (Rulíšek and Halvas 2000) and therefore these differences might be the ability of the Hg(II)-amino acid complex to mimic an endogenous substrate, which would then be actively transported into the cell. For example, histidine and threonine resulted in enhanced uptake of Hg(II) under anaerobic conditions (Figure 3.11), which is in agreement with the active transport of histidine and threonine complexes with Cu(II) in rat liver and kidney cells (Neumann and Silverberg, 1966).

In various mammals mechanisms of facilitated uptake of Hg are well documented, and generally involve the complexation of Hg with small thiol ligands. These Hg-thiol complexes appear to mimic endogenous substrates, which are subsequently taken up by cell. For example, MeHg has been demonstrated in vitro (Mokrzan et al. 1995) and in vivo (Kerper et al. 1992) to cross the blood brain barrier by forming a complex with the amino acid cysteine. This MeHg-cysteine complex

Table 3.2. Hydropathy index for the amino acids tested in the anaerobic bioreporter assays.

Amino acid	Hydropathy index*
Threonine	-0.7
Arginine	-4.5
Proline	-1.6
Histidine	-3.2
Leucine	3.8
Glycine	-0.4
Phenylalanine	2.8
Asparagine	-3.5
Isoleucine	4.5
Valine	4.2

* Values from Kyte and Doolittle (1982)

structurally mimics the amino acid methionine and is subsequently taken up by neutral amino acid transporters (Mokrzan et al. 1995, Clarkson 1994, Kerper et al. 1992).

While organic Hg complexes tend to accumulate in the brain, mercuric ions accumulate predominantly in the kidneys of mammals (reviewed in Zalups 2000). At least two mechanisms are involved in the uptake of mercuric ions by proximal tubular epithelial cells. One of the uptake systems is located on the luminal plasma membrane and the other is localized at the basolateral membrane. Luminal uptake of Hg first involves Hg complexation with glutathione, which can occur either in the proximal tubular lumen or in the plasma (Zalups 2000). However, this Hg-glutathione complex is not directly transported across the luminal plasma membrane. Alternatively, the uptake of mercury-glutathione conjugates requires the activity of gamma-glutamyltransferase (Cannon et al. 2000). This enzyme cleaves the gamma-glutamylcysteine bond in glutathione resulting in cysteinylglycine, which is further degraded by dehydropeptidases to cysteine (Zalups 2000). Evidence suggests that the mercuric ion remains bound to the cysteinyl residue of the glutathione molecules that are being degraded (Naganuma et al. 1988). Mercuric conjugates of cysteine, in particular dicysteinylmercury, are the primary species of mercury that are taken up at the luminal plasma membrane. At least two amino acid transport systems are involved in the luminal uptake of mercuric conjugates of cysteine (Cannon et al. 1999). In addition, luminal uptake of dicysteinylmercury (Km = 36.3 μM) can occur through transporter(s) involved in the luminal absorption of cystine, via a mechanism involving molecular homology (Bridges et al. 2004, Cannon et al. 2000).

The second mechanism involves the uptake of Hg across the basolateral membrane, which can account for 50-60% of the total amount of Hg accumulation in the kidneys (Zalups 1998). This mechanism of Hg uptake involves the dicarboxylate and organic anion transport system OAT1 (Zalups and Ahmad 2004, Zalups et al. 2004, Aslamkhan et al. 2002, Zalups and Barfuss 2002). The movement of α-ketoglutarate out of the cell in exchange for the uptake of an Hg-organic anion from the plasma is the general basis for this mechanism (reviewed in Zalups 2000), which correlates with the enhancement of Hg(II) uptake in *V. anguillarum* in the presence of carboxylic acids (Figure 3.20 and 3.22). Hg conjugates of cysteine, cystine, N-acetylcysteine, and homocysteine formed in the plasma are all plausible species for the basolateral uptake of Hg by this transporter. For example, in MDCK cells transfected with hOAT1 (human organic anion transporter) the K_m for the uptake of the mercury conjugate 2-Amino-3-(2-amino-2-carboxy-ethylsulfanyl-mercuricsulfanyl)-propionic acid (Cys-S-Hg-S-Cys) was 91.7 μM (Zalups et al. 2004).

The transport and accumulation of Hg in mammals is obviously more complicated than in single celled prokaryotes. However, at least 21 families of secondary carriers, 11 families of ABC transporters, and three families of channel proteins have been distinguished in prokaryotes that mediate the transport of amino acids, peptides, and their derivatives in or out of the cell (Saier 2000). Therefore, the possibility of Hg(II)-organic acid transport via molecular mimicry in bacteria should also be considered in future studies. Evidence for this was provided by Selifonova and Barkay (1994) that demonstrated that Na^+ ions significantly enhanced the uptake,

and potentially toxicity, of Hg(II) using a *mer-lux* bioreporter and Hg^{203}. One of the major functions of Na^+ is the cotransport of a variety of inorganic and organic solutes, including organic and amino acids (Maloy 1990, Dimorth 1987, Scott 1987, Skulachev 1985, Lanyi 1979). Therefore, sodium coupled transport of Hg(II)-organic acid complexes might be an important mechanism of Hg(II) uptake in cells not expressing the Hg(II)-specific transport system of the *mer* operon.

Hg(II) mimics reactive oxygen species (ROS) in its interaction with cellular ligands. If we take into consideration the relation of redox-sensing regulators, OxyR and SoxR, with MerR, and the relation of mercuric reductase with glutathione reductase (Williams et al. 1982, Brown et al. 1983), it might be possible that interactions of Hg(II) with ROS-sensing and redox homeostasis systems would play an important role (Barkay et al. 2003). In *E. coli* the in vivo concentration of glutathione been estimated at 6-7 mM (Apontoweil and Berends 1975), and therefore, the potential role of glutathione in Hg complexation and transport needs to be further investigated in bacteria.

The enhanced uptake of Hg(II) in the presence of organic acids would provide an additional explanation for why MeHg concentrations are increased following reservoir construction (St. Louis et al. 2004) and in acidified lakes (Miskimmin et al. 1992, Winfrey and Rudd 1990, Xun et al. 1987). For example, following flooding the decomposition of vegetation would result in an increase in the concentration of organic acids in the water, which in turn lowers the pH and O_2 levels. Together this could increase the proportion of Hg(II) available for uptake in bacteria responsible for Hg(II)-methylation, and hence increased rates of MeHg production. Interestingly, the

addition of carboxylic acids that lowered the pH of the assay medium below 6 appeared to have the largest effect (Table 3.1), suggesting a possible correlation of pH with Hg(II) uptake, which is further discussed in chapter 6.

3.4.3. Use of anaerobic Hg(II) bioreporters in natural samples The ability of the bioreporter to respond to Hg(II) in anaerobic sediment pore water demonstrated that it could be applied to study Hg(II) bioavailability in environmental anaerobic samples. However, the anaerobic bioreporter was complicated by the fact that trace Hg(II) concentration did not appear to be bioavailable in the standards unless the medium was supplemented with a low molecular weight organic acid. The effects that these organics might have on the bioavailability of Hg(II) in sediment pore water samples could be large, as demonstrated using histidine and fumarate, and therefore the results obtained would be difficult to interpret. Further work with the bioreporter in sediment pore water should also include complete water chemistry analysis (e.g. sulfide concentration, pH, DOC, Hg(II) concentration etc…) and better collection techniques (e.g. core slices).

Aquatic Hg(II) research has predominantly focused on how differences in water chemistry affect the chemical speciation of Hg(II) in the bulk solution, correlating the formation of lipophilic species in the water with bioavailability. However, differences in water chemistry would also affect the physiology of the cell, which subsequently could have a significant effect on the rates of Hg(II) uptake. This was demonstrated with the difference in the sensitivity of the bioreporter in the presence and absence of O_2, the addition of specific organic acids, and possibly with

pH. Such differences in Hg(II) uptake would not be expected on the basis of passive diffusion alone and therefore, additional mechanisms of Hg(II) uptake might also exist. Without knowledge of the mechanism(s) of Hg(II) uptake in bacterial cells the application of bioreporters and chemical speciation models to measure/predict bioavailable Hg(II) in the environment is limited.

Chapter 4

Reevaluation of the FIAM and passive diffusion as models for predicting Hg(II) bioavailability

4.1 Introduction

4.1.1 Derivation of the FIAM Historically toxicity bioassays were the first to demonstrate that total metal concentrations were relatively poor indicators of metal toxicity, which was further exemplified by the introduction of chemical equilibrium models in the 60s and 70s. Using chemically defined media and synthetic ligands with known stability constants a convincing body of evidence, over the period of 1976-1983, evolved to support the notion that the toxicity and uptake of metals was dependent on the concentration of the free-metal ion (Reviewed in Campbell 1995, Tessier 1994). From these experiments, the free-ion-activity model (FIAM) for metal-organism interactions was formulated (Morel 1983).

The premises for the FIAM is that the interaction of a metal with a cell surface site/transporter (X_{cell}), involving either the free-metal ion (M^{z+}) or a kinetically labile metal complex (ML) as the reactive species, can be represented in terms of the formation of M-X_{cell} complexes. In case where the free-metal ion (M^{z+}) is the species reacting at the X_{cell} site:

Solution equilibria

(1) $M^{z+} + L \xleftrightarrow{K_1} ML$

(2) $K_1=[ML]/([M^{z+}][L])$

Surface reaction of M^{z+}

(3) $M^{z+} + X_{cell} \xleftrightarrow{K_2} M\text{-}X_{cell}$

(4) $[M\text{-}X_{cell}] = K_2[X_{cell}]\,[M^{z+}]$

where K_1 and K_2 are condition equilibrium constants, and L represents a ligand in solution.

A similar situation occurs however if a kinetically labile metal complex (ML) is the species reacting with X_{cell}:

Surface reaction of ML

(5) $ML + X_{cell} \xleftrightarrow{K_3} MX_{cell} + L$

(6) $[M\text{-}X_{cell}]=K_3 ([X_{cell}][ML])/[L]$

By rearranging equation (2), one obtains

(7) $[ML]/[L] = K_1[M^{z+}]$

Substitution of equation (7) into (6) then yields

(8) $[M\text{-}X_{cell}] = K_3K_1[X_{cell}][M^{z+}]$

which shows the same dependency on M^{z+} as equation (4). According to Campbell (1995) a number of key assumptions underlie the FIAM:

1. the X_{cell} is the primary site for metal interactions;
2. interaction with X_{cell} can be described as a surface complexation reaction, forming $M\text{-}X_{cell}$;
3. metal transport in solution, towards the X_{cell}, and the subsequent surface complexation reaction occur rapidly, such that equilibrium is established between metal species in the bulk solution and those at the biological surface;
4. the biological response is strictly dependent on the concentration of the $M\text{-}X_{cell}$ surface complex;
5. the concentration of free sites, X_{cell}, remains virtually constant and variations in $M\text{-}X_{cell}$ follow those of $[M^{z+}]$ in solution;
6. the metal does not induce any changes in the nature of the cell;

7. the metal does not form lipophilic species in solution that might traverse the membrane without first forming a surface complex.

4.1.2 The passive diffusion model for Hg(II) In contrast to other heavy metals, the proposed mechanism of Hg(II) uptake and toxicity in bacteria is via passive diffusion. Passive diffusion is dependent on the formation of lipophilic species formed in the bulk solution, which subsequently traverse the membrane without first forming a surface complex, and would therefore be an exception to the FIAM. The assumption of passive diffusion is based primarily on the permeability of mM concentrations $HgCl_2$ across eukaryotic based bilayer membranes, which was calculated to be between 2.6×10^{-3} (Bienvenue et al. 1984) and 1.3×10^{-2} cm s^{-1} (Gutknecht 1981), and one study involving the diatom *Thalassiosira weissflogii*, which concluded that the passive diffusion of the neutral species, $HgCl_2$ and CH_3HgCl, were primarily responsible for accumulation and toxicity (Mason et al. 1996). Primarily on the basis of these three studies it has been assumed that the primary mechanism of Hg(II) uptake and toxicity in bacteria is via passive diffusion of lipophilic Hg species across the cytoplasmic membrane (e.g. Jay et al. 2002, Benoit et al. 2001, Benoit et al. 1999, Morel et al. 1998, Selifonova et al. 1993).

In Chapter 3 however, differences were observed in the concentration of Hg(II) required to induce the *mer* operon under aerobic and anaerobic conditions and that the addition of specific amino acids enhanced Hg(II) uptake under anaerobic conditions (Figure 3.11). However, on the basis of passive diffusion no differences in the diffusion of Hg(II) across the cytoplasmic membrane should have been observed in

the presence or absence of O_2. Also, a number of probable hydrophilic Hg(II) amino acid complexes formed in the bulk solution, which requires experimental verification, should have significantly reduced the uptake of Hg(II). In addition to passive diffusion models, the addition of these amino acids would also have a significant effect on the free-ion concentration of Hg(II), which further delineates Hg(II) uptake with the FIAM. These results were in contradiction with both the current view of passive diffusion for Hg(II) uptake and with the FIAM. Therefore, the ability of these two models to predict Hg(II) bioavailability in bacteria was reevaluated. With the aid of chemical speciation models we were able to manipulate the inorganic and organic speciation of Hg(II) to determine the effect that neutral and/or free-ion Hg^{2+} concentrations had on the uptake and toxicity under aerobic and anaerobic conditions.

4.2 Results

4.2.1 Uptake of inorganic Hg(II) Under anaerobic conditions the *mer-lux* bioreporter did not induce in the presence of trace Hg(II) concentration (5-250 pM) unless select organic acids were added to the assay medium (see Table 3.1). However, increasing concentration of Hg(II) (> 250 pM) in Assay medium A (Table 4.1) enabled an anaerobic response (Figure 4.1). This anaerobic response might have been the result of either increasing the concentration of Hg^{2+} or the neutral $HgCl_2$ and $Hg(OH)_2$ species in the bulk solution (see Table 4.1), which results from the higher concentrations of total Hg(II) used. Therefore, to ascertain whether neutral Hg(II) species or Hg^{2+} might have been responsible, we manipulated the concentration of reagents in the assay medium to alter the proportion of neutral species, $Hg(OH)_2$ and

Table 4.1. Calculated speciation of Hg(II) in the defined assay media (pH=7).

Assay Medium	Medium components [A]	$Hg(NH_3)_2^{2+}$ %	$Hg(OH)_2$ %	$HgCl_2$ %	Hg^{2+} %
A	9 mM $(NH_4)_2SO_4$	**97**	1.7	1.8×10^{-6}	5.5×10^{-8}
B	0.9 mM $(NH_4)_2SO_4$	24	**48**	6.0×10^{-5}	1.2×10^{-6}
C	0.9 mM $(NH_4)_2SO_4$ and 3.4 mM NaCl	2.3	4.4	**62**	1.2×10^{-7}

[A] Also contained 1 μM NaCl, 6.7 mM PO_4 and 5 mM Glucose.

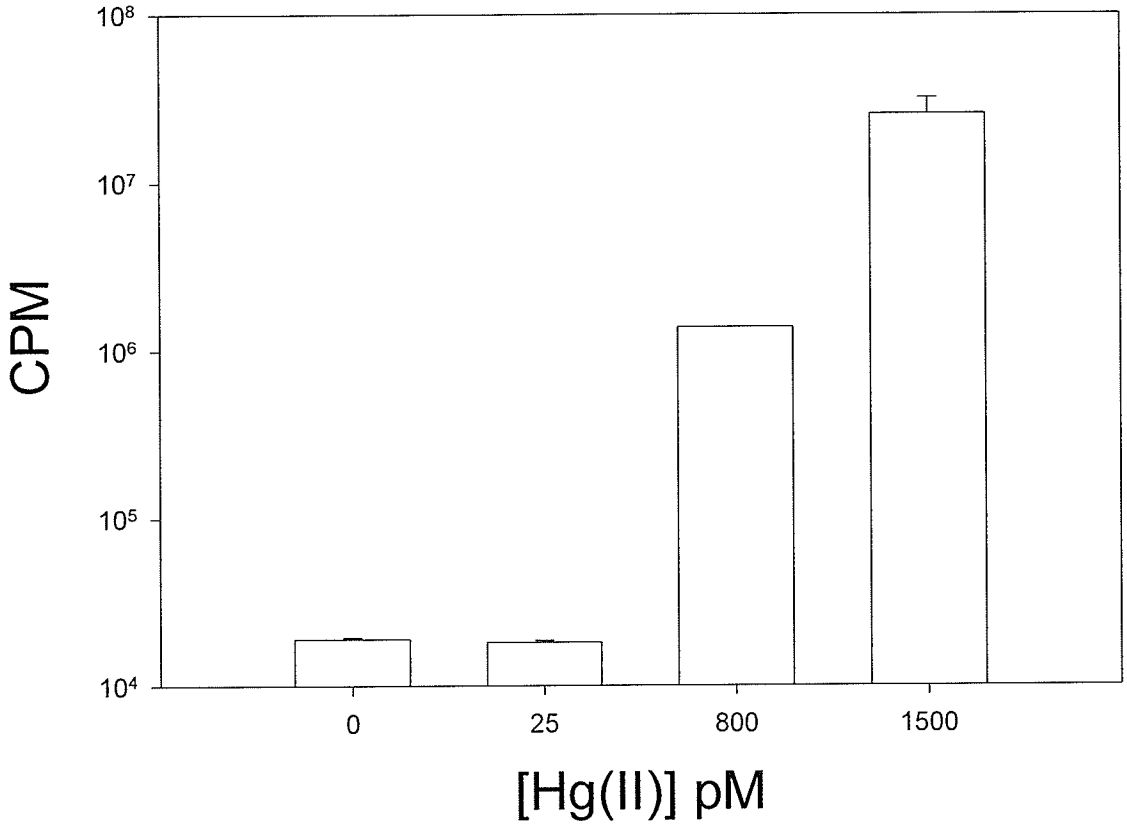

Figure 4.1. Effect of increasing concentrations of Hg(II) on the induction of *mer-lux* in *V. anguillarum* (pRB28) under anaerobic conditions in Assay medium A (see Table 4.1 for medium composition and speciation calculations). Error bars represent the standard deviation of duplicate samples.

$HgCl_2$, and Hg^{2+} independently of one another in order to determine their effect on Hg(II) uptake and toxicity in *V. anguillarum* (pRB28) and (pRB27) respectively.

The differences of medium composition in Assay medium A,B, and C (Table 4.1) did not have significant effects on the growth rates of *V. anguillarum* under anaerobic and aerobic conditions (Table 4.2). Despite growth in all three media types, low Hg(II) concentration (< 500 pM) did not enable an anaerobic bioreporter response (Figure 4.2A). At higher Hg(II) concentrations (> 500 pM) the response was proportional to the total concentration of Hg(II) for all the media types tested (Figure 4.2A). The Hg(II)-induced response was not proportional to the concentrations of the neutral species $Hg(OH)_2$ (Figure 4.2B) and $HgCl_2$ (Figure 4.2C). This was most evident in the manipulation of Hg(II)-chloride species. In these experiments the proportion of the "lipophilic" $HgCl_2$ species in the various assay media was varied over 8 orders of magnitude, however the inductions of the bioreporter were similar (Figure 4.2C). The response was also not proportional to the positively charged species $Hg(NH_3)_2^{2+}$ (Figure 4.2D) nor with the calculated concentration of Hg^{2+} (Table 4.1).

Under aerobic conditions the bioreporter is more sensitive and therefore, lower concentrations of Hg(II) were required to prevent saturation of the light response. As with the anaerobic assays, at any specific Hg(II) concentration tested the amount of light produced was not affected by the inorganic speciation of Hg(II) in the assay medium (Figure 4.3A and Table 4.1). On the basis of lipophilicity (Table 3.1) and passive diffusion, the rates at which $HgCl_2$ diffuses into the cell would be much greater than $Hg(OH)_2$. However, the slopes of induction were virtually

Table 4.2. Mean generation times of *V. anguillarum* in defined assay media. Average of 3 independent samples are shown.

Assay medium*	Aerobic g (hours/generation)	Anaerobic g (hours/generation)
A	4.7	5.4
B	4.2	5.6
C	4.8	5.5
D	4.7	4.8

* See Table 4.1 and 4.3 for medium composition

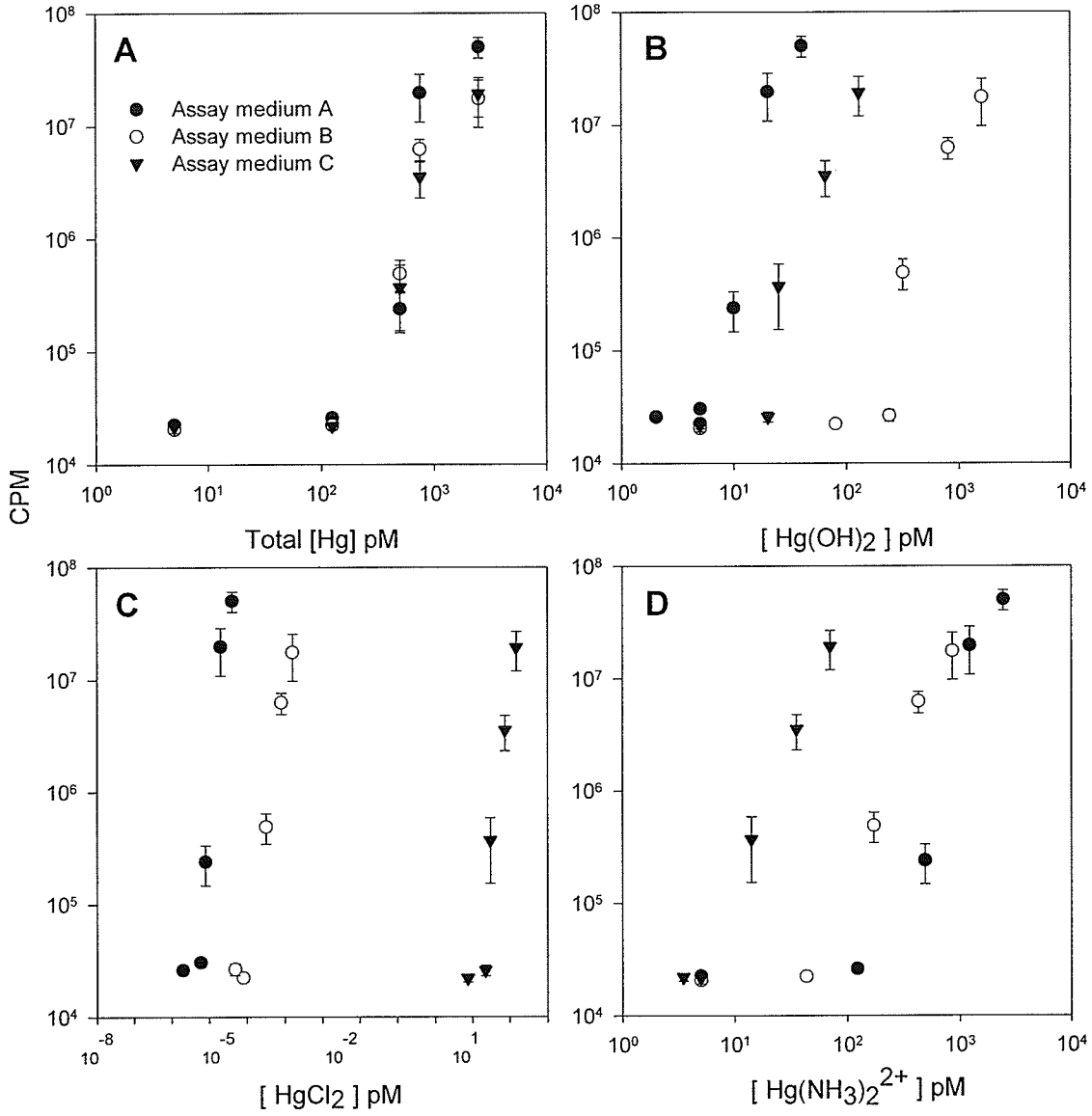

Figure 4.2. Effect of varying inorganic Hg(II) speciation on Hg(II) uptake in *V. anguillarum* (pRB28) under anaerobic conditions. Concentrations of reagents in the assay media were manipulated to promote $Hg(NH_3)_2^{2+}$ (Assay medium A), $Hg(OH)_2$ (Assay medium B), and $HgCl_2$ (Assay medium C) as the predominant species (see Table 4.1 for speciation calculations). Induced light production in the varying assay media was measured after 80 minutes of Hg(II) exposure and was plotted against A) total Hg concentration and the calculated concentration of B) $Hg(OH)_2$, C), $HgCl_2$ and D) $Hg(NH_3)_2^{2+}$. Error bars represent the standard error of duplicate samples of three independent experiments.

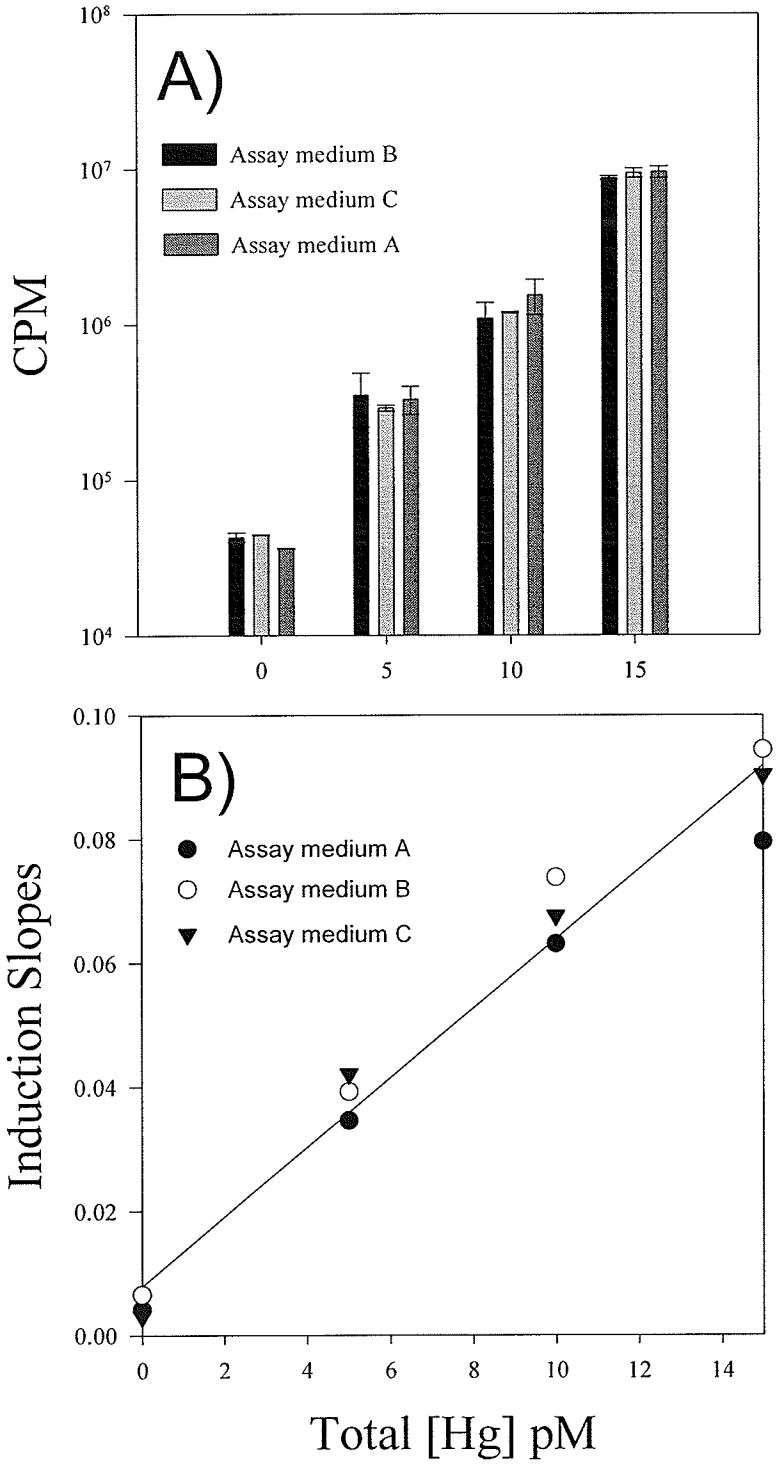

Figure 4.3. Effect of varying inorganic Hg(II) speciation on A) Hg(II) uptake and B) slopes of induction in *V. anguillarum* (pRB28) under aerobic conditions. Induced light production was measured after 80 minutes of Hg(II) exposure in assay media containing predominantly $Hg(NH_3)_2^{2+}$ (Assay medium A), $Hg(OH)_2$ (Assay medium B), or $HgCl_2$ (Assay medium C) (see Table 4.1 for speciation calculations). Error bars represent the standard error of duplicate samples of three independent experiments.

identical under all the different conditions tested, including Assay medium A that contained virtually no neutral charged species and was dominated by charged $Hg(NH_3)_2^{2+}$ species (Figure 4.3B). Thus, uptake of Hg(II) at these concentrations tested did not have the characteristics of passive diffusion. However, concentrations of Hg(II) used in the bioreporter assays might have been too low for passive diffusion to be relevant, as previous studies supporting passive diffusion were in the μM-mM range (Gutknecht 1981, Bienvenue et al. 1984).

4.2.2 Toxicity of inorganic Hg(II) Bioluminescence from *V. anguillarum* (pRB27) was used as a quantitative marker of toxicity, in an attempt to determine if neutrally charged species or Hg^{2+} were capable of predicting Hg(II) bioavailability at higher concentrations of Hg(II). Under both aerobic and anaerobic conditions (Figure 4.4A and B) bioluminescence was similar in samples containing up to 1 nM Hg(II). A significant decrease in light production was observed for all 3 different media types containing 2 nM Hg(II), and was completely eliminated in samples containing 4 nM Hg(II), regardless of the predominate species of Hg(II) in the bulk solution (Table 4.1), or the presence or absence of O_2. Therefore, even at toxic levels of Hg(II) it appears that neutrally charged Hg(II)-species and the Hg^{2+} concentration were generally poor predictors of toxicity.

4.2.3 Uptake and toxicity of Hg(II)-histidine complexes The growth rates of *V. anguillarum* in the presence of 1mM histidine were the same as in unsupplemented medium under aerobic conditions, but there was a notable small increase under

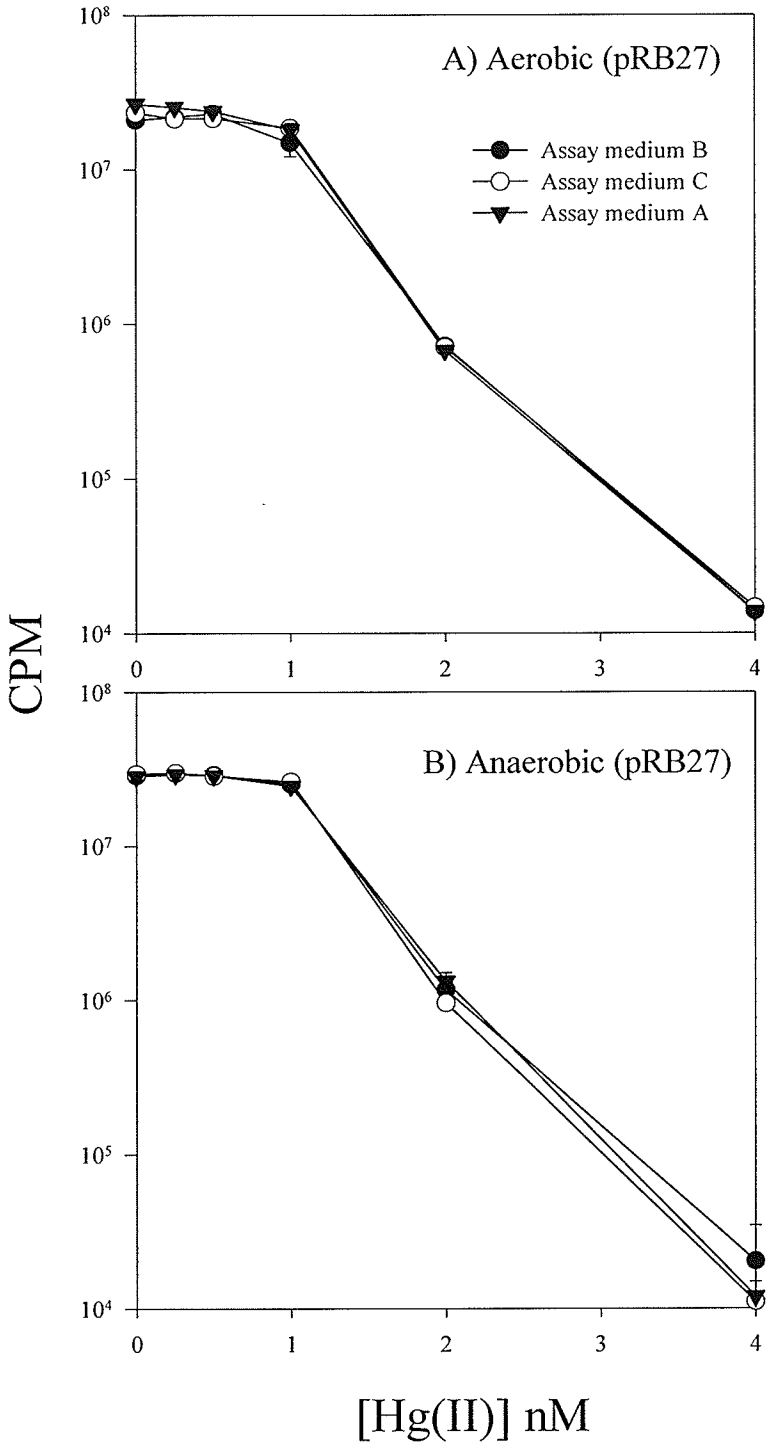

Figure 4.4. Effect of varying inorganic speciation of Hg(II) on toxicity under A) aerobic and B) anaerobic conditions in *V. anguillarum* (pRB28). Concentration of reagents in the assay media were manipulated to promote $Hg(NH_3)_2^{2+}$ (Assay medium A), $Hg(OH)_2$ (Assay medium B), and $HgCl_2$ (Assay medium C) as the predominant species (see Table 4.1 for speciation calculations). Error bars represent the standard deviation of triplicate samples.

anaerobic conditions (Table 4.2). Considering that the time frame of the assays was only 80 minutes, this small increase in growth rates was likely not significant. The addition of histidine resulted in the formation of predominantly hydrophilic Hg(II)-histidine complexes and a substantial reduction in the "free-ion" concentration and the chemically labile inorganic species such as $HgCl_2$, $Hg(OH)_2$, and $Hg(NH_3)_2^{2+}$ (Table 4.3). Despite these significant decreases, the addition of 1 mM histidine substantially increased Hg(II) uptake in *V. anguillarum* pRB28 under anaerobic conditions (Figure 4.5A) and was not required nor did it decrease the uptake of Hg(II) under aerobic conditions (Figure 4.5B). In contrast to the uptake of Hg(II) however, the addition of 1 mM histidine alleviated toxicity up to 50 nM Hg(II) under both aerobic and anaerobic conditions (Fig. 4.6A and B).

Table 4.3. Calculated speciation of Hg(II) using MINEQL+ in defined assay media +/- 1 mM histidine (pH=7).

Assay Medium	Components [A]	$Hg(NH_3)_2^{2+}$ %	$Hg(OH)_2$ %	$HgCl_2$ %	$Hg2^+$ %	Hg-Histidine complexes [B] %
A	9 mM $(NH_4)_2SO_4$	97	1.7	1.8×10^{-6}	5.5×10^{-8}	NA*
D	9 mM $(NH_4)_2SO_4$ and 1 mM Histidine	2.5×10^{-5}	4.3×10^{-7}	4.7×10^{-13}	1.4×10^{-14}	> 99

[A] Also contained 1 μM NaCl, 6.7 mM PO_4 and 5 mM Glucose.

[B] Total of all Hg-histidine complexes including HgHis, HgHHis, $Hg(His)_2$, and $Hg(HHis)_2$.

*NA = non-applicable

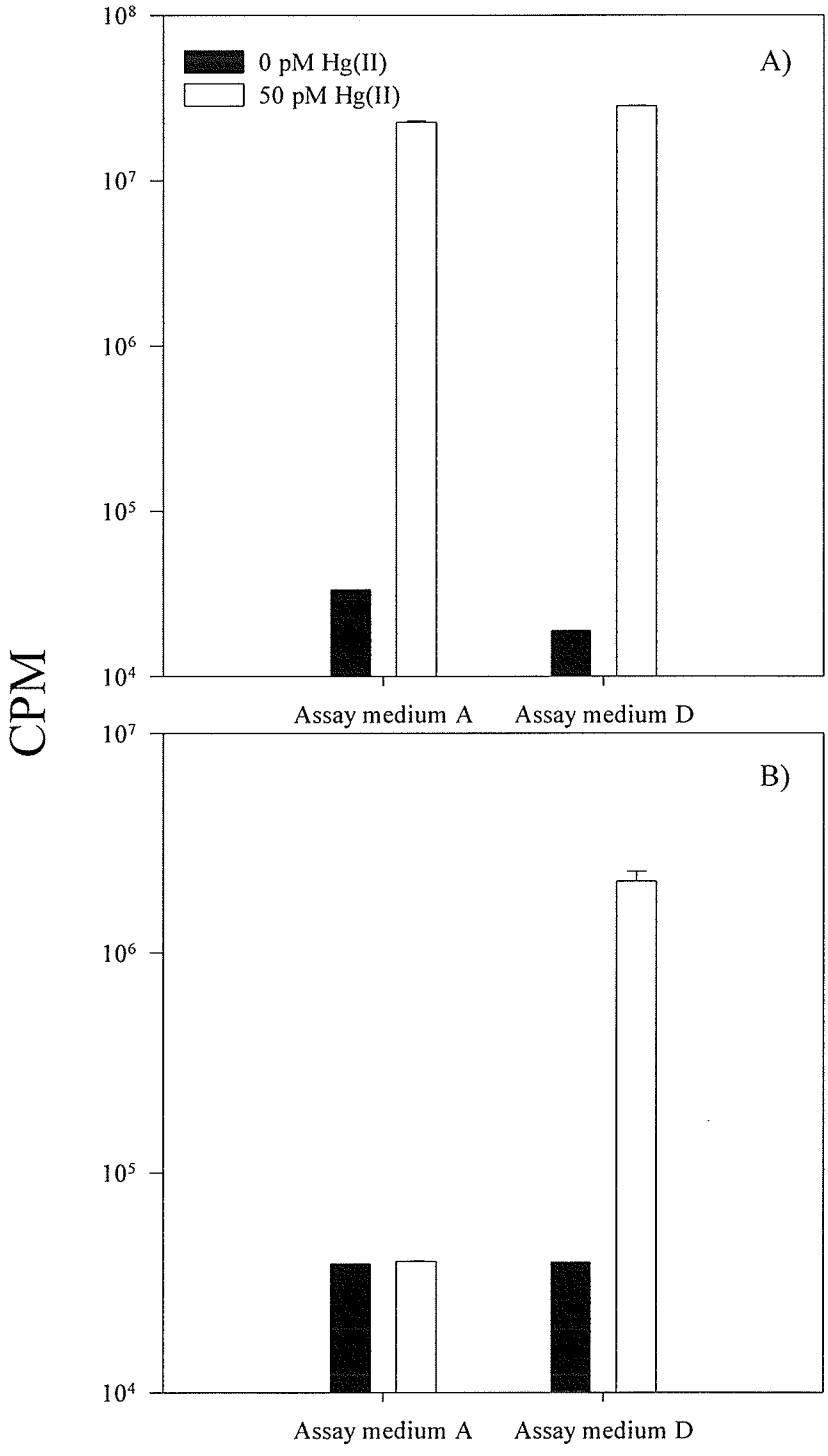

Figure 4.5. The effect of histidine on the induction of *mer-lux* in *V. anguillarum* (pRB28) under A) aerobic and b) anaerobic conditions. Light production was measured following 80 minutes of Hg(II) exposure in Assay medium A and D, in which the latter contained 1 mM histidine (see Table 4.2 for media composition and speciation calculations). Error bars represent the standard deviation of duplicate samples.

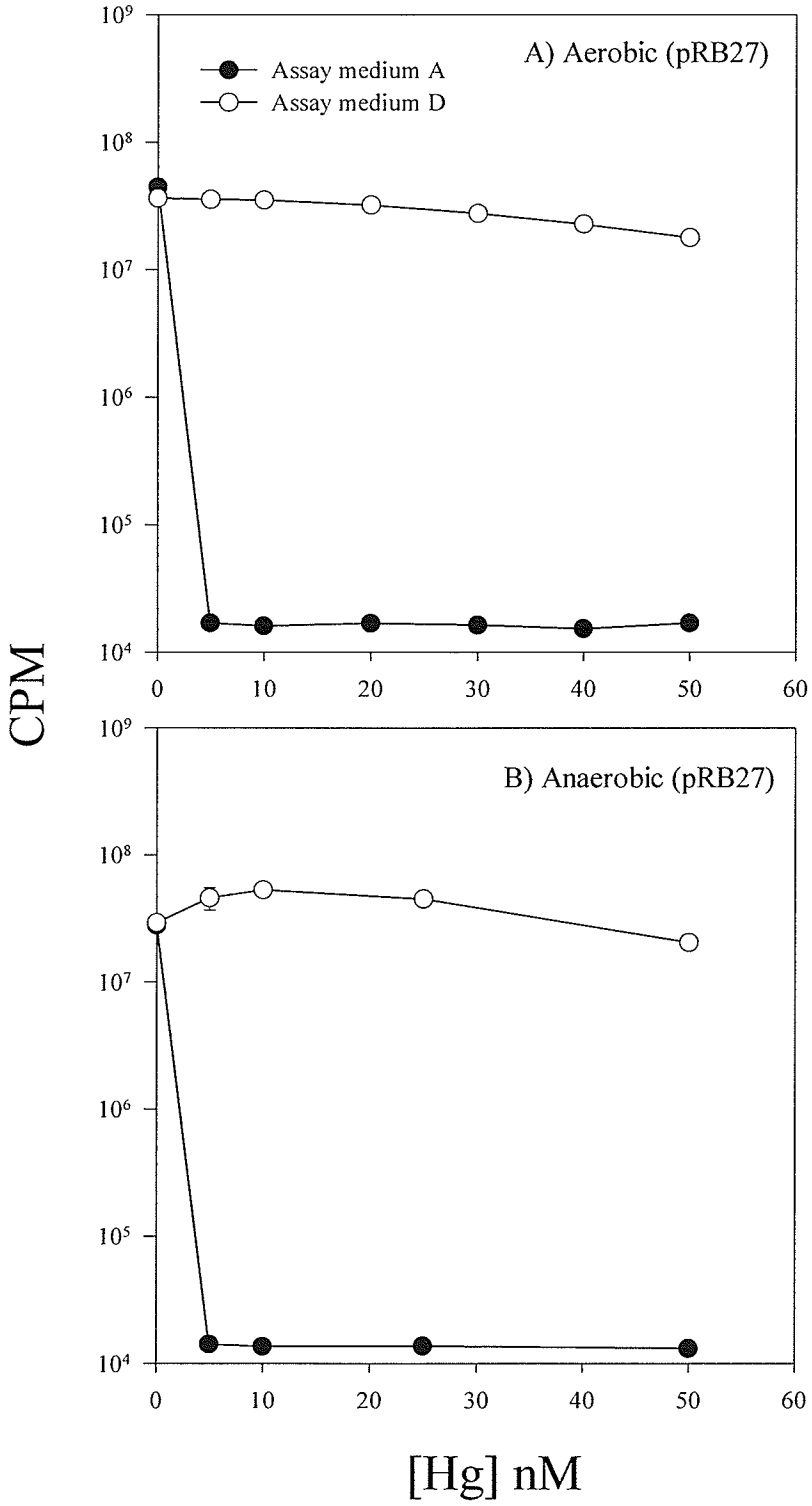

Figure 4.6. The effect of histidine on the toxicity of Hg(II) in *V. anguillarum* (pRB27) under A) aerobic and B) anaerobic conditions. Light was measured following 80 minutes of Hg(II) exposure in Assay medium A and D, in which the latter contained 1 mM histidine (see Table 4.2 for media composition and speciation calculations). Error bars represent the standard deviation of triplicate samples.

4.3 Discussion

4.3.1 Toxicity assays as predictors of Hg(II) uptake Toxicity assays were the first to demonstrate that total metal concentrations were poor predictors of metal toxicity. However, this has also led many to assume that in order for a metal to exert a toxic effect it must be in a form that is accessible by the test organism for uptake into the cell (i.e. bioavailable), whereas high metal concentrations that are not toxic are likely in a form that is non-available to the cell (e.g. Najera et al. 2005, Ritchie et al. 2001, Vulkan et al. 2000, Stumm and Morgan 1996, Hughes and Poole 1991). Using constitutive bioluminescence as an indicator of toxicity, the concentrations of Hg(II) required to observe a toxic effect under anaerobic and aerobic conditions were similar (Figure 4.4). Therefore, on the premises that toxicity assays could be used as an indicator of uptake into the cell, we would predict that the uptake of Hg(II) would also be similar under these two conditions. However, using the *mer-lux* bioreporter as an indicator of Hg(II) uptake, there was a significant increase in the concentration of Hg(II) required to induce the bioreporter under anaerobic conditions (Figure 4.2A), in comparison to the aerobic assays (Figure 4.3A). The differences observed in Hg(II) uptake under these two conditions would not have been predicted using toxicity assays alone.

The differences in Hg(II) uptake and toxicity is especially prevalent when assayed in the presence of 1 mM histidine. Under both anaerobic and aerobic conditions the addition of 1 mM histidine alleviated Hg(II) toxicity up to 50 nM (Figure 4.6). We would therefore predict that histidine would severely mitigate the

uptake of Hg(II) into the cell. On the contrary however, histidine appeared to significantly enhance Hg(II) uptake into the cell under anaerobic conditions (Figure 4.5B), and was shown not to decrease the uptake of Hg(II) under aerobic conditions (Figure 4.5A). Therefore, while toxicity assays might be able to tell us something about the general adsorption of Hg(II) to the cell, these data suggest that they should be used with caution as a predictor of Hg(II) uptake into the cytoplasm of the cell.

4.3.2 Evidence not supporting passive diffusion Lipid solubility can be estimated by the octanol-water partition coefficient (K_{ow}), which ranges from 0 for very hydrophilic molecules to 10^8 for very hydrophobic ones (Morel 1998) (see Table 3.1 for comparison with other lipophilic compounds). However, the measured K_{ow} of $HgCl_2$ and $Hg(OH)_2$ was only 3.3 and 0.5 respectively (Mason et al. 1996). Despite this relatively low lipophilicity, $HgCl_2$ was shown to diffuse rapidly across lipid bilayers, whereas $Hg(OH)_2$ did not permeate lipid bilayers at a significant rate (Gutknecht 1981). Therefore, if passive diffusion were the predominant mechanism in bacteria, uptake should have been highest in Assay medium C, where $HgCl_2$ dominated in the bulk solution, followed far behind by Assay medium B, which contained predominately $Hg(OH)_2$. However, the manipulation of the inorganic speciation of Hg(II) demonstrated that there was no significant difference in the uptake of Hg(II) under anaerobic (Figure 4.2A) and aerobic (Figure 4.3A, K.Scott Ph.D. dissertation University of Manitoba) conditions in *V. anguillarum* (pRB28).

These results coincide with the indiscriminate uptake of $HgCl_2$ and $Hg(OH)_2$ in *E. coli* HMS174 (pRB28), whereas increasing chloride concentration, resulting in the

formation of negatively charged Hg(II)-chloride species, $HgCl_3^-$ and $HgCl_4^{2-}$, were shown to decrease the uptake of Hg(II) (Barkay et al. 1997). It was therefore proposed that the neutral species, $HgCl_2$ and $Hg(OH)_2$, were likely diffusing passively into the cell and the charged complexes were not available to the cell (Barkay et al. 1997). However, we showed that Hg(II) is also taken up equivalently in Assay medium A, which contained virtually no neutral species and was dominated by a positively charged $Hg(NH_3)_2^{2+}$ species (Figure 4.2A and 4.3). Therefore, the indiscriminate uptake of trace concentrations of $Hg(OH)_2$, $HgCl_2$, and $Hg(NH_3)_2^{2+}$ observed under aerobic and anaerobic conditions did not support the model of passive diffusion.

Chloride and hydroxide could be important in Hg(II) speciation in natural oxic waters, but the chemistry of Hg(II) differs substantially in anoxic sediments. For example, sulfide has a very high affinity for binding Hg(II) and therefore plays a major role in the speciation of Hg(II) (Jay et al. 2000). Therefore, once again on the basis of passive diffusion, it was hypothesized that the formation of neutral Hg(II)-sulfide species would likely be responsible for the uptake of Hg(II) by bacteria in anoxic sediments (Benoit et al. 1999). In support of this, correlations were found between an optimal sulfide concentration, which promoted the formation of a neutral HgS species, and mercury methylation rates in *Desulfobulbus propionicus* (Benoit et al. 2001, Benoit et al. 1999). Whereas higher sulfide concentrations, resulting in the formation of negatively charged HgS_2H^- and HgS_2^{2-}, were shown to reduce methylation rates.

One has to consider however, that the formation and concentration of HgS is significant only under "ideal" sulfide concentrations. Since Hg(II)-methylation occurs at significant rates under a variety of environmental conditions, the overall relevance of the formation and passive diffusion of HgS remains to be determined. An alternative hypothesis is that the surface of bacterial cells is negatively charged, which would ultimately repel negatively charged Hg(II) species. This would be consistent with the apparent reduced bioavailability of $HgCl_3^-$ and $HgCl_4^{2-}$ in *mer-lux* bioreporter studies (Barkay et al. 1997) and HgS_2H^- and HgS_2^{2-} in studies involving rates of Hg(II)-methylation (Benoit et al. 2001). The repulsion of negatively charged Hg species by the cell surface provides an alternative hypothesis to the decreased bioavailability of Hg(II), which was previously only related to the low lipophilicity of these negatively charged Hg(II) complexes (Benoit et al. 1999). This charged surface could be important for electrostatic interactions with Hg in solution however, and surface complexation models of Hg(II) with gram-negative bacterial cells need to be investigated to confirm this.

4.3.3 Evidence not supporting the FIAM Our only data that was consistent with the FIAM was that the addition of 1mM histidine alleviated Hg(II) toxicity (Figure 4.6), as would be expected from the relatively high affinity of Hg(II) for histidine (Van der Linden and Beers 1973) and the substantial decrease of Hg(II) free-ion concentration and labile Hg(II) species in Assay medium D (Table 4.2). Results from experiments such as this, demonstrating that toxicity was correlated with free-ion concentration, ultimately resulted in the formulation of the FIAM, which some have

extended to make predictions regarding metal uptake into the cell. However, using a *mer-lux* bioreporter it was shown that the addition of histidine had no effect on Hg(II) uptake under aerobic conditions (Figure 4.5A) and substantially enhanced the uptake of Hg(II) under anaerobic conditions (Figure 4.5B), which is in complete contrast with what the toxicity data and the FIAM would have predicted. One possible reason for these differences could be that Hg(II)-histidine complexes shields Hg(II) from binding to weaker cellular ligands, which might have otherwise resulted in toxicity. This coincides with the finding of intracellular histidine reducing the toxicity of Cu^{2+}, Co^{2+}, and Ni^{2+} in *Saccharomyces cerevisiae* (Pearce and Sherman 1999) and Ni^{2+} in plants (Kramer et al. 2000). However, despite not being toxic to the cell, Hg(II)-amino acid complexes could mimic an essential substrate that is accidentally co-transported, which has been demonstrated for the uptake of Hg(II) in mammalian cells (see discussion in Chapter 3, section 3.4.2). The ability of the bioreporter to respond to these complexes would likely stem from the very high affinity of MerR for Hg(II) (Ralston and O'Halloran 1990).

The indiscriminate uptake of $Hg(OH)_2$, $HgCl_2$, $Hg(NH_3)_2^{2+}$, and Hg^{2+} did not support the model of passive diffusion or the FIAM, but was consistent with the model of kinetically controlled facilitated uptake (Hudson et al. 1994). This model proposes that a variety of "labile" species interact with a surface associated transport ligand ($X_{transporter}$). As long as the affinity of the $X_{transporter}$ is stronger than the affinity of Hg(II) to the extracellular complex, and provided the rate of entry of Hg(II) is fast compared with the rate of formation of Hg(II)-$X_{transporter}$, Hg(II) uptake should be relatively insensitive to Hg(II) speciation in the external medium (Hudson 1998).

The uptake of Hg(II) into the cell would therefore likely involve ligand exchange reactions with sulfhydryl groups present in transport systems. For example, the uptake of Hg(II) into the cell might involve transport systems specific for other heavy metals, as demonstrated previously for the uptake of other non-essential heavy metals (see discussion in Chapter 1, section 1.5.2). If the expression of these systems are effected by the presence or absence of O_2, then the differences in Hg(II) uptake under aerobic and anaerobic conditions could potentially be explained. Whereas sites of toxicity outside or on the cytoplasm might not be affected under these two conditions, which would also explain why no substantial differences in toxicity were seen.

4.3.4 Cellular limitations for the FIAM In addition to cellular limitations to passive diffusion (discussed in Chapter 1, section 1.5.1), the structure of the bacterial cell also poses challenges to the FIAM that need to be addressed. For the free ion activity model, only one parameter is usually considered, which in the case of transport would be the K_M of the transporter. However, taking into consideration the entire bacterial cell, there are seemingly endless possible sites where Hg(II) interactions can occur before it even reaches the transport site. Such cellular interactions would ultimately affect the diffusion rates of metals and metal complexes to the transporter, which may lead to non-equilibrium conditions (Pinheiro and Van Leeuwen 2001). Secondly, the periplasm is separated from the surrounding environment by the outer membrane, and therefore should be treated as a separate microenvironment. Therefore, internally bound metals in the periplasm would not be in thermodynamic equilibrium with the external medium (Williams and Frausto da Silva 2000).

The potential strong binding capacity of the periplasm for Hg would affect the kinetics of ligand exchange with transporter sites. For the free ion model, the dissociative mechanism of ligand exchange would only be significant if the ligand is weakly bound (Bell et al. 2002). However, the speciation of Hg in the periplasm would likely be controlled by thiol containing molecules, such as glutathione and cysteine. As the affinity of potential ligands for Hg(II) increases, an associative mechanism of ligand exchange becomes more probable (Bell et al. 2002). Therefore, the relative rates of ligand exchange would be dependent on the relative binding strengths of Hg(II) for the ligand, L1, and $X_{transporter}$.

In the periplasm, Hg(II) binding can result in additional steric, or spatial factors, which can also affect the rate of metal ligand exchange (Bell et al. 2002). For example, if L1 is a large spatially demanding ligand, such as a periplasmic protein, then it may be restricted in making a bond with the transporter and the reaction rate would ultimately decrease. Not considered in any kinetic models however, is the binding of metals by specific periplasmic proteins, metallochaperones, which aid in the transport of the metal to cytoplasmic membrane. These metallochaperones actually increase the rates of metal uptake by increasing the likelihood that the metal will interact with a transport system in the cytoplasmic membrane (reviewed in O'Halloran and Culotta 2000).

MerP, which is encoded by the *mer* operon, is one example of a Hg(II) metallochaperone. MerP contains a cysteine metal binding motif (Powlowski and Sahlman 1999), which is also found in a number of other metal binding proteins including cadmium and copper (DeSilva et al. 2002), and acts as a scavenger for

Hg(II) in the periplasm (Hamlett et al. 1992). Following binding of Hg(II), MerP then chaperones the metal to the cytoplasmic transport system MerT, which also contains cysteine residues that are essential for ligand exchange and transport (Morby et al. 1995). MerP exchanges the bound Hg ion with MerT by an associative mechanism involving the sulfhydryl groups of the cysteine residues (Figure 1.2). Following ligand exchange, Hg is then transported into the cytoplasm where additional cysteine thiol ligands in mercuric reductase binds Hg(II) (Engst and Miller 1999) and detoxifies it by reduction to the volatile Hg(0) species (Fox and Walsh 1982). Therefore, Hg(II) is never "free" inside the cell, but is rather controlled by thiol-thiol ligand exchange reactions. The expression of a periplasmic chaperone for the transport of Hg(II) demonstrates the potential diffusion limitations and sequestering capabilities of the periplasm. Therefore, the kinetics of the transport systems alone would not account for the overall rates of metal uptake in the cell. The study of metallochaperones is still in its infancy, but poses yet another challenge for the FIAM in bacterial cells.

The FIAM also assumes that the rates of dissociation and formation of metals with the transporter are fast in comparison to the uptake of the metal. However, heavy-metal ions, both essential and non-essential, can be taken up by constitutively expressed transport systems that are unspecific and fast (Nies et al. 1999). For such transport systems, the internalization rates would be much larger than the dissociation rate (Stumm and Morgan 1996). In this case, the affinity of the transporter is controlled by kinetics rather than the equilibrium constant of the transporter site (Hudson 1998), which has severe implications for the FIAM (Hudson 2005).

Additional types of metal transport systems have high substrate specificity, slower uptake rates, and are only expressed by the cell in times of need, starvation or a special metabolic situation (Nies et al. 1999). Therefore, these transport systems are inducible and the concentration of $X_{transporter}$ would not be constant, but would be dependent on the physiological needs of the bacterial cell. Therefore, differences in water chemistry affect not only the speciation of metals, but also the physiology of the organism under study. Physiological considerations have often been overlooked in speciation models, but would have a significant impact on the types and amounts of different transport systems and hence, kinetics of metal uptake.

The ability to predict Hg(II) uptake into the cytoplasm of the bacterial cell is very important since this is where the enzymes for the bacterial mediated transformations of Hg(II) are located. In bacterial cells however, numerous sites for Hg(II) toxicity could potentially exist outside of the cytoplasm, which could include Hg(II) interactions with the outermembrane, within the periplasm, and at the cytoplasmic membrane. Hg(II) bound to any of these sites outside of the cytoplasm would not be available to the enzymes responsible for Hg(II) transformations, including the *mer-lux* bioreporter. Therefore, predictions regarding Hg(II) uptake on the basis of toxicity data alone might not be applicable for our purpose of predicting Hg(II) uptake into the cell. Therefore, we suggest that the results obtained solely from toxicity assays be used with caution as predictors of uptake into the cell. Since our results implicated facilitated uptake of Hg(II) and not passive diffusion, the mechanism(s) of Hg(II) uptake and how different environmental parameters might affect this process(es) need to be addressed in bacteria before accurate and more

reliable predictions can be made in future modeling studies.

Chapter 5

Evidence for secondary transport systems for the "accidental" uptake of Hg(II) in *E. coli* HMS174

5.1 Introduction

Much effort has been made in predicting the bioavailability of Hg to bacteria in aquatic ecosystems on the basis of modeling the chemical speciation of Hg in the bulk solution. However, in Chapter 4 we provided evidence against two widely accepted models, passive diffusion and the FIAM, for predicting metal availability in aquatic ecosystems. Taking into consideration the complexity of gram-negative bacteria, we can speculate that the passive diffusion of "labile" lipophilic and the 'free ion" species formed in the bulk solution would be less important than previously believed. The mechanism(s) of Hg uptake needs to be determined in bacteria not expressing the *mer*TPC transport system. This in turn would greatly increase our predictive ability of Hg(II) in aquatic ecosystems and could possibly provide alternative remediation strategies for Hg contaminated ecosystems.

A number of essential metabolic processes in bacteria require μM concentrations of heavy metal ions. However, many of these essential metals are only present in nM concentrations in the environment. Therefore, the ability to accumulate metal ions, in often-metal limiting environments, is of uttermost importance. Satisfying the metal requirements of the bacterial cell is achieved by a variety of transport systems. The first type of system is highly specific for the metal, generally ATP-dependent, and is only induced during times of need (Nies and Silver 1995). The second type of system is constitutively expressed, driven by the chemiosmotic gradient, and relatively non-specific for metal ions. Together these systems are capable of accumulating high concentration of metals (mM) inside the cell at fast rates (Nies and Silver 1995). The constitutive non-specific systems are

important in satisfying a wide range of metal requirements of the cell. However, high intracellular concentrations of heavy metal ions can be deleterious to the cell. In addition, the non-specificity of these systems can result in the transport of toxic non-essential heavy metals. Since these transport systems are not regulated, they are a likely reason why heavy metal ions are toxic to microorganisms (Nies and Silver 1995).

To alleviate the potential toxicity of metal ions, bacteria have evolved detoxification systems to maintain metal homeostasis. The most common detoxification mechanism involves P-type ATPase efflux pumps, which extrude metals such as Pb(II), Cd(II), Zn(II), Cu(I), and Co(II) from the cell (reviewed in Rosen 2002, Nies 1999, Silver and Phung 1996). Regulation of these systems involves transcriptional regulators of the MerR family, including CoaR for the Co(II) efflux system CoaT (Rutherford et al. 1999), ZntR for the Zn(II) efflux system ZntA (Brocklehurst et al. 1999), CadR for the Cd(II) efflux system CadA (Lee et al. 2001), and CueR for the Cu(I) efflux system CopA (Stoyanov et al. 2001). As with MerR, these regulators contain helix-turn-helix DNA binding motifs, act on promoters with extended spacer regions, and are inducible by the presence of specific intracellular metals.

The *mer* operon shares many similarities to these detoxification systems, but instead of efflux pumps Hg(II) is transported into the cell and then reduced by mercuric reductase. As with other metal detoxification systems, Hg(II) must first enter the cell to activate MerR, which then induces expression of the transport and detoxification genes. Hobman and Brown (1997) have suggested that the initial

accumulation of Hg(II) might be accomplished either by low background levels of expression of transport systems of the *mer* operon or via passive diffusion of lipophilic species. Another possibility, however, is that secondary low affinity uptake systems for Hg(II) also exist.

Transport systems of many essential heavy-metal ions contain metal binding motifs consisting of cysteine and histidine residues. Binding of Hg to these ligands could potentially be too strong, which can result in the abolition of essential metal transport. However, if Hg binding does not interfere with the structural integrity of the protein, it might also result in the non-specific uptake of Hg. NMR spectroscopy data shows that Hg has the ability to bind to a variety of different cysteine configurations in vitro (DeSilva et al. 2002). Due to the higher affinity of Hg for these residues it seems logical that transport systems specific for other metals would also bind Hg, but as to whether these systems can also transport Hg is unknown in bacterial cells.

Hg(II) has neither been considered nor examined in bacterial metal transport studies that demonstrated non-specific uptake of other metals. This might be a reflection of the assumption that Hg only diffuses passively into the cell, the high-cost of Hg isotopes, or the difficulty in working with Hg because of its high affinity for cellular ligands. Another difficulty is that Hg(II) is very toxic at concentrations in which other metals are studied during transport experiments. Therefore, because of the limitation of detection using conventional means (e.g. radiolabelling) Hg uptake might not be detectable below its toxicity threshold.

In an attempt to determine if secondary transport systems for Hg(II) exist in bacteria we investigated the effects that the chemiosmotic uncoupler CCCP, and various competing metal ions have on Hg(II) uptake. However, *V. anguillarum*, which was the focus of my initial research, is a natural isolate that has not been well characterized in the laboratory. Therefore, *E. coli* was chosen as the model organism to study Hg(II) uptake for the remainder of my research, since the entire genome has been sequenced (Blattner et al. 1997) and the genetics and physiology of this bacterium have been intensively studied.

5.2 Additional Methods

The aerobic and anaerobic *mer-lux* bioreporter and toxicity assays in *E. coli* HMS174 were carried out as described in Chapter 2 (section 2.5). In the uncoupler experiments however, Hg(II) was added last to initiate the assays. This was done to allow time for CCCP to first dissipate the H^+ gradient in *E. coli* before being exposed to Hg(II). For all the assays presented in this chapter the assay medium was pH 7 and consisted of 5 mM glucose, 9 mM $(NH_4)_2SO_4$, and 6.7 mM PO_4, unless otherwise stated in the figure legend. Metals used in the toxicity and mitigation experiments are outlined in Table 5.1.

5.3 Results

5.3.1 Effect of CCCP on Hg(II) uptake Many transport systems are driven by the chemiosmotic gradient across the cytoplasmic membrane (Nies 1999). The protonophore CCCP is a commonly used uncoupler that has been used to establish the

Table 5.1. List of metals used in the toxicity and bioreporter assays.

Metals	Brand	Grade
$FeSO_4 \cdot 7H_2O$	BDH	Analytical
$CaCl_2 \cdot 2H_2O$	BDH	Analytical
$NiCl_2 \cdot 6H_2O$	BDH	Analytical
$ZnSO_4 \cdot 7H_2O$	BDH	Analytical
$MnCl_2 \cdot 4H_2O$	BDH	Analytical
$Na_2MoO_4 \cdot 2H_2O$	BDH	Analytical
$CdCl_2$	Sigma	ACS
$AlK(SO_4)_2$	Sigma	Ultra
Na_2SeO_3	Sigma	Ultra
Na_2SeO_4	Sigma	Ultra
$CuSO_4 \cdot 5H_2O$	Sigma	ACS
$MgSO_4 \cdot 7H_2O$	Fisher	ACS

transport of solutes across biological membranes that derive their energy from the H^+ proton gradient. Therefore, to determine if the energetics of Hg(II) uptake in bacteria was dependent on the H^+ proton gradient we tested the effect that varying concentrations of CCCP had on the uptake of Hg(II) under anaerobic and aerobic conditions.

Under anaerobic conditions CCCP concentrations of 0.125 – 1 μM resulted in a significant decrease in light production from *E. coli* HMS174 (pRB28) (Figure 5.1A). However, light production from *E. coli* HMS174 (pRB27) also decreased significantly using these same concentrations of CCCP (Figure 5.1A). Therefore, the lessened Hg(II)-dependent light response to 15 pM Hg(II) under anaerobic conditions appeared to be due to a decrease in cellular energetics required for light emission, and not with decreased Hg(II)-uptake in the presence of CCCP. Under aerobic conditions however, concentrations up to 1 μM CCCP did not affect constitutive light production from *E. coli* HMS174 (pRB27) whereas CCCP concentrations as low as 0.0625 μM had a significant effect on the uptake of 15 pM Hg(II), as indicated from *E. coli* HMS174 (pRB28) (Figure 5.1B).

5.3.2 Metal toxicity The potential requirement of a H^+ gradient for Hg(II) uptake under aerobic conditions, along with the differences observed between aerobic and anaerobic uptake (see Chapter 3 and 4), and the inorganic speciation data not supporting passive diffusion (see Chapter 4), all strongly support facilitated mechanism(s) for Hg(II) transport in *E. coli*. In an attempt to determine possible transport systems that might be involved in Hg(II) uptake, competition experiments involving various concentrations (0.0001 μM – 100 μM) of various metals and 10 pM

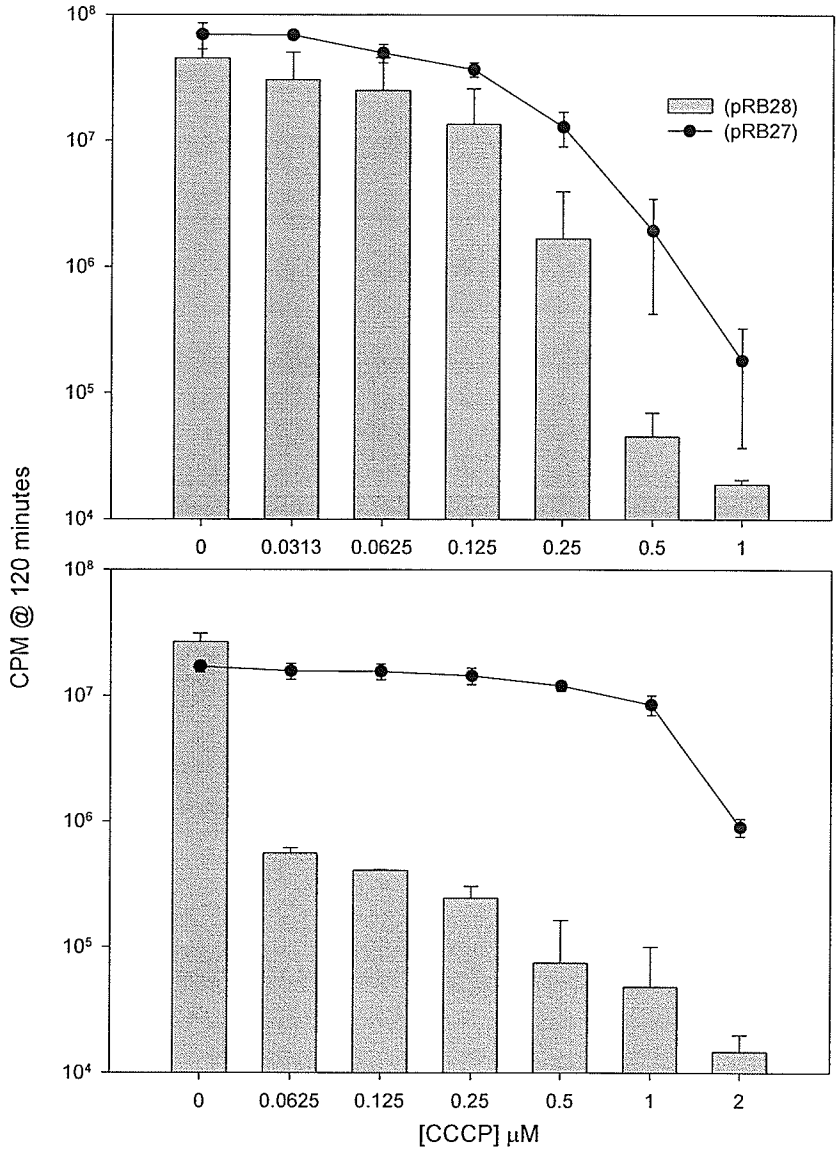

Figure 5.1. Effect of varying concentrations of CCCP on Hg(II) induced light production (pRB28) and constitutive light production (pRB27) under A) anaerobic and B) aerobic conditions in *E. coli* HMS174. Samples were exposed to 15 pM Hg(II) for 120 minutes prior to light measurement. Error bars in the anaerobic assay represent the standard deviation of 4 independent assays for (pRB28) (n=8) and 3 independent assays for (pRB27) (n=9). In the aerobic assays the error bars represent the standard deviation of 5 independent assays for (pRB28) (n=10) and 4 independent assays from (pRB27) (n=12).

Hg(II) were performed using *E. coli* HMS174 (pRB28). Constitutive light production from *E. coli* HMS174 (pRB27) was first used as an indicator of toxicity of the varying concentration of metals to be used in the mitigation studies. The following metals did not appear toxic to *E. coli* HMS174 (pRB27): Mg(II) (Figure 5.2A), Fe(III) (Figure 5.3A), Ca(II) (Figure 5.4A), Al(III) (Figure 5.5A), Se(VI) (Figure 5.6A), Se(IV) (Figure 5.7A), and Mo(VI) (Figure 5.8A), as light production was unperturbed in samples containing up to 100 μM. At a concentration of 100 μM there was an observed decrease in light production for Mn(II) (Figure 5.9A), Zn(II) (Figure 5.10A), and Cd(II) (Figure 5.11A), whereas this concentration resulted in substantial decrease in light production for Cu(II) (Figure 5.12A) and Ni(II) (Figure 5.13A). Hg(II) was by far the most toxic and completely eliminated light production in medium containing concentrations as low as 3 nM (Figure 5.14). Ni(II) was the second most toxic of the metals tested, as reduction in light production was observed using concentrations as low as 0.1 μM (Figure 5.13A).

5.3.3 Metal mitigation of Hg(II) uptake Using *E. coli* HMS174 (pRB28) as an indicator of Hg(II) uptake we found that increasing concentration of a variety of metals appeared to mitigate the uptake of 10 pM Hg(II). Substantial reductions in a Hg(II)-dependent light response were observed using concentrations as low as 0.001 μM Cu(II) (Figure 5.12B) and Mn(II) (Figure 5.9B), 0.01-0.1 μM Mg(II) (Figure 5.2B), 0.1 μM Ni(II) (Figure 5.13B), 1 μM Fe(III) (Figure 5.3B), and 10 μM Ca(II) (Figure 5.4B). For Al(III) (Figure 5.5B) and Se(IV) (Figure 5.7B), a slight reduction was observed using a concentration of 100 μM. No decrease was observed using concentrations up to 100 μM for Mo(VI) (Figure 5.8B) or Se(VI) (Figure 5.6B).

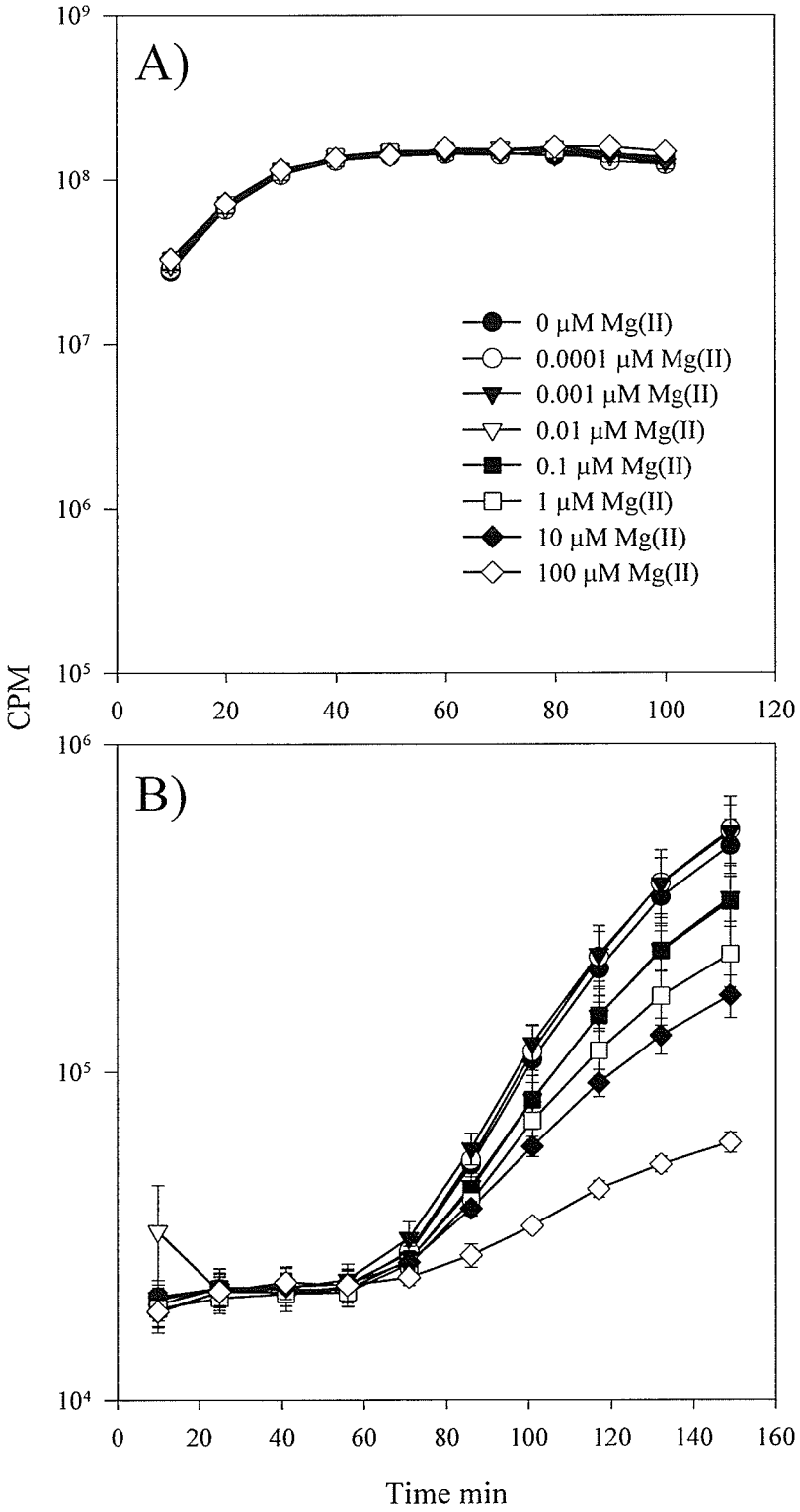

Figure 5.2. Effect of varying the concentration of Mg(II) on A) constitutive light production in *E. coli* HMS174 (pRB27) and B) Hg(II)-induction in *E. coli* HMS174 (pRB28) in assay medium containing 10 pM Hg(II). Error bars represent the standard error of two independent assays (n=6).

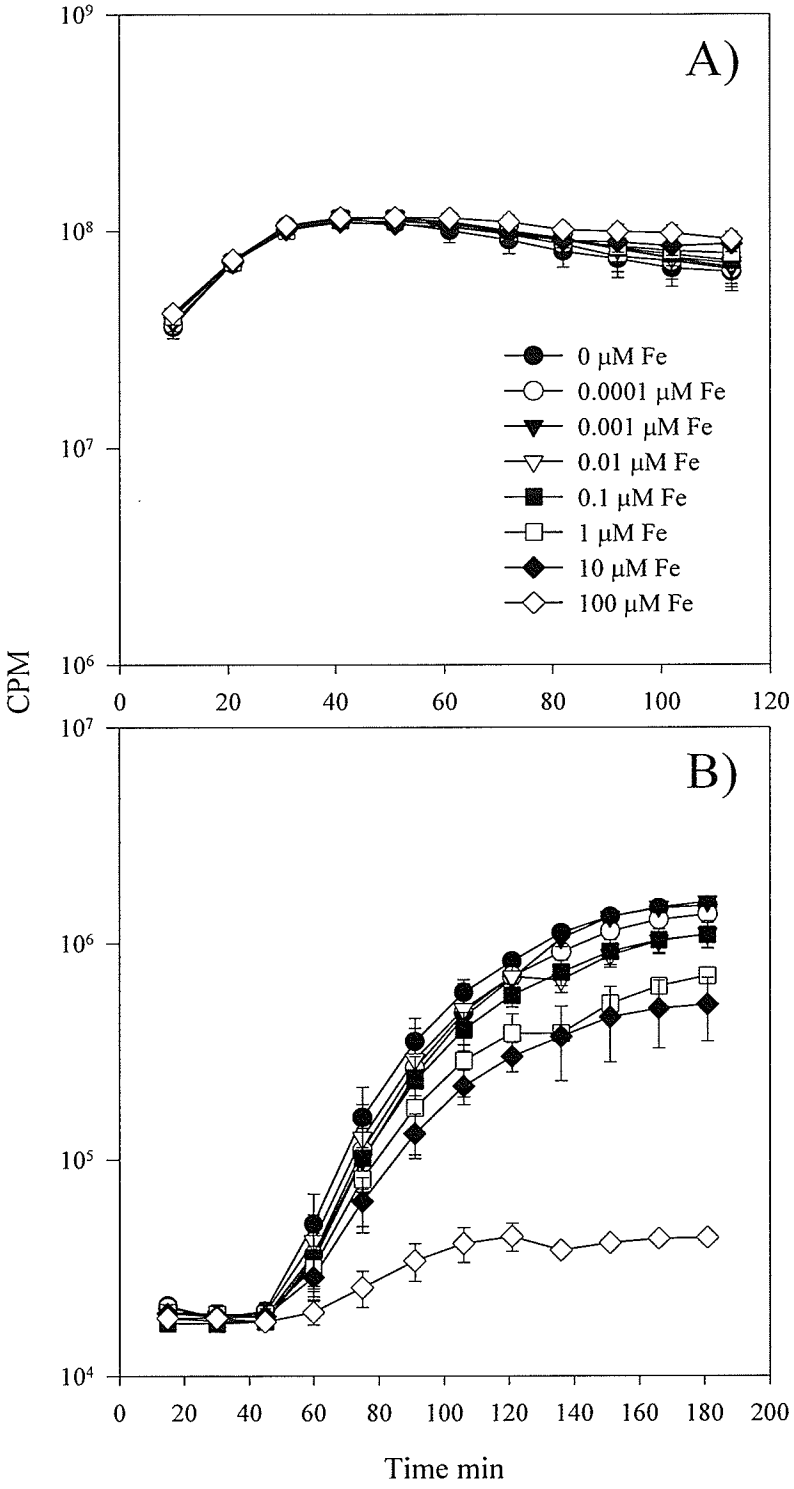

Figure 5.3. Effect of varying the concentration of Fe(III) on A) constitutive light production in *E. coli* HMS174 (pRB27) and B) Hg(II)-induction in *E. coli* HMS174 (pRB28) in assay medium containing 10 pM Hg(II). Error bars represent the standard error of two independent assays (n=6).

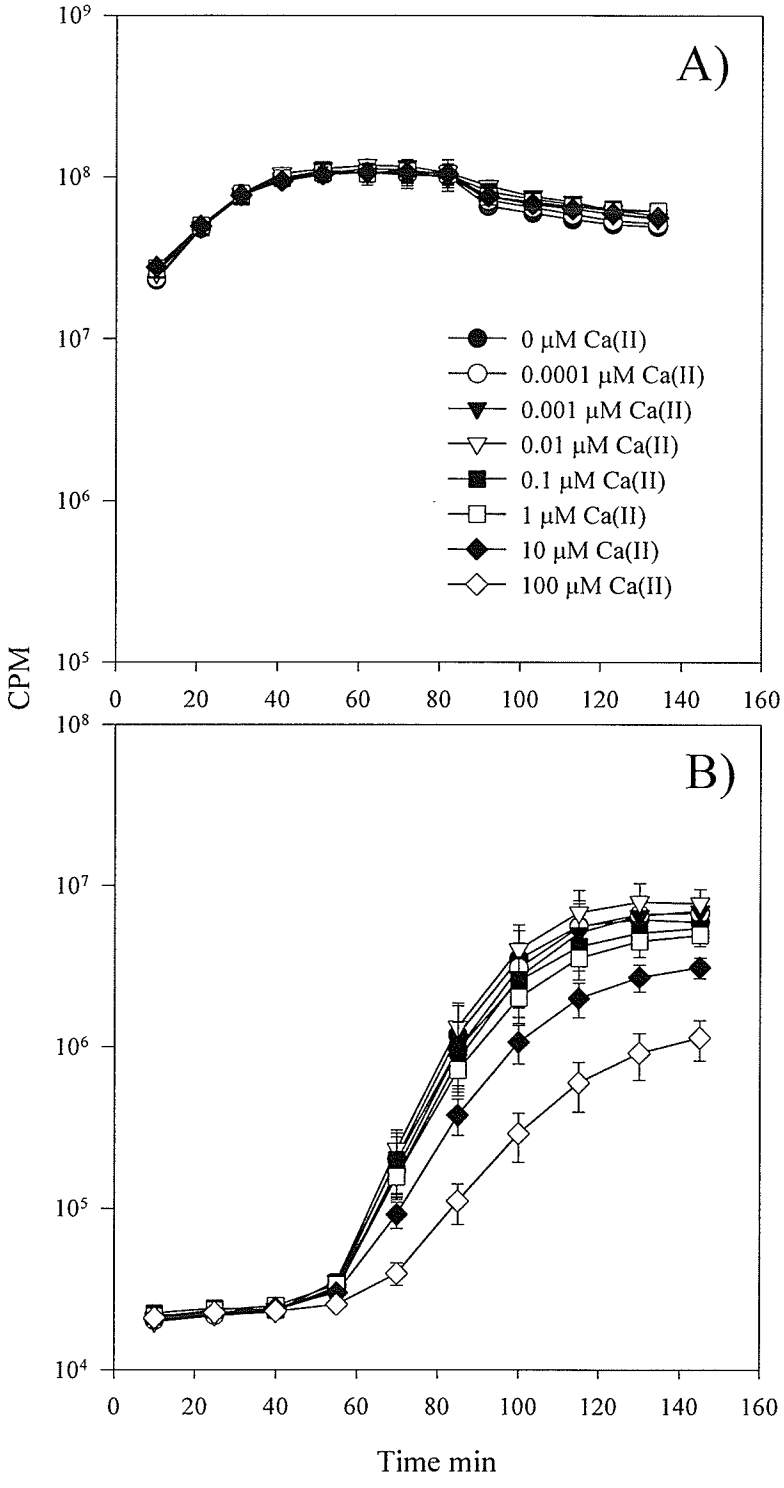

Figure 5.4. Effect of varying the concentration of Ca(II) on A) constitutive light production in *E. coli* HMS174 (pRB27) and B) Hg(II)-induction in *E. coli* HMS174 (pRB28) in assay medium containing 10 pM Hg(II). Error bars represent the standard error of two independent assays (n=6).

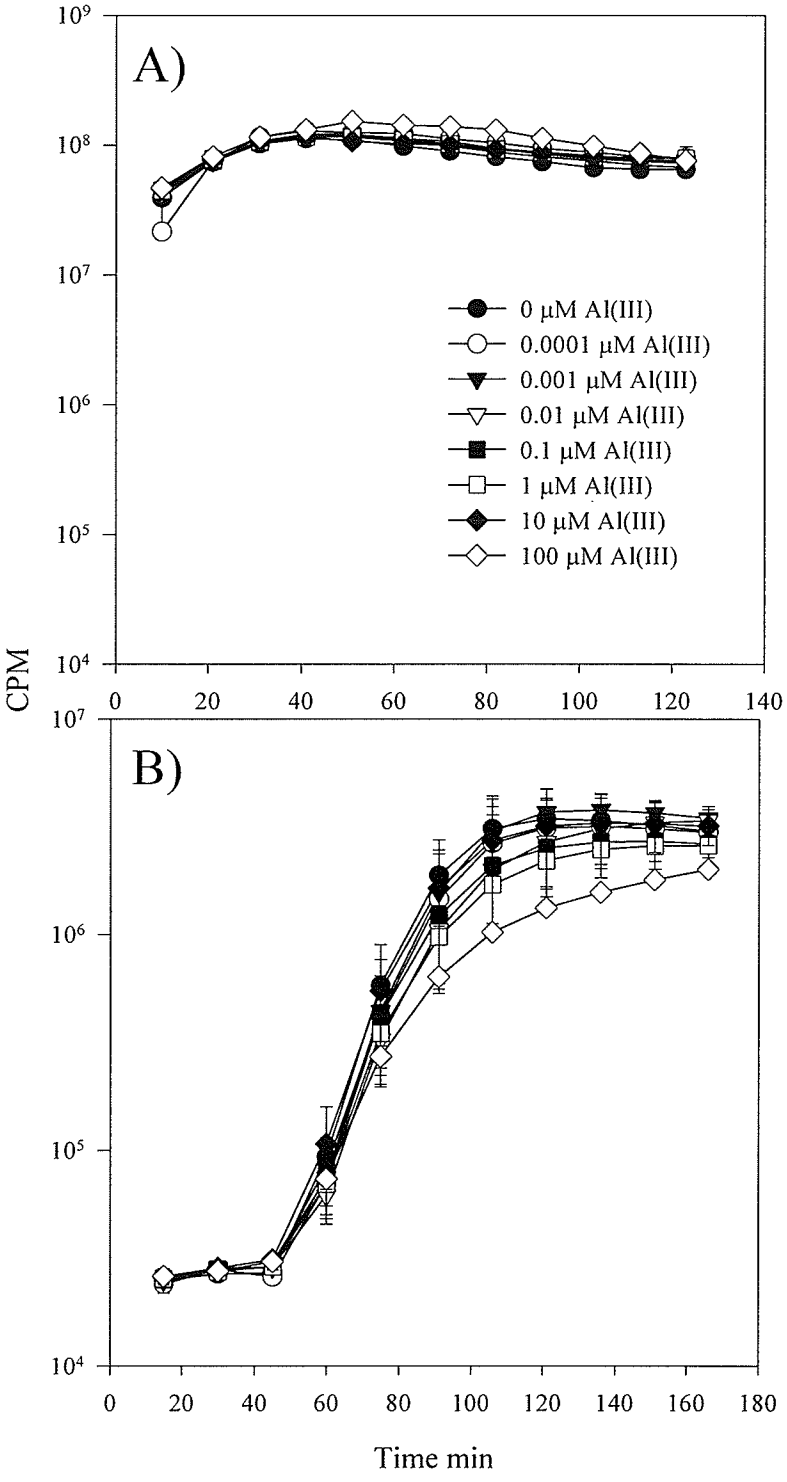

Figure 5.5. Effect of varying the concentration of Al(III) on A) constitutive light production in *E. coli* HMS174 (pRB27) and B) Hg(II)-induction in *E. coli* HMS174 (pRB28) in assay medium containing 10 pM Hg(II). Error bars represent the standard error of two independent assays (n=6).

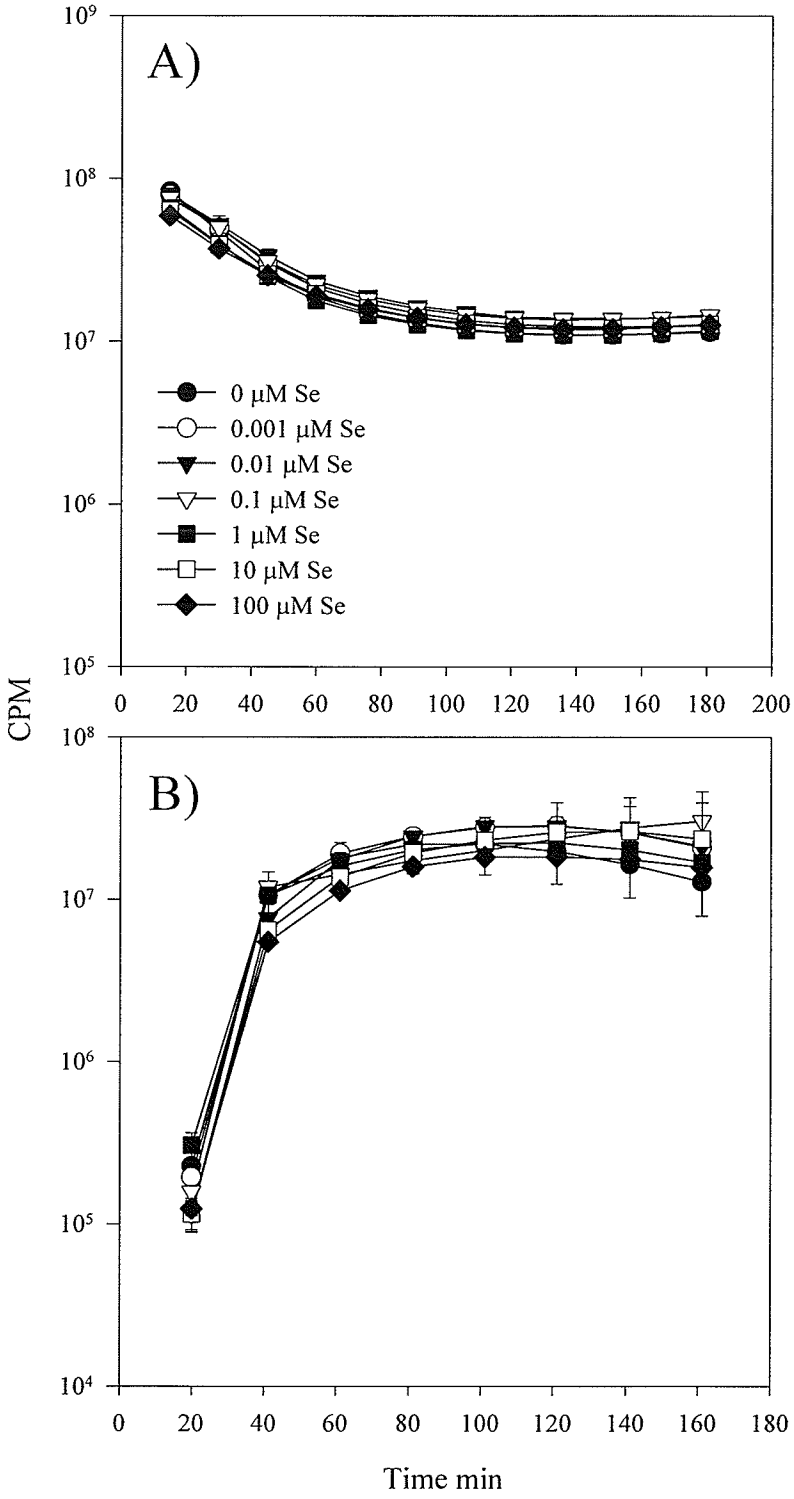

Figure 5.6. Effect of varying the concentration of Selenate on A) constitutive light production in *E. coli* HMS174 (pRB27) and B) Hg(II)-induction in *E. coli* HMS174 (pRB28) in assay medium containing 10 pM Hg(II). Error bars represent the standard error of triplicate samples.

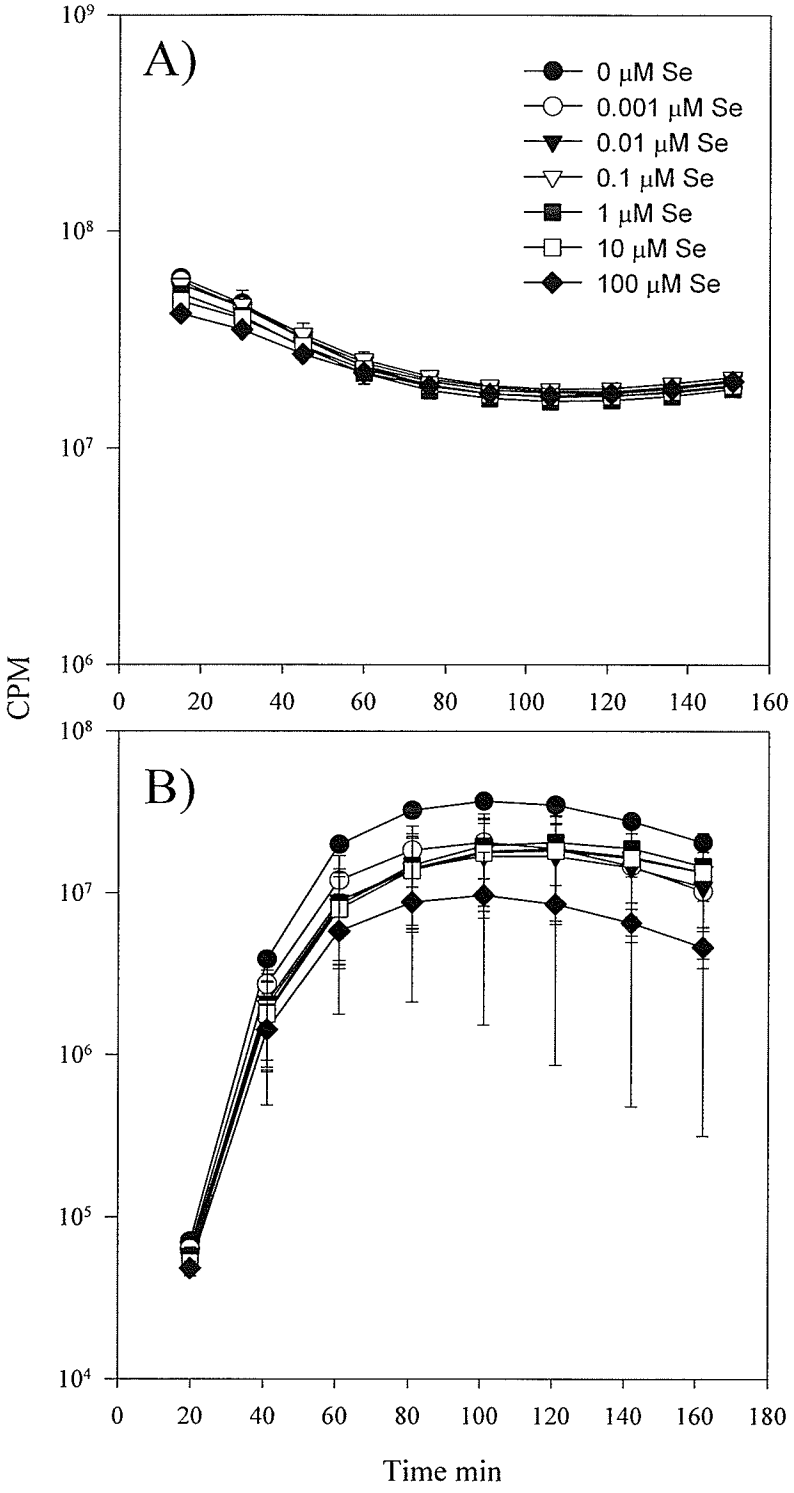

Figure 5.7. Effect of varying the concentration of selenite on A) constitutive light production in *E. coli* HMS174 (pRB27) and B) Hg(II)-induction in *E. coli* HMS174 (pRB28) in assay medium containing 10 pM Hg(II). Error bars represent the standard error of triplicate samples.

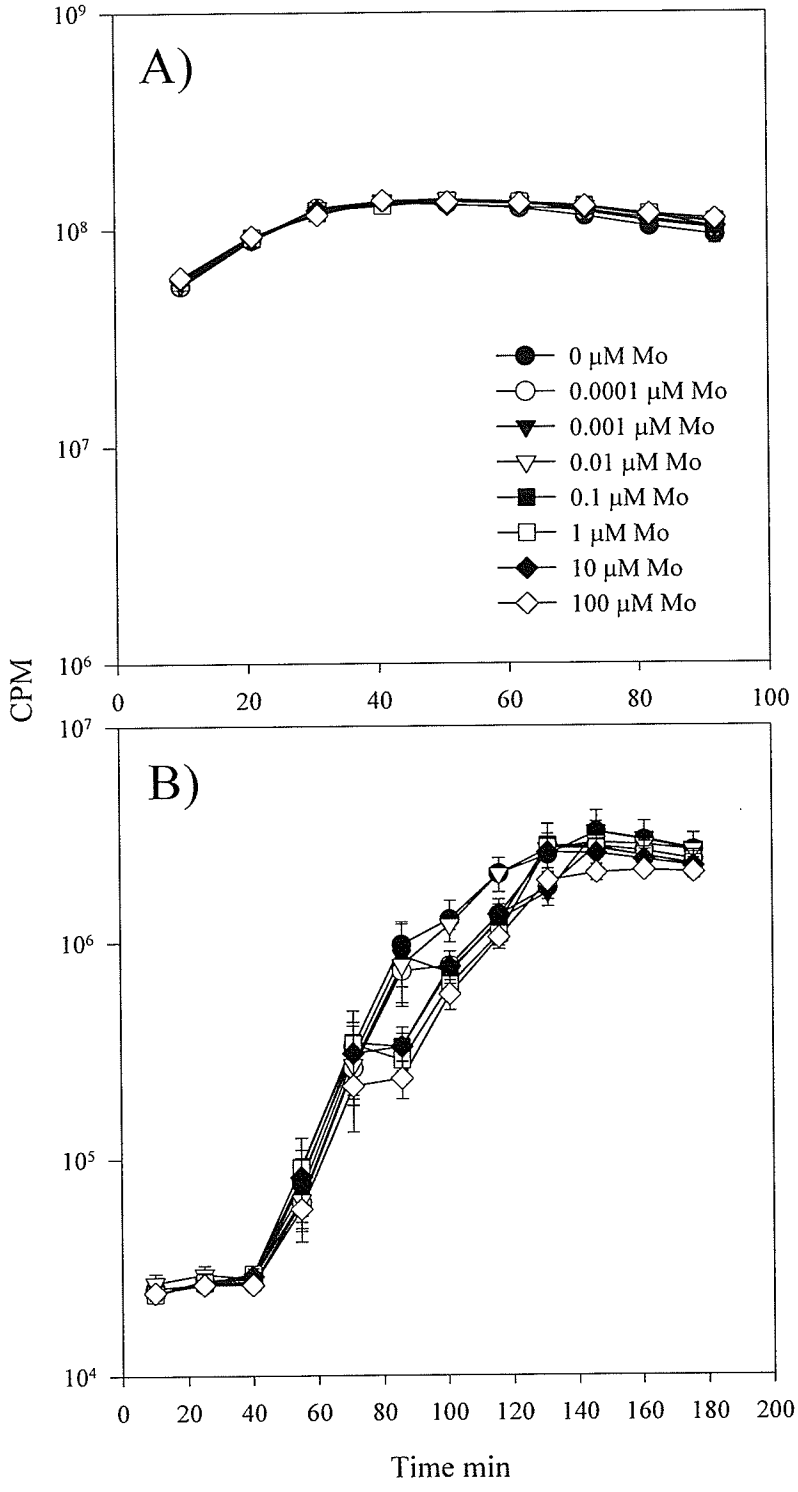

Figure 5.8. Effect of varying the concentration of Mo on A) constitutive light production in *E. coli* HMS174 (pRB27) and B) Hg(II)-induction in *E. coli* HMS174 (pRB28) in assay medium containing 10 pM Hg(II). Error bars represent the standard error of two independent assays (n=6).

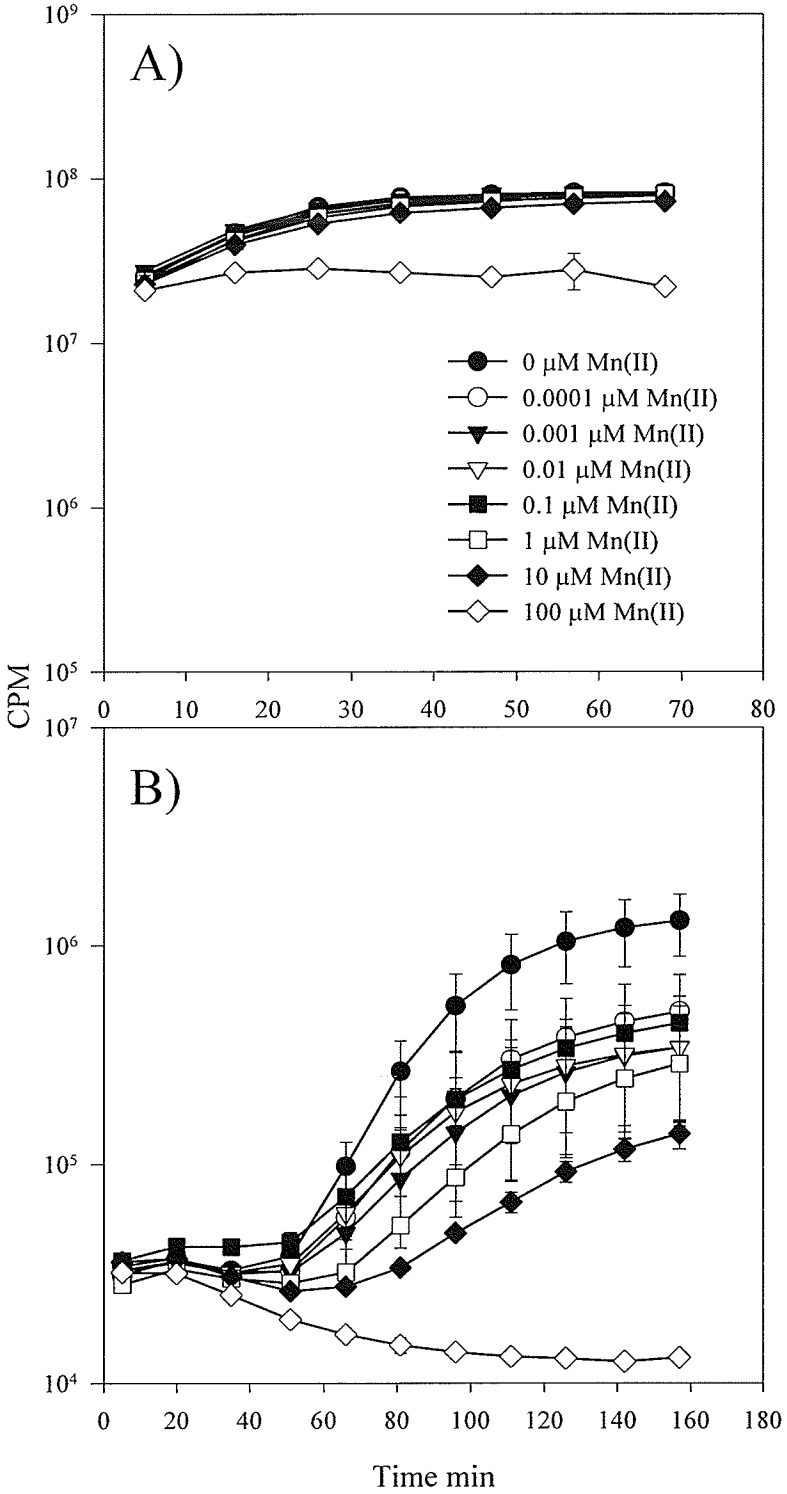

Figure 5.9. Effect of varying the concentration of Mn(II) on A) constitutive light production in *E. coli* HMS174 (pRB27) and B) Hg(II)-induction in *E. coli* HMS174 (pRB28) in assay medium containing 10 pM Hg(II). Error bars represent the standard error of two independent assays (n=6).

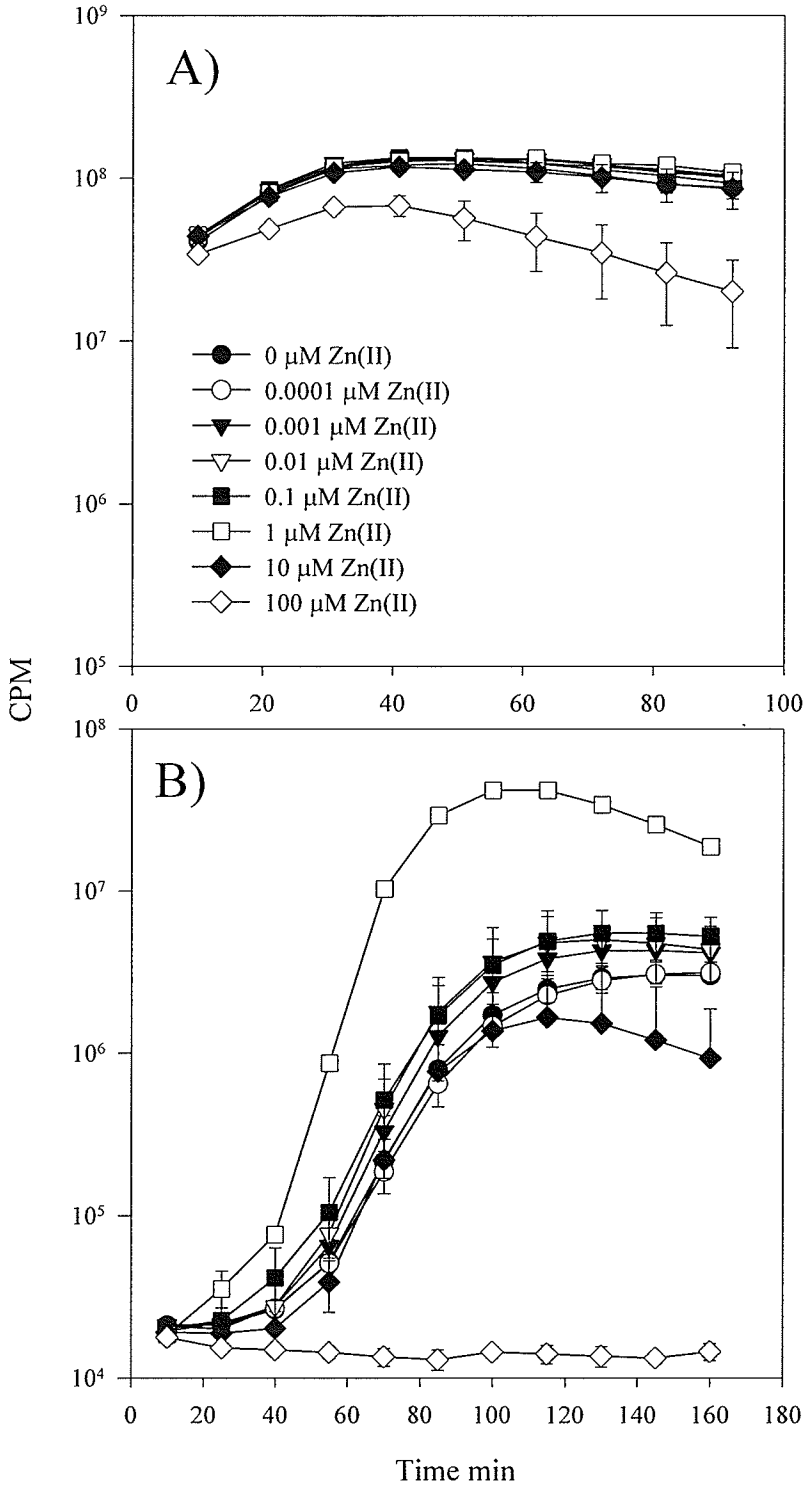

Figure 5.10. Effect of varying the concentration of Zn(II) on A) constitutive light production in *E. coli* HMS174 (pRB27) and B) Hg(II)-induction in *E. coli* HMS174 (pRB28) in assay medium containing 10 pM Hg(II). Error bars represent the standard error of two independent assays (n=6).

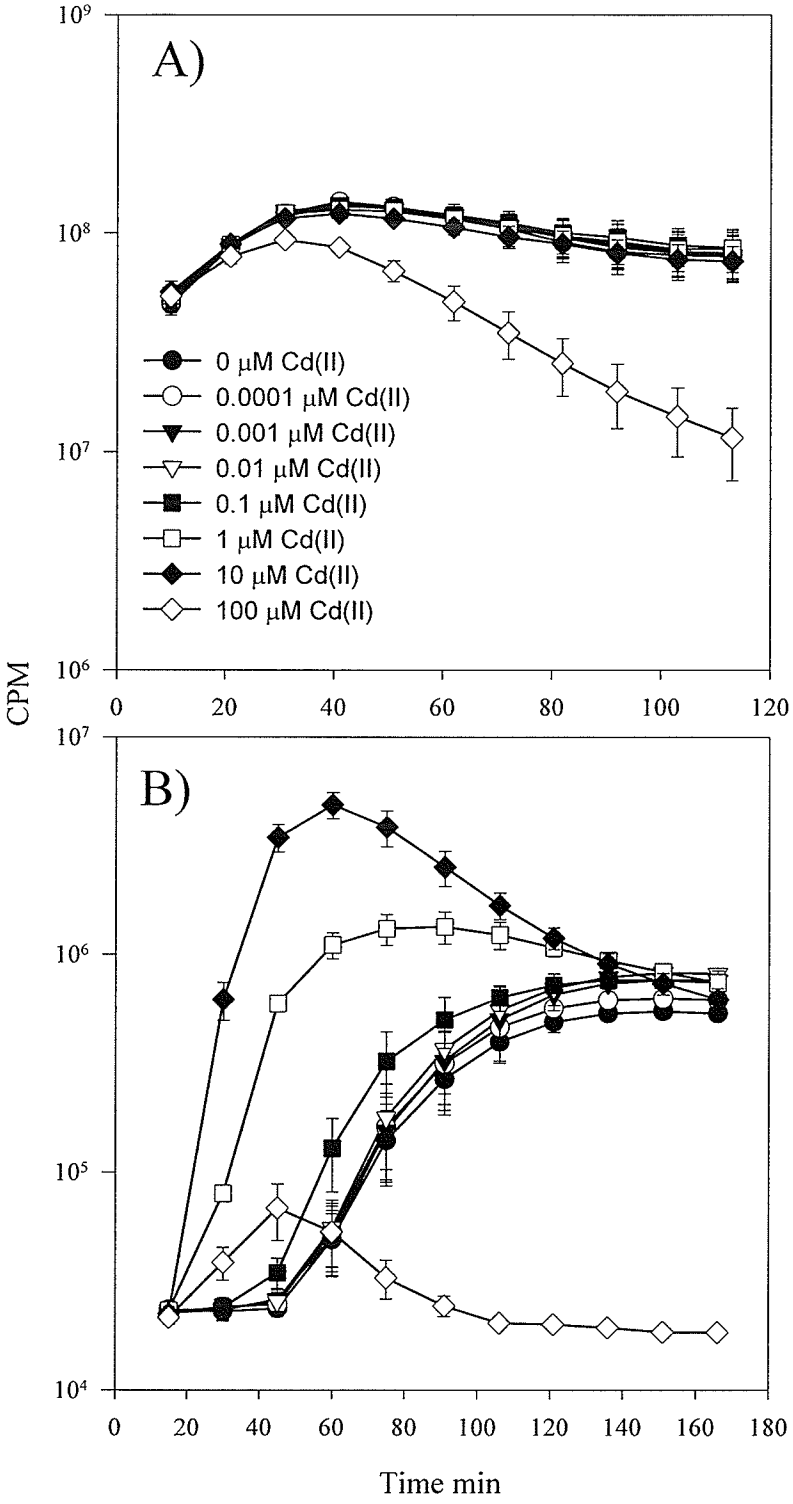

Figure 5.11. Effect of varying the concentration of Cd(II) on A) constitutive light production in *E. coli* HMS174 (pRB27) and B) Hg(II)-induction in *E. coli* HMS174 (pRB28) in assay medium containing 10 pM Hg(II). Error bars represent the standard error of two independent assays (n=6).

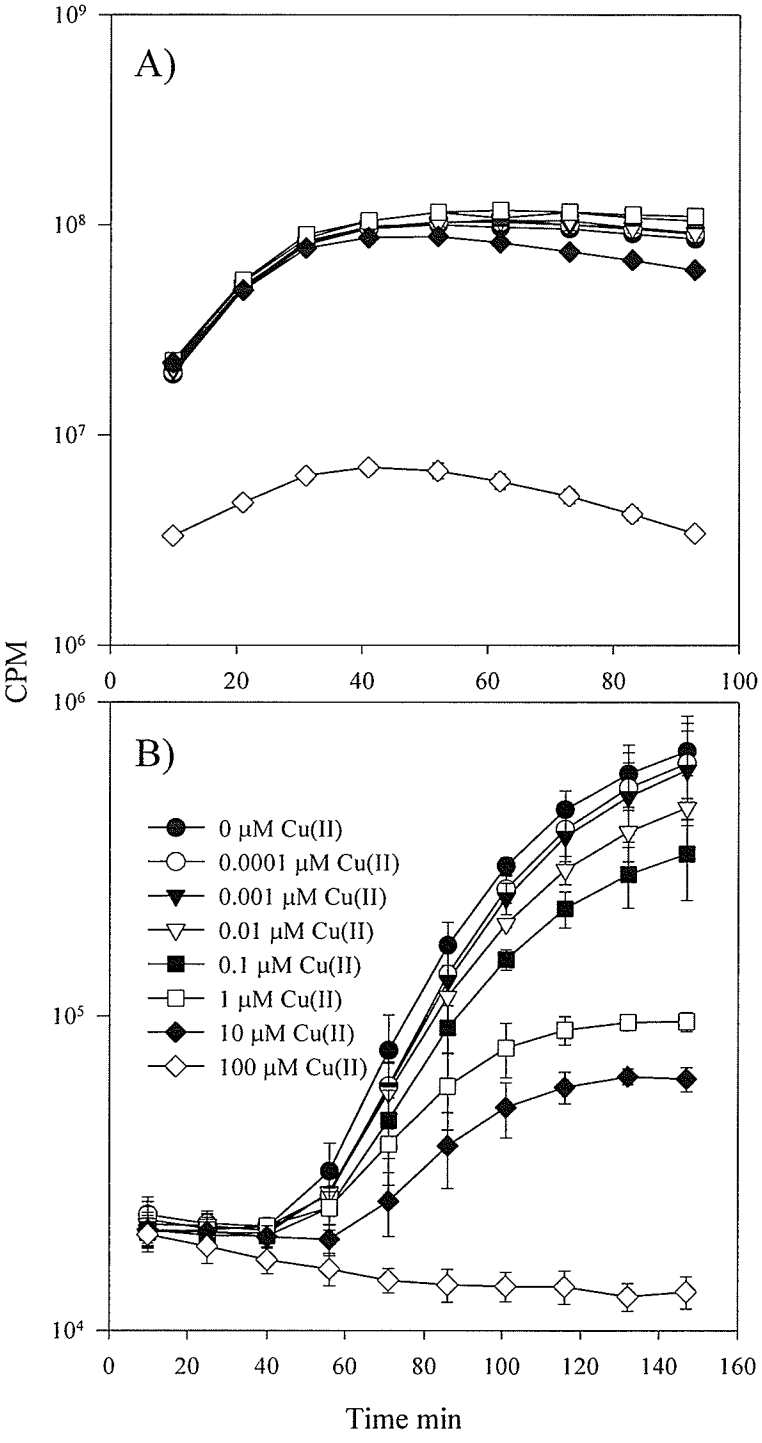

Figure 5.12. Effect of varying the concentration of Cu(II) on A) constitutive light production in *E. coli* HMS174 (pRB27) and B) Hg(II)-induction in *E. coli* HMS174 (pRB28) in assay medium containing 10 pM Hg(II). Error bars represent the standard error of two independent assays (n=6).

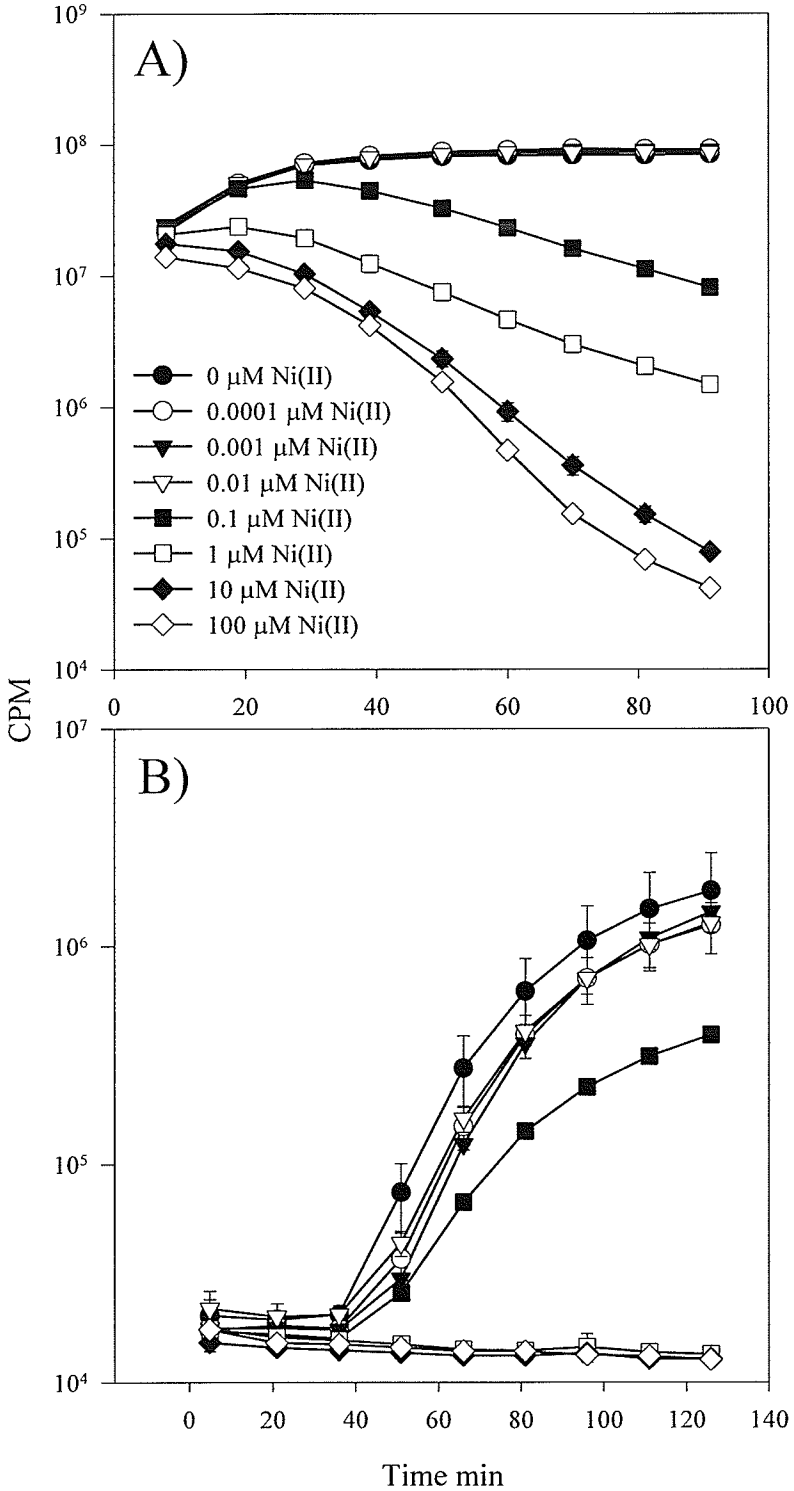

Figure 5.13. Effect of varying the concentration of Ni(II) on A) constitutive light production in *E. coli* HMS174 (pRB27) and B) Hg(II)-induction in *E. coli* HMS174 (pRB28) in assay medium containing 10 pM Hg(II). Error bars represent the standard error of two independent assays (n=6).

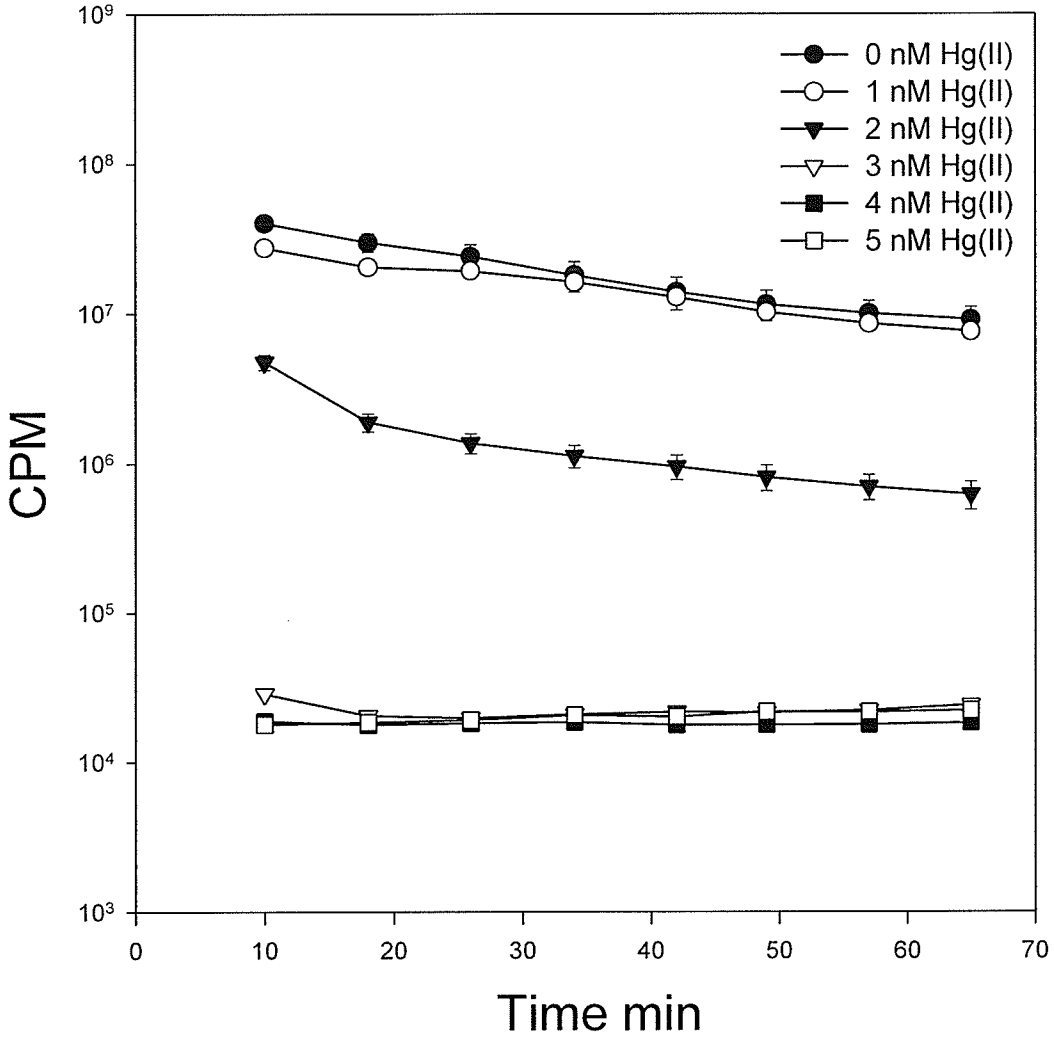

Figure 5.14. The effect of varying concentration of Hg(II) on light production in *E. coli* HMS174 (pRB27). Error bars represent the standard deviation of triplicate samples.

Caution must be used for the interpretation of the results from the bioreporter (pRB28) using metal concentrations that were shown to reduce light production from the constitutive control (pRB27). For example, in the bioreporter assays 0.1 μM Ni(II) drastically decreased the Hg(II)-dependent light response, whereas Ni(II) concentrations ranging from 1-100 μM completely eliminated the response (Figure 5.13B). However, these concentrations were somewhat toxic to the cell, indicated by decreased light emission from the constitutive control (pRB27) (Figure 5.13A).

Mitigation of Hg(II) uptake in the presence of either Cd(II) or Zn(II) was inconclusive since these two metals stimulated the bioreporter response. Concentrations of 0.1 – 10 μM for Cd(II) (Figure 5.11B) and 0.1 – 1 μM Zn(II) (Figure 5.10B) increased light production in samples containing 10 pM Hg(II). Cd(II) concentration of 100 μM also resulted in an increase in the time required for induction, but quickly decreased after 40 minutes to background levels. Zn(II) concentration of 10 μM decreased the bioreporter response after 90 minutes, and completely eliminated any response in samples containing 100 μM.

5.3.4 Anaerobic metal toxicity and mitigation of Hg(II) uptake Since we observed differences in the effect of CCCP on the uptake of Hg(II) under aerobic and anaerobic conditions, it might be possible that different transport systems that are regulated in response to O_2 are involved in the "accidental" uptake of Hg(II). For example, in *E. coli* high affinity Ni(II) transport is mediated by the genes encoded within the *nikABCDE* operon (reviewed in Mulrooney and Hausinger 2003). The regulation of the *nik* operon is under Fnr control. Fnr is a global regulator that

controls expression of genes in response to O_2 levels and has been shown to affect the regulation of up to 125 genes in *E. coli* (Sawers et al. 1998). Another metal specifically regulated in response to O_2, but not under Fnr control, is Cu (Outten et al. 2000). In *E. coli* the primary aerobic system for Cu tolerance involves the *cue* operon, which includes *copA* and *cueO*, but under anaerobic growth the *cus* Cu exporter is required (Outten et al. 2001). Therefore, the toxicity of Ni(II) and Cu and their potential to mitigate Hg(II) uptake was also investigated under anaerobic conditions. A Ni(II) concentration of 10 μM was required for a reduction in light from the constitutive control (Figure 5.15), whereas 10 μM Cu(II) was toxic and eliminated all light production (Figure 5.16). For the mitigation experiments, Ni(II) concentrations of 1 μM had a small effect on Hg(II)-induced light production and 10 μM resulted in a significant decrease (Figure 5.15). Increasing concentration of Cu(II) resulted in a significant decrease in the Hg(II)-dependent light response (Figure 5.16).

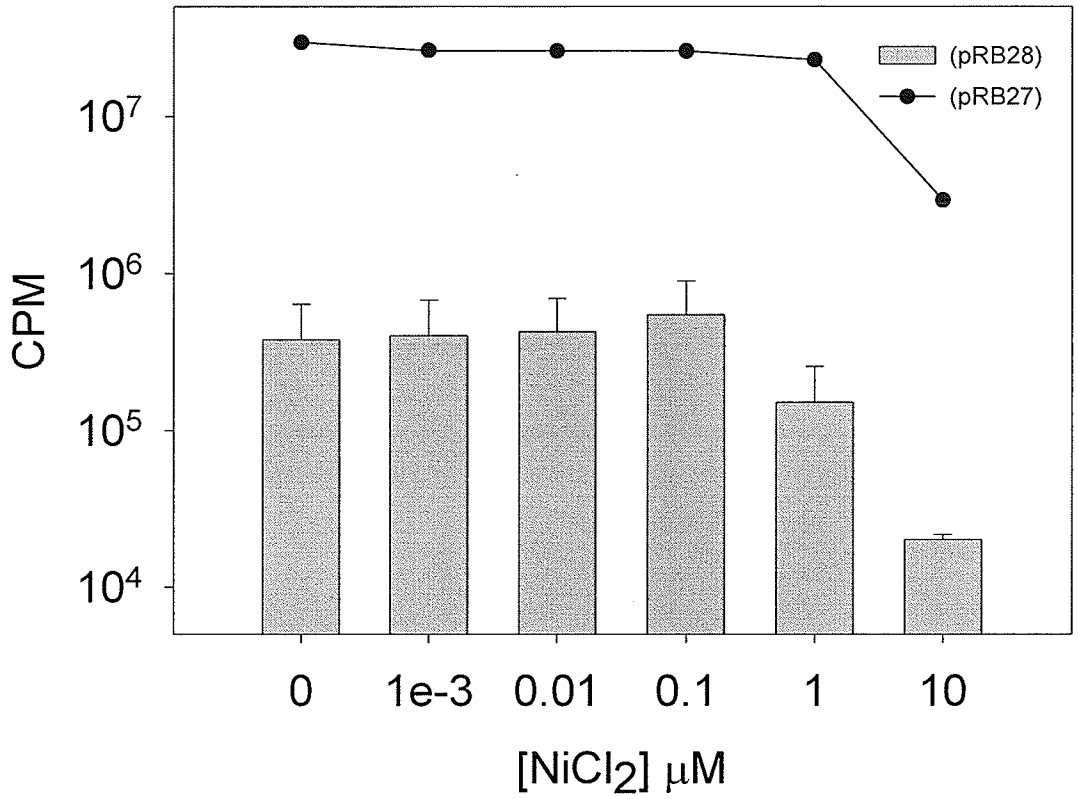

Figure 5.15. The effect of varying concentration of Ni(II) on Hg(II) induction (pRB28) and constitutive light production (pRB27) under anaerobic conditions in *E. coli* HMS174. 5 pM Hg(II) was added to the medium (pH=5.8) and incubated 120 minutes prior to light measurement. Error bars represent the standard error of two independent experiments (n=6).

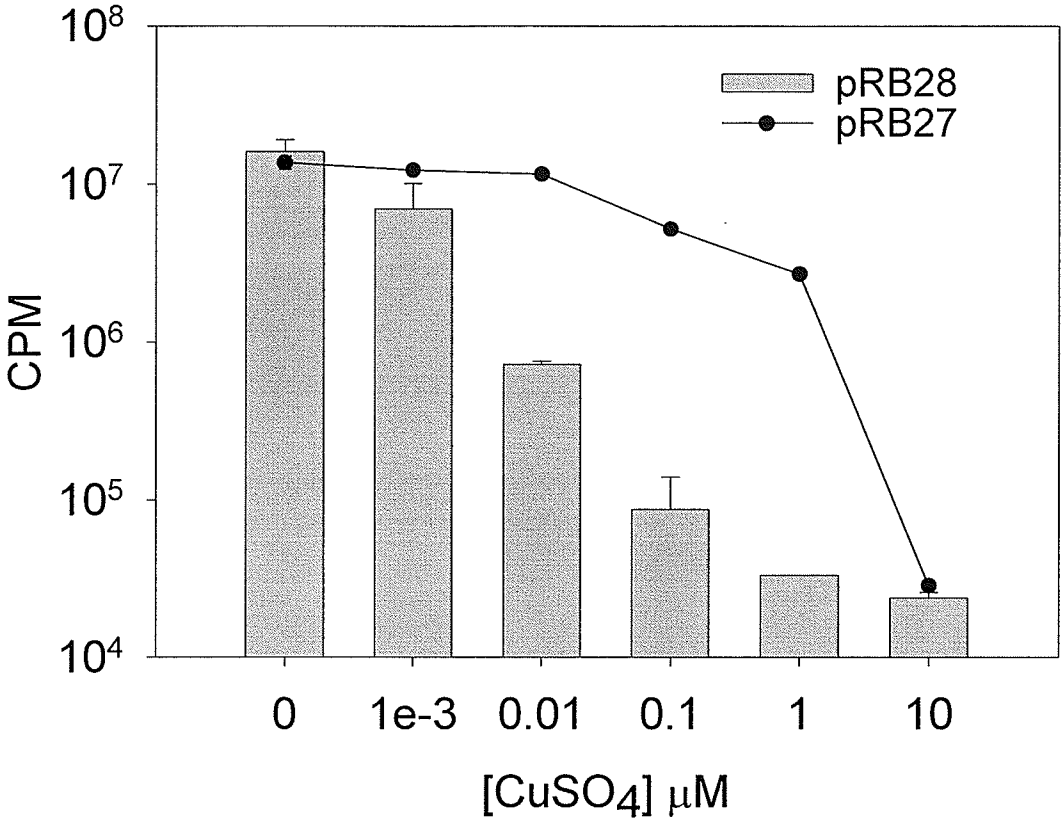

Figure 5.16. Effect of varying the concentration of Cu(II) on Hg(II) induction (pRB28) and constitutive light production (pRB27) under anaerobic conditions in *E. coli* HMS174. 5 pM Hg(II) was added to the medium (pH=5.8) and incubated 120 minutes prior to measurement. Error bars represent the standard error of two independent experiments (n=6).

5.4 Discussion

5.4.1 Metal toxicity The use of bioluminescence in *E. coli* HMS174 (pRB27) was a very sensitive method for the determination of metal toxicity, and also provided some advantages over other methods previously used. For example, these toxicity assays can be monitored in "real time" under aerobic conditions. This is also the first report of an anaerobic toxicity bioassay, which can also be applied for direct comparisons of aerobic and anaerobic toxicity in the same bacterial host, and would enable toxicity data to be obtained without compromising the chemistry of anaerobic samples by pre-exposure to O_2.

The order of increasing metal toxicity under aerobic conditions, based on the levels of reduction in light emission, can be summarized as Hg(II) >> Ni(II) > Cu(II) > Cd(II) > Zn(II) > Mn(II). With the exception of Ni(II), which was more toxic in *E.coli*, these results are in concordance with metal toxicity testing using the commercially available Microtox® (reviewed in Kong et al 1995), an isolated strain of *Pseudomonas* from activated sludge (Ren et al. 2003), and the freshwater organism *Vibrio qinghaiensis* (Ma et al. 1999). Therefore, *E. coli* HMS174 (pRB27) provides a sensitive test for Ni(II) toxicity under aerobic conditions.

Under anaerobic conditions *E. coli* HMS174 (pRB27) was much more tolerant to Ni(II) (Figure 5.15). This could be a result of the Fnr activation of the *nik* operon, which encodes a high affinity Ni(II) ATP-dependent transport system (reviewed in Mulrooney and Hausinger 2003). The induction of this system under anaerobic conditions might result in less "free" Ni(II) in the periplasm and/or cell to bind cellular sites, which could result in toxicity. Cu(II) toxicity on the other hand

appeared to increase under anaerobic conditions, which is consistent with the different Cu(II) detoxification systems predominating under aerobic and anaerobic conditions (Outten et al. 2001).

5.4.2 Metal mitigation of Hg(II) uptake The decreased light production observed in some of the bioreporter assays might be the result of metal toxicity and not Hg(II) bioavailability. On this basis, we cannot confidently interpret the results for 100 μM of Mn(II), Zn(II), Cd(II), and Cu(II). Lower metal concentrations that did not inhibit light production from the constitutive control, but still reduced the bioreporter response, are more likely a result of decreased Hg(II) uptake. On this assumption, the relative order of mitigation of Hg(II) uptake would be Mn(II) > Cu(II) > Mg(II) > Fe(III) > Ca(II) > Zn(II) > Selenite > Al(III) > Se(IV). At certain concentrations the addition of Cd(II) and Zn(II) stimulated induction of the *mer-lux* bioreporter. This is unlikely a synergistic effect enhancing Hg(II) uptake since these concentrations of Cd(II) and Zn(II) have previously been reported to induce the *mer* operon (Ralston and O'Halloran 1990). These additional metals should have no effect on the passive diffusion of Hg across the cytoplasmic membrane.

Since Cd(II) and Zn(II) stimulated the bioreporter response one could argue that these metals are competing with Hg(II) for binding to MerR, and not with a specific transport site. However, MerR has a very high affinity for Hg(II), which lies in its unusual trigonal binding site (Zeng et al. 1998). It would therefore be expected that Hg(II) should easily displace other metals that are bound to MerR. The in vitro affinity of MerR for other heavy metals has not been examined (Zeng et al. 1998), however Cd(II) would be expected to have the highest affinity for MerR followed by

Zn(II) (Ralston and O'Halloran). In an experiment presented in the next chapter (Figure 6.9), we provide some evidence that Cd(II), the metal with potentially the second highest affinity for Hg(II), does not compete with Hg(II) for MerR, as induction of *mer-lux* was additive in the presence of these two metals.

5.4.3 Energetics of Hg(II) uptake The energetics of Hg(II) uptake in bacteria containing the transport proteins of the *mer* operon has only been addressed in a few studies (Nakahara et al. 1979, Summers et al. 1982), but to date remains unclear. Since no membrane-associated proteins for ATP hydrolysis have been identified with the *mer* operon, Sahlman and Johnson (1992) proposed that the transport of Hg(II) across the membrane could be dependent on the H^+ gradient. For bacteria not containing the *mer* encoded transport systems the source of energetics required for transport is unknown. Under aerobic conditions we provided evidence that Hg(II) uptake required energy derived from the proton motive force (PMF), as the addition of CCCP decreased Hg(II) uptake (Figure 5.1B). Formation constants were not available for Hg(II) and CCCP, but the PMF has also been implicated as the driving force of uptake for phenylmercury in *Pseudomonas* K-62 (Uno et al. 1997, Kiyono et al. 2000). Under anaerobic conditions however, CCCP did not appear to have an effect on Hg(II) uptake (Figure 5.1A). Therefore if the effect of CCCP was on Hg(II)-binding we would have expected the same decreased response under anaerobic conditions. It might be possible that multiple transport systems are involved in the uptake of Hg(II). For example, one might be regulated in response to O_2, which would coincide with the differences of Hg(II) uptake observed under aerobic and anaerobic conditions in Chapters 3 and 4. In one previous study, evidence for Na^+

ions enhancing the uptake of Hg(II), and possibly toxicity, suggested the possibility of sodium-coupled transport of Hg(II) as a potential mechanism of Hg(II) uptake in cells not containing the transport system of the *mer* operon (Selifonova and Barkay 1994). Therefore, it is possible that two or more transport systems are participating under aerobic conditions, where one system is driven by the Na^+ gradient and the other by H^+. If passive diffusion was the predominant mechanism of Hg(II) accumulation then it would not be affected by diminishing either one of these gradients.

5.4.4 Possible transport systems involved in the uptake of Hg(II) The hypothesis of multiple transport systems involved in the non-specific transport would not be unique for a toxic metal. For example, Cd(II) is also a non-essential heavy metal that contains no specific transport systems for uptake. However, the accumulation of Cd(II), to potentially toxic levels in the cell, can occur via non-specific transporters such as CorA (Nies and Silver 1989), MntH (Kehres et al. 2000), and potentially PitA (Beard et al. 2000). To alleviate Cd(II) toxicity, the MerR family regulatory protein, CadR, binds Cd(II) and induces the expression of the CadA-ATPase, which then actively extrudes Cd(II) from the cell (Lee et al. 2001). Similar to Cd(II), transporter systems specific for essential metals could potentially accumulate Hg inside the cell, which then induces the *mer* operon for detoxification.

Transport system(s) responsible for the "accidental" uptake of Hg(II) could not be elucidated from this study, however possible examples sharing similarities with our findings also include CorA, MntH, and PitA, which are discussed in more detail in Chapter 1 (Section 1.5.2). CorA is a constitutively expressed transport

system that is capable of accumulating a broad spectrum of metal ions. The MntH transporter is stimulated by H^+, which is consistent with CCCP mitigating Hg(II) uptake in *E. coli* (Figure 5.1B), and is also regulated by multiple transcriptional control systems, including OxyR (Kehres et al. 2000), which could explain the differences in Hg(II) uptake observed in *E. coli* under aerobic and anaerobic conditions. PitA represents a low affinity system that is capable of transporting a wide range of divalent metal ions and is driven by a H^+ gradient. These transporters are just a few examples of identified systems that could be responsible for the uptake of Hg(II) in *E. coli*. The genome of *E. coli* contains many additional proteins containing a CXXC metal binding motifs whose function remains to be determined, but could potentially play a role in the accidental uptake and trafficking of Hg(II).

Cu(II) was interesting in that it mitigated Hg(II) uptake under aerobic (Figure 5.12) and anaerobic conditions (Figure 5.16). Unfortunately, the mechanism of Cu(II) uptake in *E. coli* remains unknown. In *Enterococcus hirae* Cu(I) uptake is mediated by the *cop* operon. CopZ is primarily responsible for copper trafficking in the cell and interacts specifically with the P-type ATPase CopA (Multhaup et al. 2001) and the regulatory protein CopY (Cobine et al. 2002, Cobine et al. 1999). Interestingly, the GMXCXXC motif found in the bacterial copper chaperone CopZ (Banci et al. 2001) is also present in the periplasmic MerP protein, which has a high affinity for Hg(II), and therefore presents a possible candidate for Hg(II) binding and non-specific uptake in bacterial cells.

Copper chaperones homologous to MerP and CopZ have also been identified in plants, humans, and yeast (Himelblau et al. 1998, Lin et al. 1997, Klomp et al.

1997). The yeast copper chaperone, Atx1, is responsible for the delivery of copper to the P-type ATPase transporter Ccc2 (reviewed in Rosenzweig 2001, O'Halloran and Culotta 2000). The crystal structure of Atx1 demonstrated that Hg, as well as Cu, could bind to the metal binding site (Rosenzweig et al. 1999). In addition to binding, this chaperone is capable of transferring Hg(II) to a domain of Ccc2 (Rosenzweig et al. 1999), demonstrating a potential mechanism for the non-specific uptake of Hg in yeast cells.

The possibility of Hg(II) uptake via such a mechanism has not been considered in environmental studies. Instead, Hg(II) research involving aquatic ecosystems has focused on the chemical speciation of Hg(II) in the bulk solution, on the premise that conditions that promote the formation of neutral Hg-species in the bulk solution would result in the highest rates of Hg(II) accumulation via passive diffusion. However, if secondary transport systems for Hg(II) exist, then alternative remediation strategies for MeHg contaminated ecosystems could be adopted. For example, increasing iron concentration was shown to reduce levels of Hg(II)-methylation in pure cultures of *Desulfobulbus propionicus* (1pr3) (Mehrotra et al. 2003), which was attributed to a decrease in the neutral HgS species (Mehrotra et al. 2003), but alternatively might involve iron competition with Hg(II) for transport.

The mitigation of Hg(II) uptake by a variety of different metals and the potential requirement of a chemiosmotic gradient for transport cannot be explained by passive diffusion. These results were consistent with the hypothesis that a Hg(II) non-specific secondary transport systems exists in *E. coli*. Differences in bacterial physiology, as a result of differences in water chemistry, would then play a

significant role. For example, if these transport systems are inducible, then differences in aquatic chemistry would alter their expression, which would play a significant role in the rates of Hg(II) uptake. Therefore, the mechanism(s) of Hg(II) uptake, and how different environmental factors affect these processes, need to be identified in order to increase our predictive ability of Hg(II) in the environment.

Chapter 6

The effect of pH on Hg(II) uptake

6.1 Introduction

What is common among bacterial mediated transformations of Hg(II) is that they are enzymatically catalyzed. Therefore, the rates of transformations will depend on 1) the amount of enzyme in the cell and 2) the concentration of Hg(II) inside the cell. The concentration of Hg(II) inside the cell is determined by external parameters affecting the availability of Hg(II) to the cell and by cellular physiology modulating the uptake of Hg(II) into the cell.

One environmental parameter that has shown positive correlations with MeHg concentration is pH (reviewed in Ulrich et al. 2001). For example, in aquatic ecosystems where the pH tends to be low, e.g. wetlands, MeHg concentration tends to be high (Kelly et al. 1997, St. Louis et al. 1994). This increase in MeHg concentration in acidified lakes has been attributed to an increase in the rates of bacterial methylation of Hg(II) (Miskimmin et al. 1992, Gilmour and Henry 1992, Bloom et al. 1991, Xun et al. 1987). Under anaerobic conditions we observed a possible correlation with the pH of our assay medium with the uptake of Hg(II), where the addition of carboxylic acids that lowered the pH of the assay medium below 6 appeared to have the greatest effect on Hg(II) uptake (Table 3.1).

A preliminary study into the effect of pH on Hg(II) uptake in *V. anguillarum* under aerobic conditions demonstrated that even small changes in pH (7.3-6.3) resulted in large increases in Hg(II) uptake (Kelly et al. 2003). This pH dependent increase in Hg(II) concentration inside the cell might be one of the factors explaining the observed increases of CH_3Hg concentration (reviewed in Ulrich 2001) and

bacterial Hg(II) methylation rates following acidification (i.e. Miskimmin et al. 1992, Gilmour and Henry 1992, Bloom et al. 1991, Xun et al. 1987).

Since methylation of Hg(II) occurs predominantly under anaerobic conditions (Compeau and Bartha 1985) further studies into the effect of pH on Hg(II) uptake were undertaken. While initial work (Kelly et al. 2003) focused on *V. anguillarum* and a pH range of 6.3-7.3, the experiments were repeated and extended under anaerobic and aerobic conditions with *E. coli*, whose genetics and physiology are better understood, over a pH range of 5-8.

6.2 Additional Methods

The *E. coli* strains RKP2922 (formerly SJB201) and MG1655 were kindly provided by Dr. R.K. Poole and are described in Beard et al. (2000). Transformation of the bioreporter plasmids (pRB28) and (pRB27) into these strains were carried out as described in Chapter 2 (section 2.2) and are described in Chapter 1 (section 1.6). Milli-Q water was obtained from Dr. Feiyue Wang's lab (Department of Chemistry, University of Manitoba) for the experiments involving the role of Pit in Hg(II) uptake (Figure 6.16-6.18). Total Hg(II) analysis of the Hg(II)-binding experiments was kindly provided by Alexandre Poulain. Ultraclean HCl (0.0013 ng ml^{-1} Hg(II)) used in the acidification experiments was obtained from Flett Research Ltd.

6.3 Results

6.31 Effect of pH on Hg(II) uptake A decrease in added Hg(II) required to induce a *mer-lux* response under anaerobic conditions was observed in *V.*

anguillarum through the addition of specific carboxylic acids (Table 3.2). Fumarate, 1 mM, provided a large stimulation of an anaerobic Hg(II)-dependant response (Figure 3.17), but also decreased the pH of the medium most significantly (pH 5.7) in the weakly buffered medium used (6.7 mM phosphate). Further investigation (Table 6.1) has indicated that the observed effect could be achieved in a 6.7 mM and 67 mM phosphate buffered medium (pH 5.7) in the absence of fumarate. Conversely, 1 mM fumarate had no positive effect on light production when the cells were incubated at pH 7 in a strongly buffered medium (67 mM phosphate). This suggested that the observed increase in Hg(II) sensitivity under anaerobic conditions was dependent on pH, and not on the specific nature of the carboxylic acid added to the assay medium.

The effect of pH on the Hg(II)-induced light response was then directly investigated in *E. coli* HMS174 under anaerobic and aerobic conditions. Under anaerobic conditions the Hg(II)-dependent light response to 5 pM Hg(II) was detectable only when the pH of the assay medium was < 6, and Hg(II)-induced light production continued to increase as the pH was further decreased (Figure 6.1A). Under aerobic conditions (Figure 6.1B), while Hg(II) uptake was detectable throughout the pH range tested, lowering the pH of the assay medium resulted in an increase of the Hg(II)-dependent light response. Similar results were also obtained using *V. anguillarum* as the bioreporter host under aerobic (Kelly et al. 2003) and anaerobic conditions.

Differences in bacterial physiology, as a result of varying environmental parameters, can significantly alter levels of bioluminescence in bacterial cells (Dorn et al. 2003). To ensure the enhanced bioluminescence in our assays was Hg(II)-

Table 6.1. Differentiating between the effect of 1 mM fumarate and pH on the enhanced uptake of Hg(II) under anaerobic conditions in *V. anguillarum*. Samples contained 50 pM Hg(II) and were incubated 120 min prior to measurement.

Fumarate added	[Phosphate] (mM)	pH of the assay medium	Hg(II) induction
-	6.7 or 67	7.1	-
+	67	7.1	-
+	6.7	5.7	+
-	67 or 6.7	5.7	+

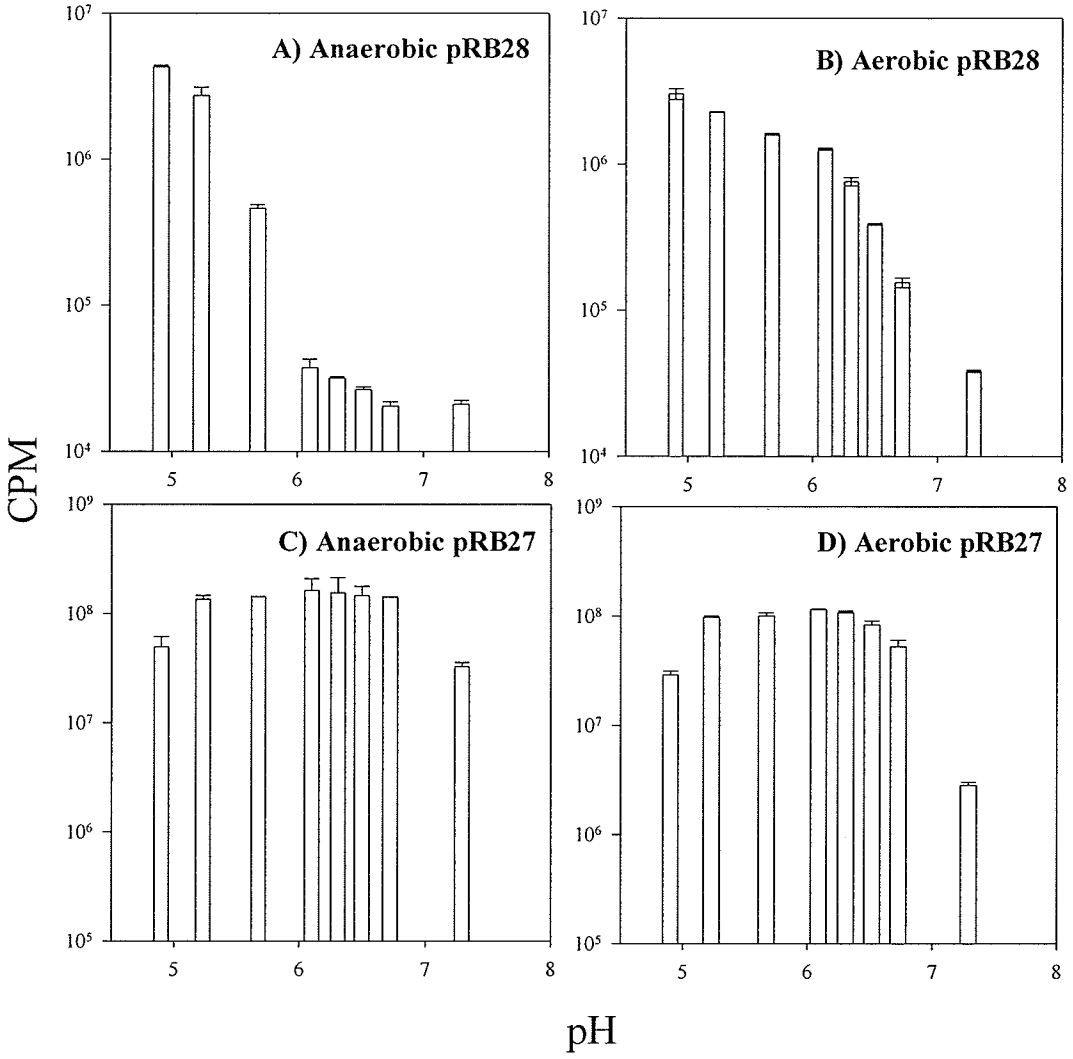

Figure 6.1 Effect of varying pH on Hg(II) uptake in *E. coli* HMS174 (pRB28) under A) anaerobic and B) aerobic conditions and on light production in *E. coli* HMS174 (pRB27) under C) anaerobic and D) aerobic conditions. Samples contained 5 pM Hg(II). Error bars represent the standard deviation of duplicate samples.

dependent, and not a result of increased light capability of the cell from decreasing the pH of the assay medium, bioluminescence from *E. coli* HMS174 (pRB27) was measured as a means to evaluate general cell fitness throughout the pH range tested. Light production from *E. coli* HMS174 (pRB27) under anaerobic (Figure 6.1C) and aerobic (Figure 6.1D) conditions was similar over the pH range of 6-7, but a decrease was observed outside of this pH range. In addition to the light producing capability of the cell, the mean generation times of *E.coli* HMS174 (pRB28) under aerobic and anaerobic conditions also decreased outside of the pH range of 6-7 (Table 6.2). Therefore, as the pH of the assay medium decreases below 6 light production from the constitutive control and growth rates also decreases. This suggests that the increased Hg(II)-induced light production observed at lower pH (Figure 6.1A and B) was Hg(II)-dependent and not the result of increased light production capability of the cell at lower pH.

Measurement of total Hg(II) in solution at the different pH's (Flett Research Ltd.) indicated that the increase in Hg(II)-response at low pH was not a result of desorption of Hg(II) from the scintillation vials in higher pH samples, leaching of potential contaminant Hg(II) from the scintillation vials at low pH, or from trace Hg(II) contamination in NaH_2PO_4, whose concentration was increased to lower the pH (Figure 6.2). Contrary to the enhanced bioreporter response observed at low pH, there was more endogenous Hg(II) in the higher pH samples in the media blanks, which could possibly be attributed to increasing concentration of K_2HPO_4 (Figure 6.2).

Table 6.2. Effect of varying pH on the mean generation times (g) of *E. coli* HMS174 (pRB28) in defined assay media. Average of triplicate samples are shown.

pH of the assay media	Aerobic g (hours/generation)	Anaerobic g (hours/generation)
5	6.6	7.2
5.5	5.9	6.1
6	5.7	5.7
6.5	4.2	4.7
7	4.0	6.5
7.5	6.9	7

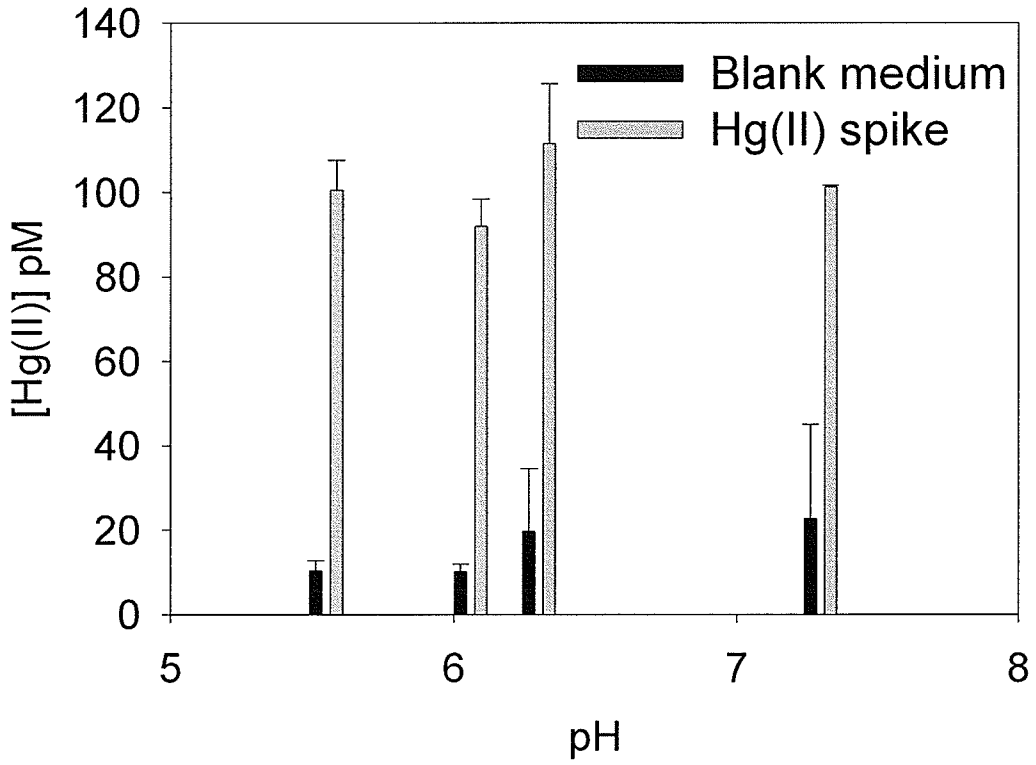

Figure 6.2. Total Hg(II) analysis of the defined assay medium of varying pH. Error bars represent the standard deviation of duplicate samples.

The difference in induction profiles under anaerobic and aerobic conditions might have been the result of the differences in Hg(II) concentration required to induce the bioreporter at pH 7. At pH 7, anaerobic *mer-lux* bioreporter assays require ~ 375 pM Hg(II) to yield equivalent light production obtained using only 5 pM Hg(II) under aerobic conditions (Figure 6.3A). Anaerobic assays were then performed using 375 pM Hg(II) over a pH range of 5 - 7.5 (Figure 6.3B). Results from this assay still displayed a sharp increase in light production as the pH decreased below pH 6, which differentiates the Hg(II)-induced response of the anaerobic and aerobic assays (Figure 6.1).

To determine whether this pH enhanced response to Hg was a consequence of the magnitude of the shift between the growth and assay pH values, the cells were preconditioned by growing at pH 6, 7, and 8 in GMM prior to the assay. Under both anaerobic (Figure 6.4A) and aerobic conditions (Figure 6.4B) the Hg(II)-induced light response of *E. coli* (pRB28) to 5 pM Hg(II) increased significantly with decreasing pH, regardless of initial growth pH. This indicated that the pH effect observed was not an inducible response, but was rather directly related to the pH of the assay medium. The increase in light response was not linear over the tested range of pH, nor was it identical under both atmospheres. Under anaerobic conditions there was no observable response to 5 pM Hg(II) above pH 6, while a response to 5 pM Hg(II) was observed aerobically up to a pH of 8. Light production from the constitutive control (pRB27) indicated that pH had little effect on light capability of the cell,

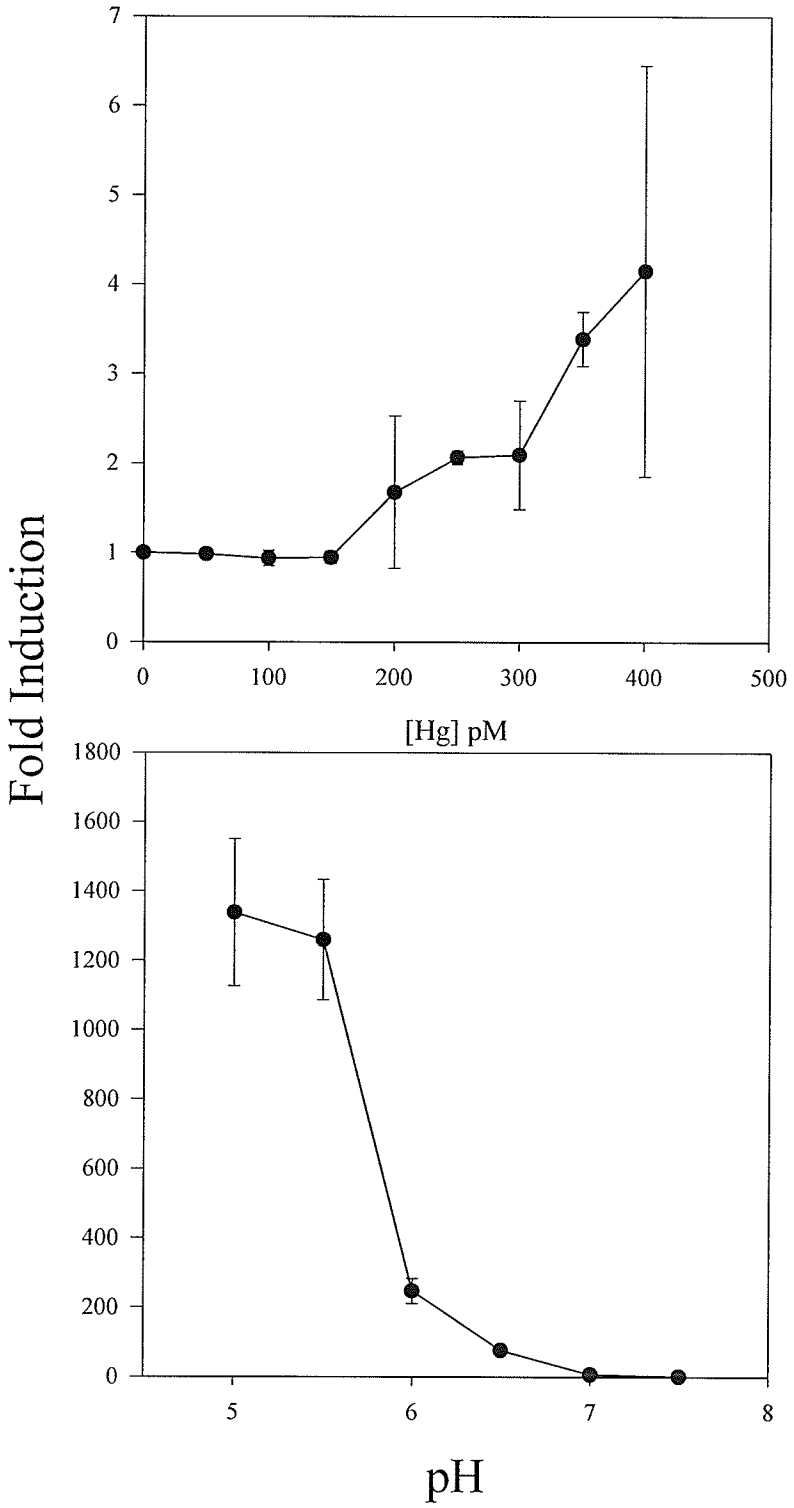

Figure 6.3. Concentration of Hg(II) required for induction under anaerobic conditions in A) pH 7 assay medium and B) the effect of varying pH on Hg(II) uptake in assay medium containing 375 pM Hg(II) in *E. coli* HMS174 (pRB28). Error bars represent the standard deviation of triplicate samples.

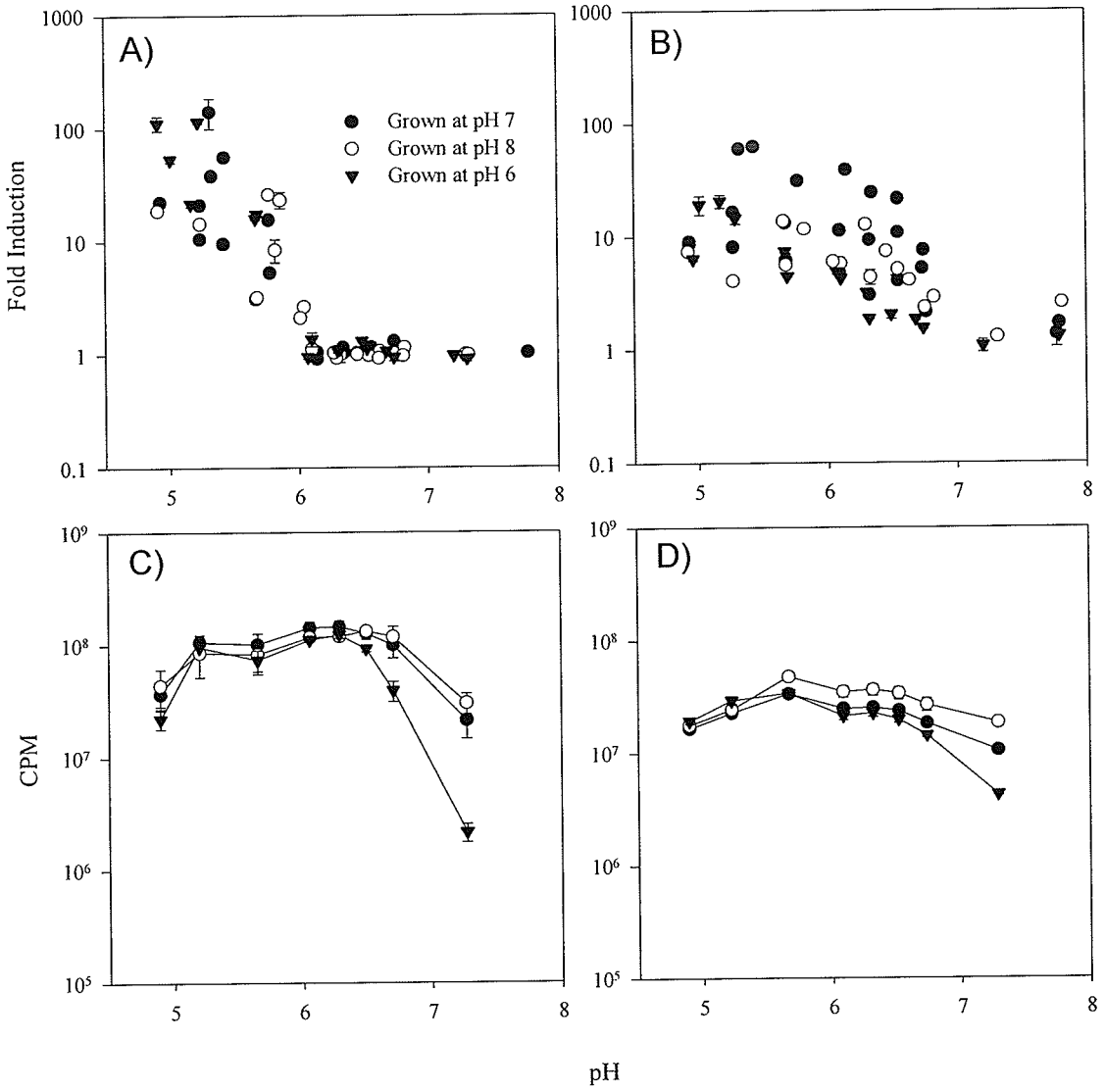

Figure 6.4. Effect of varying pH on the Hg(II)-induced light response from *E. coli* HMS174 (pRB28) under A) anaerobic and B) aerobic conditions, and on constitutive light production from *E. coli* HMS174 (pRB27) under C) anaerobic and D) aerobic conditions, using cells preconditioned in pH 6-8 growth medium. Hg(II) was added to a final concentration of 5 pM in the bioreporter assays and light production was measured following 120 minutes of incubation. Error bars for the *mer-lux* bioreporter assays represent the standard deviation of duplicate samples. Error bars for the constitutive *lux* assays represent the standard error of 3 independent assays (n=6).

except at the lowest and highest pHs tested where light production declined (Figure 6.4C and 6.4D).

The effect of acidification on cells previously exposed to Hg(II) was also investigated under aerobic conditions. In these experiments, cells were preconditioned in pH 7 assay medium containing 10 pM Hg(II) for 120 minutes, which was generally the time required for the Hg(II)-dependent light response to plateau. Selected samples were then acidified (pH ~ 5.5) using ultraclean HCl and monitored over time. In comparison to the non-acidified samples, whose Hg(II)-dependent light emission remained unchanged over time, the acidified samples displayed a significant increase in light production following a short lag (Figure 6.5A). Therefore, new protein synthesis was required for this additional light production. Indeed, the addition of chloramphenicol (Cam), which prevents further protein synthesis, resulted in a steady decrease in light production in both acidified and non-acidified samples (Figure 6.5A). This demonstrated that the increased light response following acidification was a result of increased expression of the Hg(II)-dependent reporter genes and not a general physiological response that might have enabled the cells to yield more light under conditions of low pH. This was further verified by the constitutive control (pRB27), which showed that light production was not enhanced following acidification (Figure 6.5B). As with the *mer-lux* bioreporter assays (Figure 6.5A), the addition of Cam resulted in a decrease in light production from the constitutive control in both acidified and non-acidified samples.

Hg(II)-induction of pH 7 samples, which were subsequently acidified to pH 5.5 following 180 minutes of incubation, produced an equivalent amount of light as

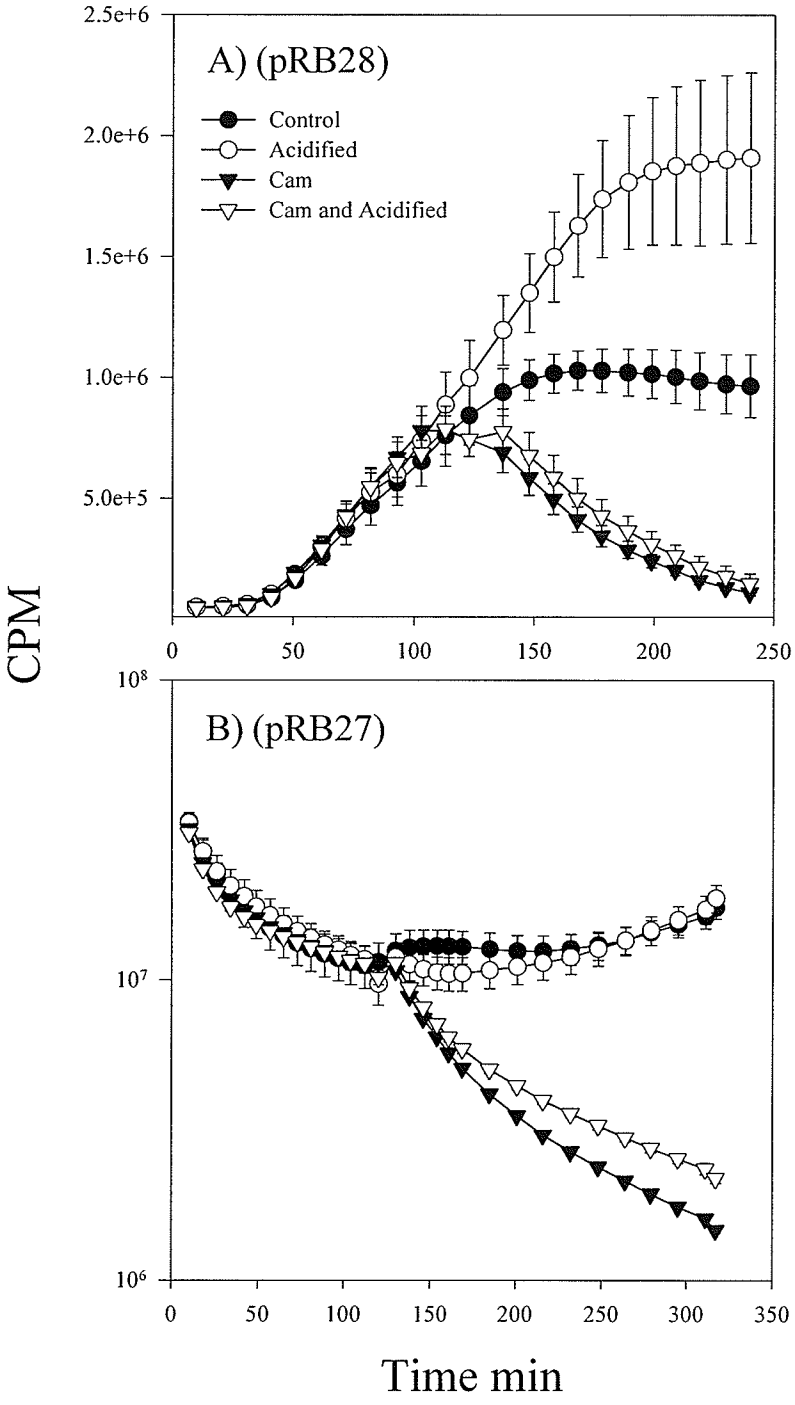

Figure 6.5. Effect of acidification (pH 5.5) and chloramphenicol (15 μg L^{-1}) on (A) Hg(II)-induced light production in *E. coli* HMS174 (pRB28) and (B) constitutive light production in *E. coli* HMS174 (pRB27) following 120 minutes of 10 pM Hg(II) exposure in pH 7 assay medium under aerobic conditions. Error bars represent the standard error of three independent assays (n = 6).

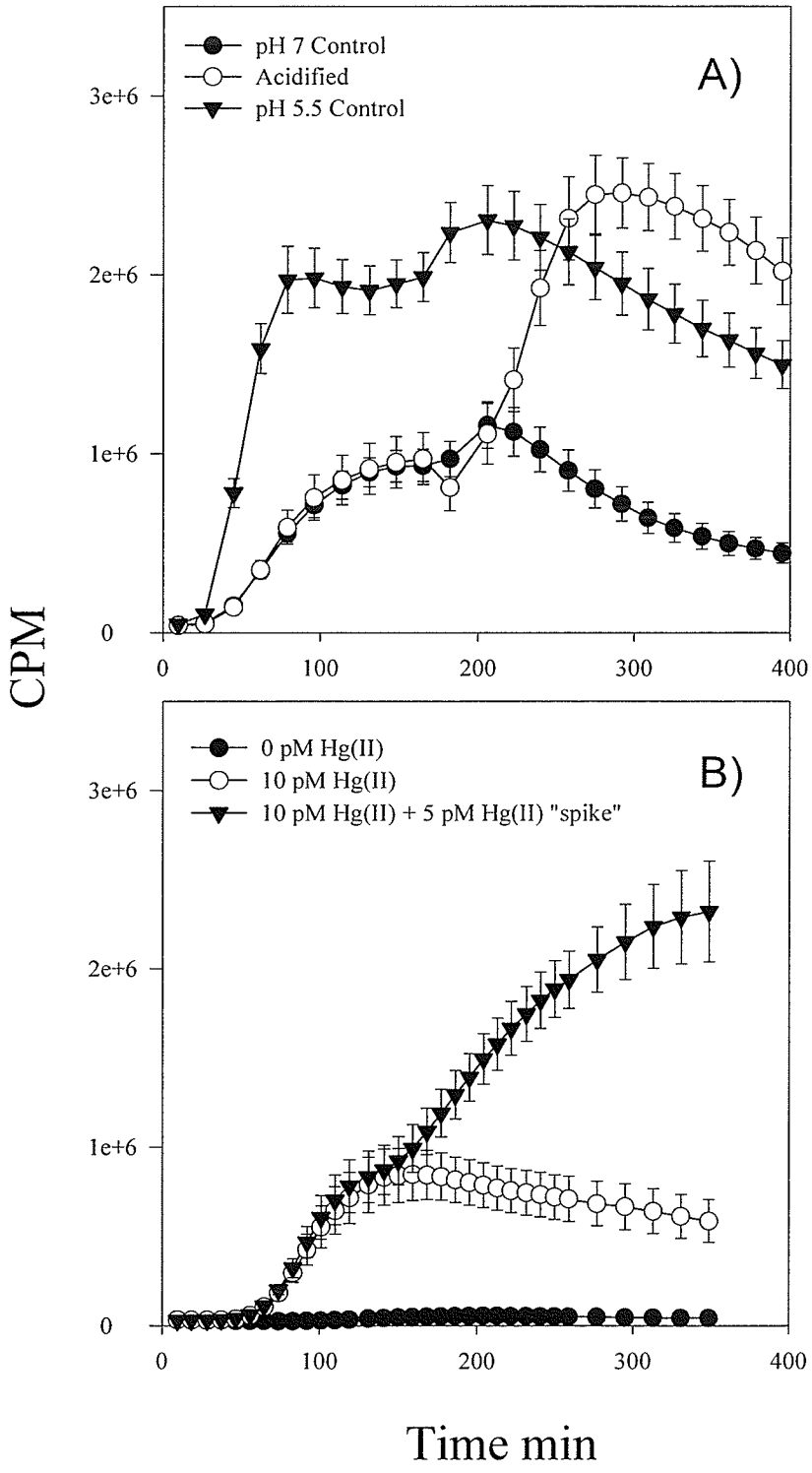

Figure 6.6. Comparison of A) Hg(II)-induced light production in pH 5.5 and pH 7 assay medium +/- acidification and B) Hg(II)-induced light production in pH 7 assay medium spiked with an additional 5 pM Hg(II) following 120 minutes of Hg(II) exposure. Error bars represent the standard error of two independent experiments (n = 6).

samples incubated throughout the assay in pH 5.5 assay medium (Figure 6.6A). This increase in the Hg(II)-dependent light response was similar to the induction profile of experiments where additional Hg(II) (5 pM) was added following pre-Hg(II) exposure (10 pM) (Figure 6.6B). These results strongly suggest that acidification results in an increase in the uptake of Hg(II) in the samples.

6.3.2 Effect of pH on Cd(II) induction of mer-lux Additional evidence that increased light production under conditions of low pH was specifically Hg(II)-dependent was provided in experiments using Cd(II). Cd(II) is a metal that induces the *mer* operon, but much higher concentrations are required for Cd(II) than for Hg(II) to cause induction (Caguiat et al. 1999, Ralston and O'Halloran 1990). Consistent with these reports, there was a requirement of higher Cd(II) concentration (0.1 – 1 μM) to induce the *mer-lux* bioreporter under anaerobic (Figure 6.7) and aerobic (Figure 5.11) conditions. Therefore, in contrast to Hg(II), the concentrations of Cd(II) required to induce *mer-lux* were similar under aerobic and anaerobic conditions.

Under anaerobic conditions, in pH 5.5 – 7.4 assay media, the *mer-lux* bioreporter response to Cd(II) (Figure 6.8A) followed a completely different pattern of induction compared to Hg(II) (Figure 6.8B). In the samples containing Cd(II), a bell-shaped curve was observed over the pH range tested with an optimal Cd(II)-induced light response at pH 6.1. In contrast to Hg(II), the Cd(II)-induced light production decreased sharply as the pH was further lowered. This same pattern was observed using Cd(II) (Figure 6.8C) and Hg(II) (Figure 6.8D) under aerobic conditions, with a slightly different pH optimum for Cd(II) uptake, compared to

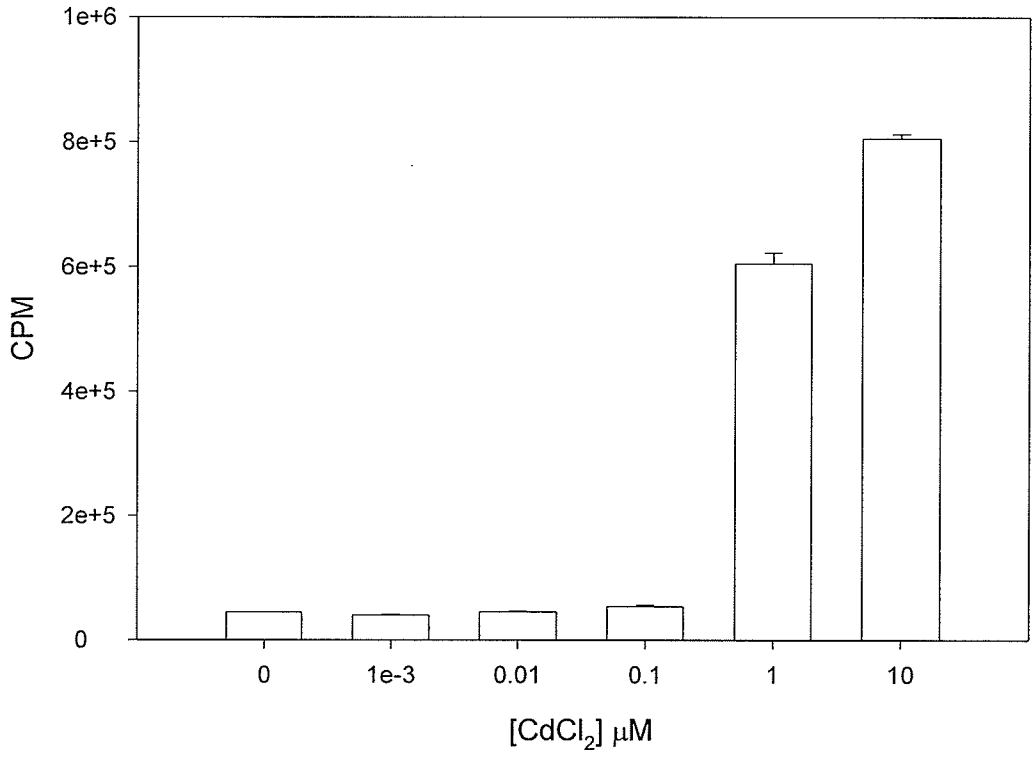

Figure 6.7. Cd(II) induction of *mer-lux* in *E. coli* HMS174 (pRB28) under anaerobic inductions. Samples were incubated 120 minutes prior to measurement. Error bars represent the standard deviation of duplicate samples.

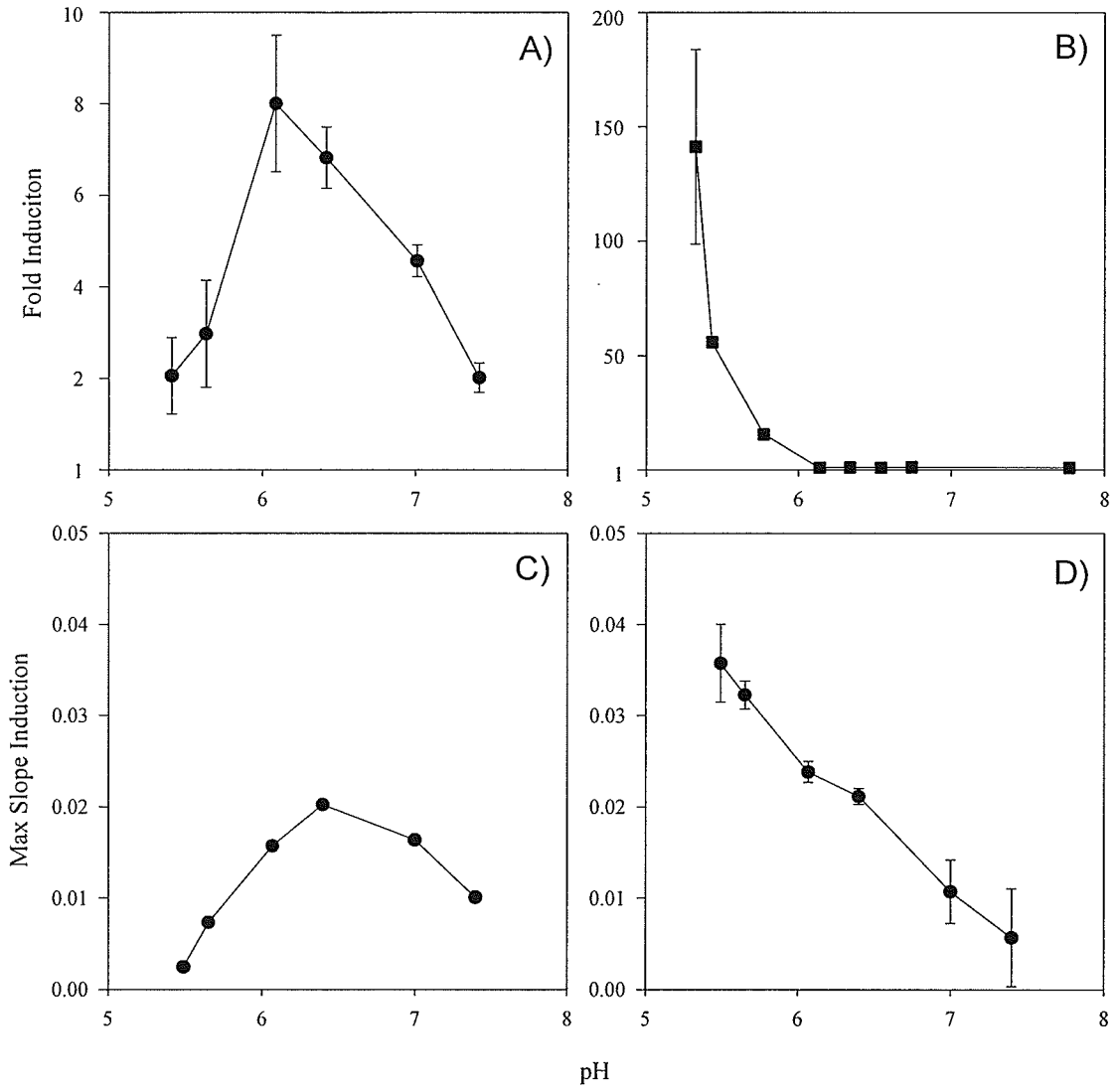

Figure 6.8. Effect of pH on A) Cd(II) and B) Hg(II)-induced light production from *E. coli* HMS174 (pRB28) under anaerobic conditions, and the max slopes of induction of C) Cd(II) and D) Hg(II) under aerobic conditions. The assay medium contained a final concentration of 6.7 mM phosphate. Cd(II) and Hg(II) were added to a final concentration of 1 μM and 5 pM respectively. Error bars represent the standard deviation of duplicate samples.

anaerobic assays. In the aerobic experiments, a pH of 6.4 resulted in the largest slope of induction for Cd(II), which sharply decreased as the pH was lowered. In contrast, the max slopes of induction for Hg(II) increased in a linear fashion as the pH was decreased. The expression of *mer-lux* in assay medium containing both Cd(II) and Hg(II) appeared additive in pH 5.4, 6.4 and 7.4 assay medium (Figure 6.9). These experiments demonstrated that the pH effect is not a general effect on MerR and that Cd(II) and Hg(II) transport systems are likely independent of one another as illustrated by the fact that Cd(II) does not mitigate Hg(II) uptake.

6.3.3 Effect of pH on Hg(II) toxicity On the basis of the *mer-lux* bioreporter results, it could be hypothesized that conditions that enhance Hg(II) uptake, should also decrease the amount of Hg(II) needed to cause a cytotoxic effect. Hg(II) toxicity can be measured as a reduction in bioluminescence from the host containing the constitutive *lux* plasmid (pRB27). For the anaerobic and aerobic assays Hg(II) concentrations of 10 and 2.5 nM were chosen to study the effect of pH on Hg(II) toxicity. These two concentrations were shown to reduce bioluminescence levels 70-80 % in comparison to the non-Hg(II)-amended samples at pH 6.8 (Figure 6.10). As expected from the bioreporter assays, the toxicity of Hg(II) was mitigated at higher pH and was enhanced with decreasing pH under anaerobic and aerobic conditions (Figure 6.11A and B respectively).

To determine the effect of pH on Hg(II) binding to *E. coli* HMS174 (pRB28) total Hg(II) concentration was measured in assay medium ranging in pH from 5.3-7.3. The total concentration of Hg(II) adsorbed by cells within the 60 minute exposure period increased slightly as the pH increased from 5.3 – 7.3 (Figure 6.12). The

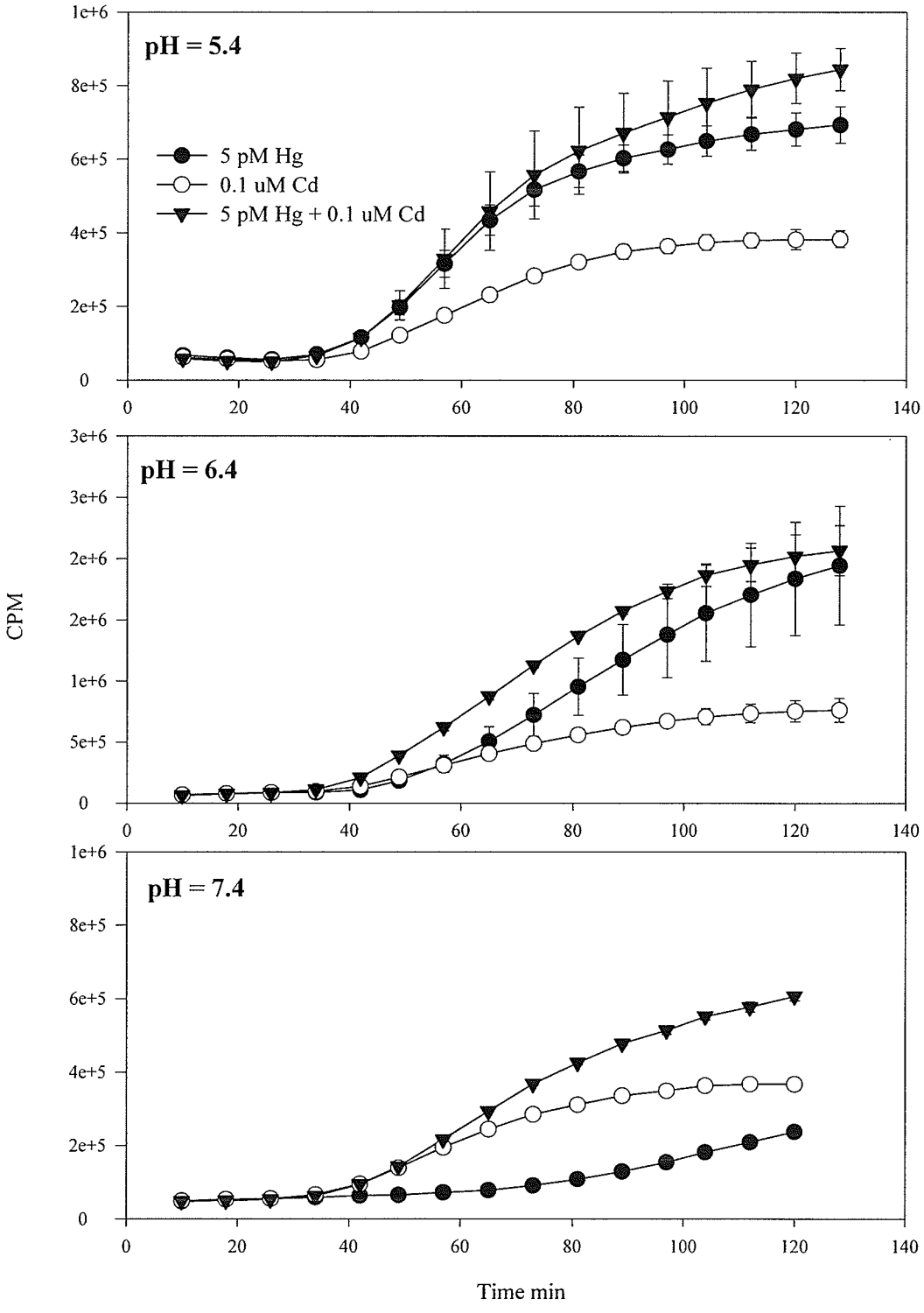

Figure 6.9. The combined effect of Hg(II) and Cd(II) on light production from *E. coli* HMS174 (pRB28) under aerobic conditions in pH 5.4, 6.4, and 7.4 assay medium. Error bars represent the standard deviation of duplicate samples.

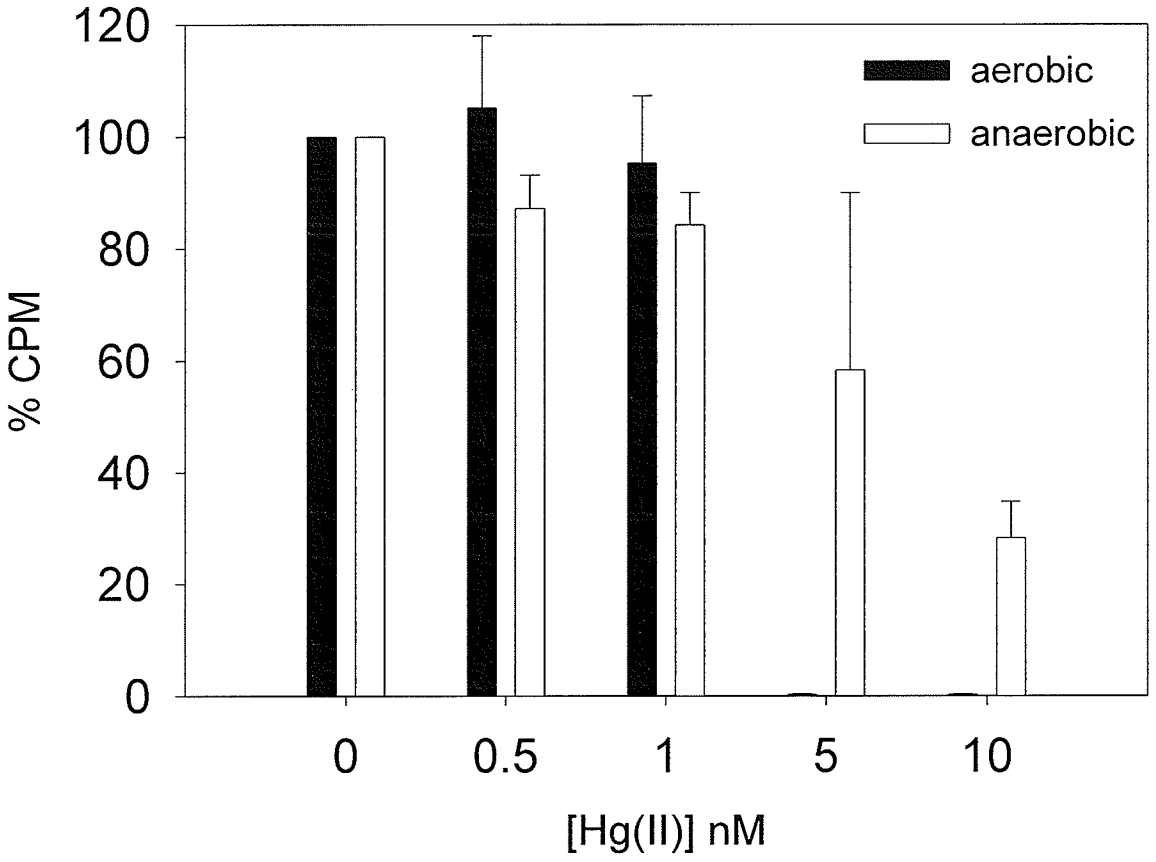

Figure 6.10. Toxicity of Hg(II) in pH 7 assay medium under anaerobic and aerobic conditions in *E. coli* HMS174 pRB27 following 120 min Hg(II) exposure. Light measurements were normalized to samples containing no Hg(II). Error bars represent the standard deviation of triplicate samples.

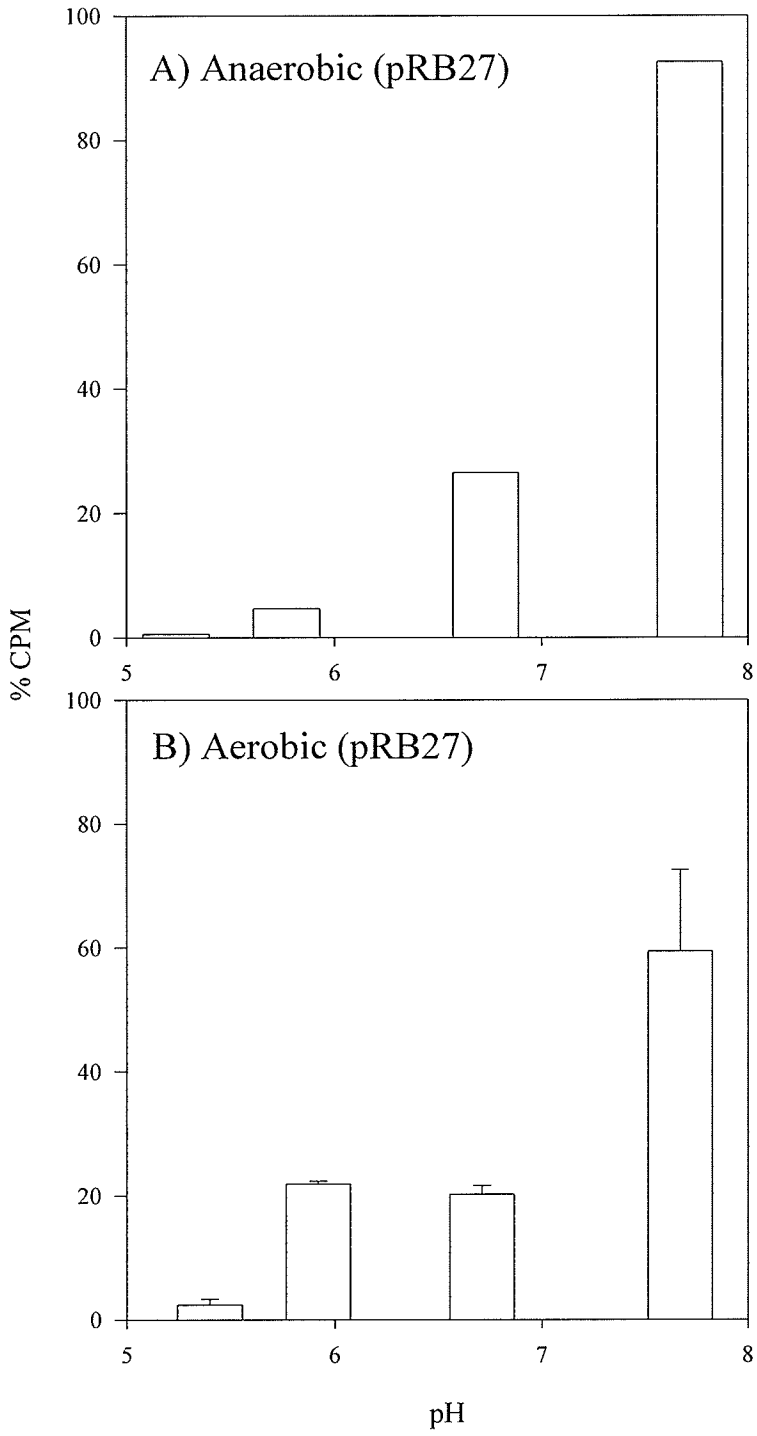

Figure 6.11. Effect of pH on Hg(II) toxicity in *E. coli* HMS174 (pRB27) under (A) anaerobic and (B) aerobic conditions. Mercury was added to a final concentration of 10 and 2.5 nM for the anaerobic and aerobic assays respectively. Light measured at each pH was normalized to light emission in samples containing no Hg(II) at each tested pH. Error bars represent the standard deviation of duplicate samples.

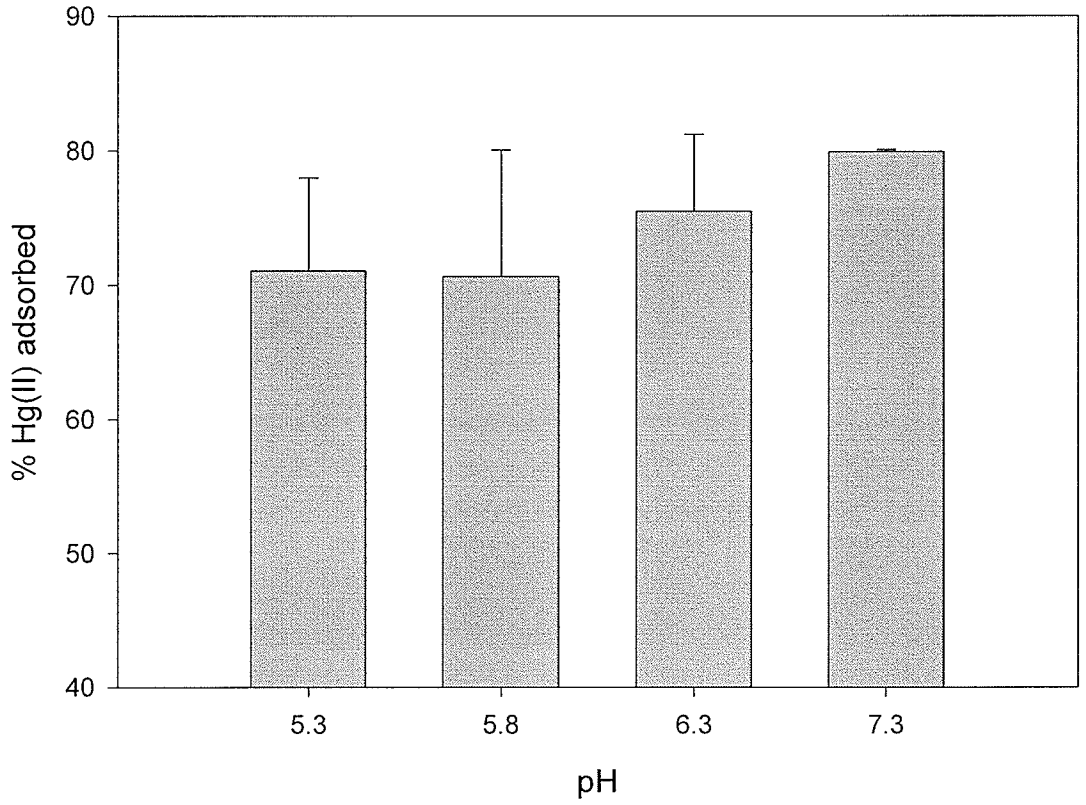

Figure 6.12. Effect of pH on Hg(II) binding to *E. coli* HMS174 (pRB28). Cells were exposed to 50 pM Hg(II) for 60 minutes, centrifuged, and then the total Hg(II) remaining in the supernatant was measured. Error bars represent the standard deviation of two independent experiments.

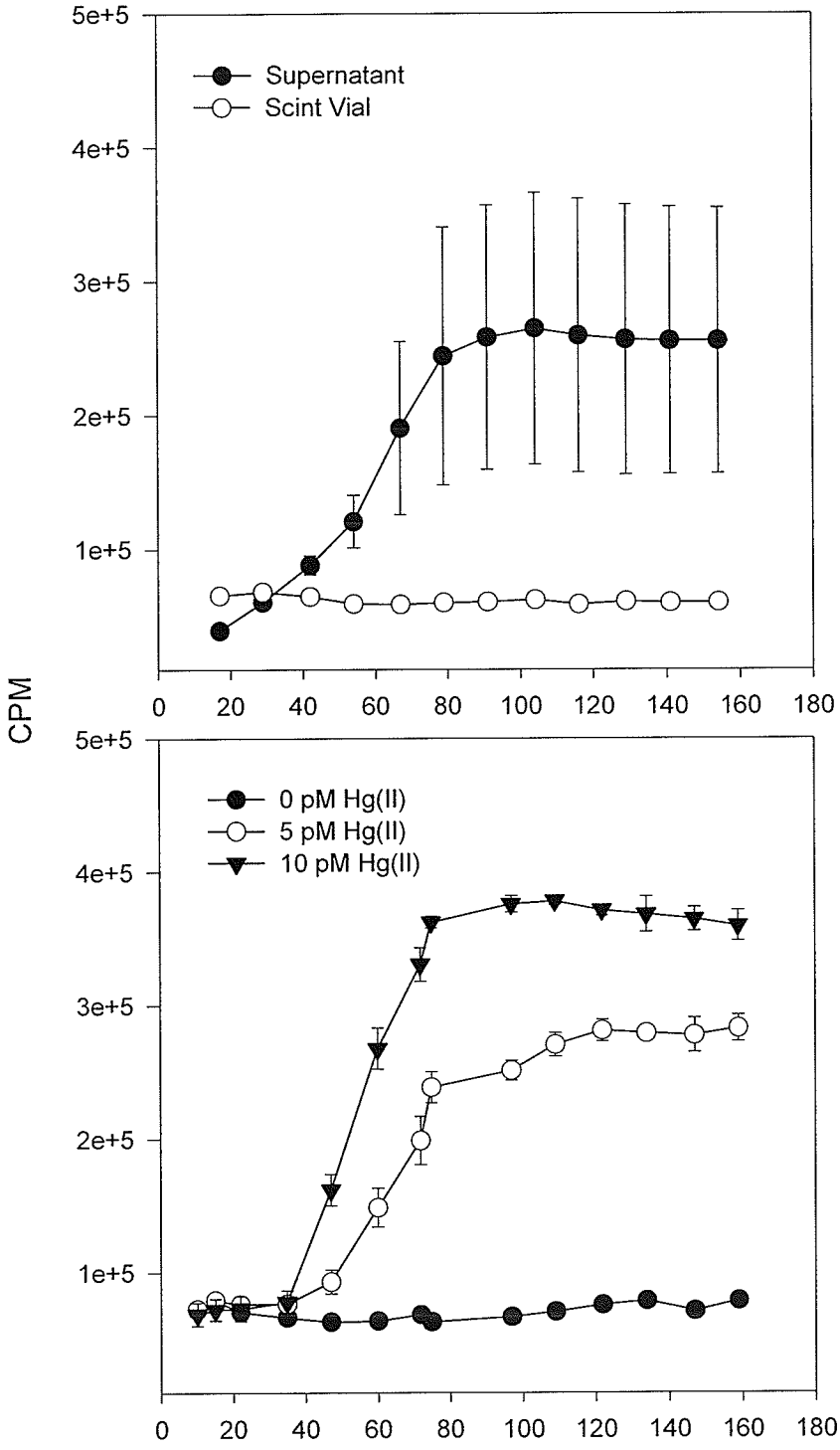

Figure 6.13. Estimation of Hg(II) remaining in the bulk solution in bioreporter assays. A) *E. coli* HMS174 (pRB28) was exposed to 50 pM Hg(II) for 60 minutes. The culture was then pelleted by centrifugation and the supernatant was re-inoculated with fresh *E. coli* HMS174 (pRB28) in a new vial. The initial vials were also re-inoculated with fresh culture and assay medium. B) Standard Hg(II)-response of *E. coli* HMS174 (pRB28). Error bars represent the standard error of triplicate samples.

adsorption of 80% Hg(II) is in concordance with bioreporter assays that were designed to estimate Hg(II) remaining in the bulk solution. In these experiments *E. coli* HMS174 (pRB28) was exposed to 50 pM Hg(II) for 60 minutes. Following the incubation the culture was pelleted and the supernatant was re-inoculated with fresh culture. No induction was evident when new medium and "fresh" *E. coli* HMS174 (pRB28) were added to the original vials containing Hg(II), suggesting that the Hg(II) was not lost to the vial (Figure 6.13A). Comparing the bioreporter response in the supernatant (Figure 6.13A) with a standard assay (Figure 6.13B), which was run in parallel, it appears that ~ 7.5 – 10 pM Hg(II) remained in the bulk solution, which corresponds to ~ 15-20% of the initial concentration.

6.3.4 Role of Hg(II) speciation at varying pH? The increase in Hg(II) uptake, as a result of decreasing pH, might be due to a change in the chemical speciation of Hg(II) in the bulk solution. At pH 7 the predominate Hg(II) species calculated by MINEQL+ is $Hg(NH_3)_2^{2+}$, which accounts for 94-97 % of the total Hg(II) in the bulk solution of the assay medium (Figure 6.14). However, as the pH decreases below 6 there is a significant increase in the formation of a neutral Hg(II)-phosphate species, $HgHPO_4$, which closely mimics the anaerobic bioreporter response (Figure 6.15).

The formation of the metal phosphate species, $MeHPO_4$, is required for uptake by the phosphate inorganic transport system (PitA). We therefore hypothesized that $HgHPO_4$ might enter the cell via PitA. PitA is a ubiquitous transport system that is constitutively expressed in *E. coli* and functions as a H+/solute symport system (reviewed in van Veen 1997). PitA represents a secondary low-affinity transporter for the divalent metal ions Mg(II), Ca(II), Mn(II), Co(II), Zn(II), and potentially

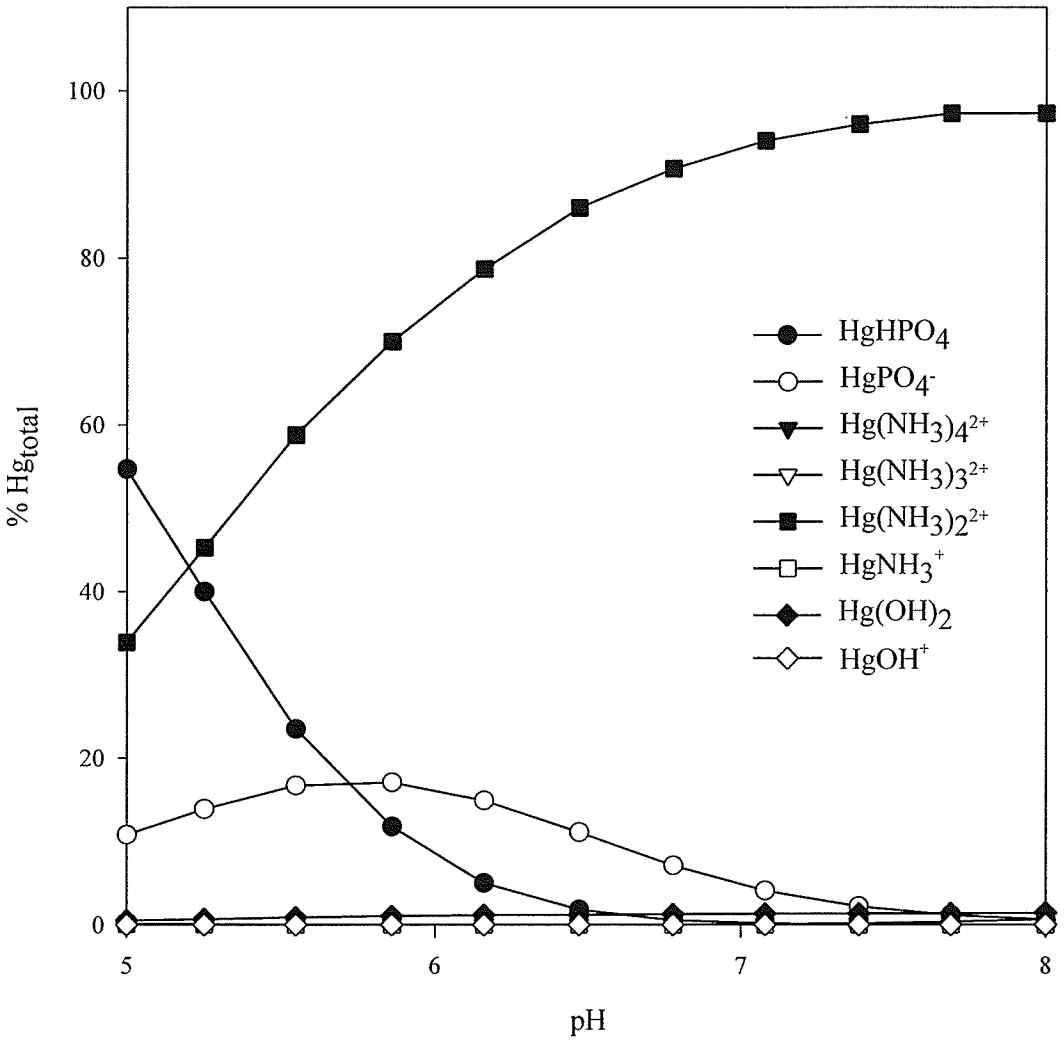

Figure 6.14. The effect of pH on Hg(II) speciation as calculated using MINEQL+ version 4.5. The assay medium consisted of 67 mM phosphate, 9 mM $(NH_4)_2SO_4$, and 5 mM Glucose.

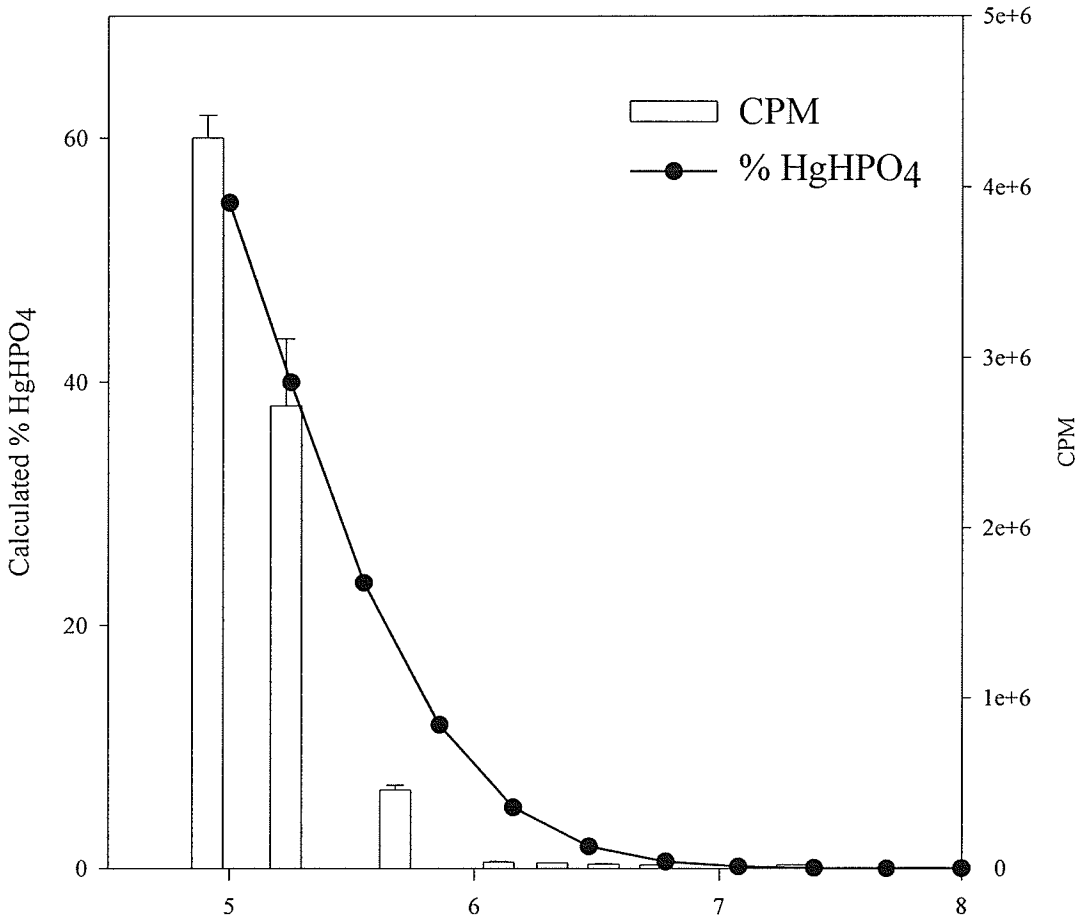

Figure 6.15. Correlation of $HgHPO_4$ with Hg(II) uptake in *E. coli* HMS174 (pRB28) under anaerobic conditions. Samples containing 5 pM Hg(II) were incubated 120 minutes prior to measurement. MINEQL+ version 4.5 was used to calculate Hg(II) speciation. Error bars represent the standard deviation (n=4).

Cd(II) (Beard et al. 2000, Van Veen et al. 1994), but the uptake of Hg(II) was not tested in these previous reports.

To determine if PitA was also involved in the uptake of Hg we obtained *E. coli* RKP2922 (formerly SJB201), which is a Tn*10*PitA $^-$ mutant, and the corresponding parental strain MG1655 (Beard et al. 2000). These two strains were transformed with the bioreporter plasmid (pRB28) and assayed for their ability to accumulate Hg(II) under varying assay conditions. Under anaerobic (Figure 6.16) and aerobic (Figure 6.17) conditions Hg(II) induced light production increased with decreasing pH in both *E. coli* RKP2922 and MG1655. Since no major differences were observed, it appears that PitA does not play a significant role in Hg(II) uptake. However, as the pH decreases the correlation with $HgHPO_4$ and Hg(II) uptake was still evident, suggesting that this species of Hg(II) might be important for uptake via an alternative uptake mechanism.

Varying the concentration of phosphate (8.75-67 mM) and $(NH_4)_2SO_4$ (2.225-9 mM) in our assay medium enabled us to manipulate the speciation of $HgHPO_4$ independently of pH. *E .coli* HMS174 (pRB28) and *E. coli* RKP2922 (pRB28) were used as indicators of Hg(II) uptake under anaerobic conditions to distinguish whether Hg(II)-phosphate speciation or pH was responsible for enhancing the bioavailability of Hg(II). Hg(II) uptake in *E. coli* HMS174 and RKP2922 did not correlate with the formation of $HgHPO_4$ at a constant pH of 5.9 (Figure 6.18). The lessened response observed in the medium containing lower concentrations of $(NH_4)_2SO_4$ appeared to be due to the energetics of the cell, as light production from the constitutive control (pRB27) was also decreased in this same medium for both strains (Figure 6.18B).

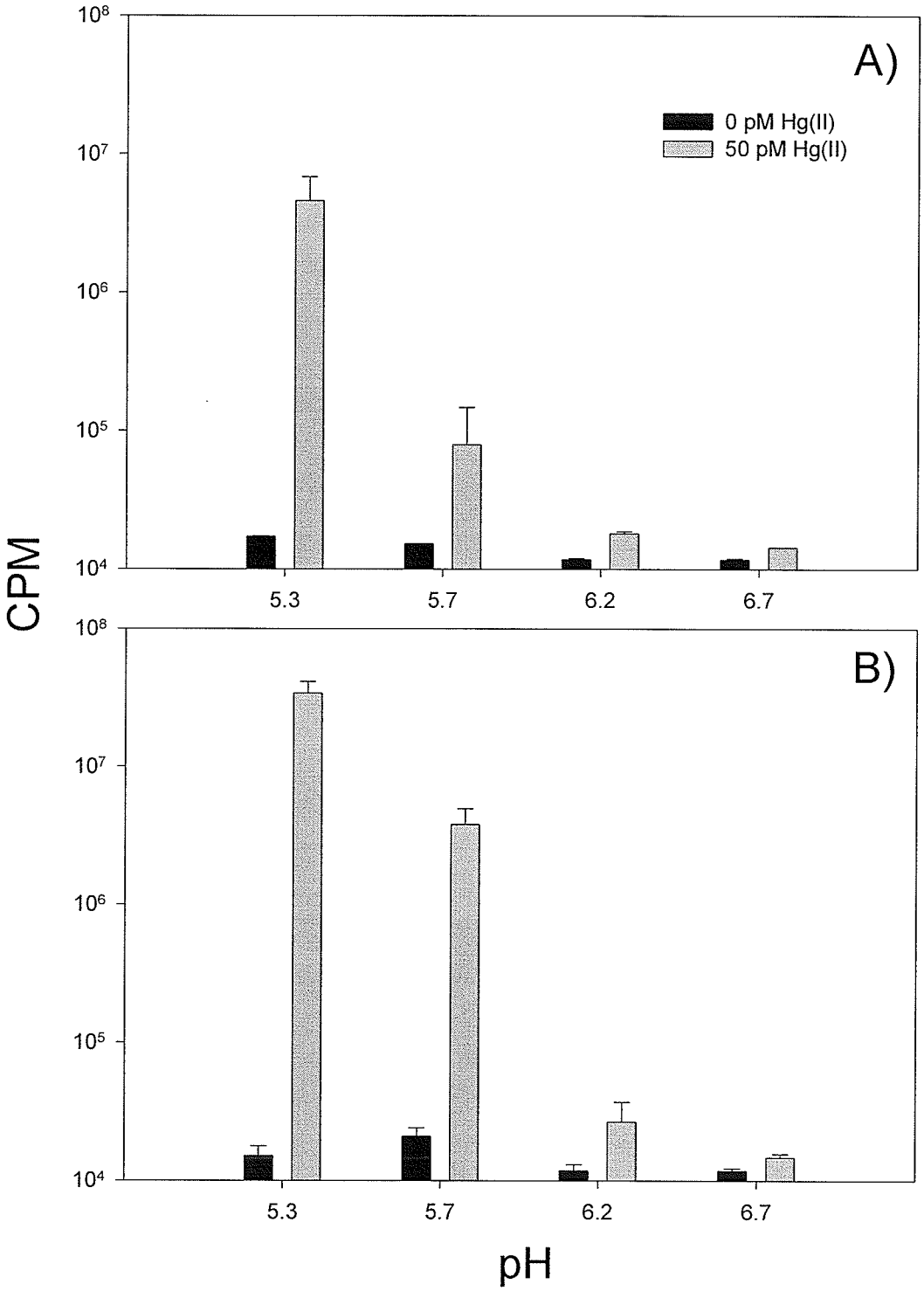

Figure 6.16. The effect of varying pH on Hg(II) uptake in A) *E. coli* MG1655 (pRB28) and B) *E. coli* RKP2922 (pRB28) under anaerobic conditions. Samples were incubated 120 min prior to measurement. Error bars represent the standard deviation of duplicate samples of two independent assays (n=4).

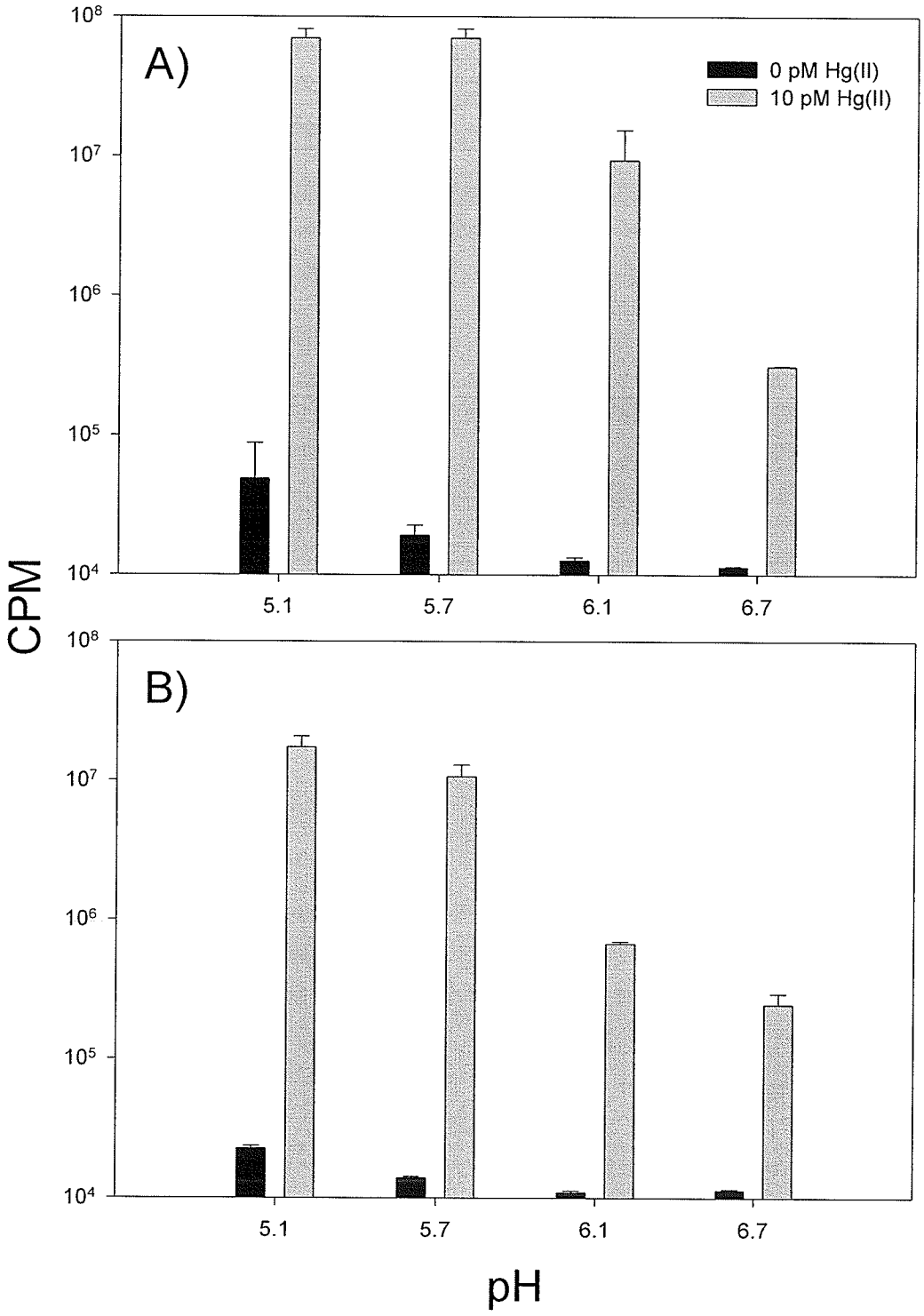

Figure 6.17. Effect of varying pH on Hg(II) uptake in A) *E. coli* MG1655 (pRB28) and B) *E. coli* RKP2922 (pRB28) under aerobic conditions. The light measurements following 120 minutes are represented. Error bars represent the standard deviation of duplicate samples of two independent assays (n=4).

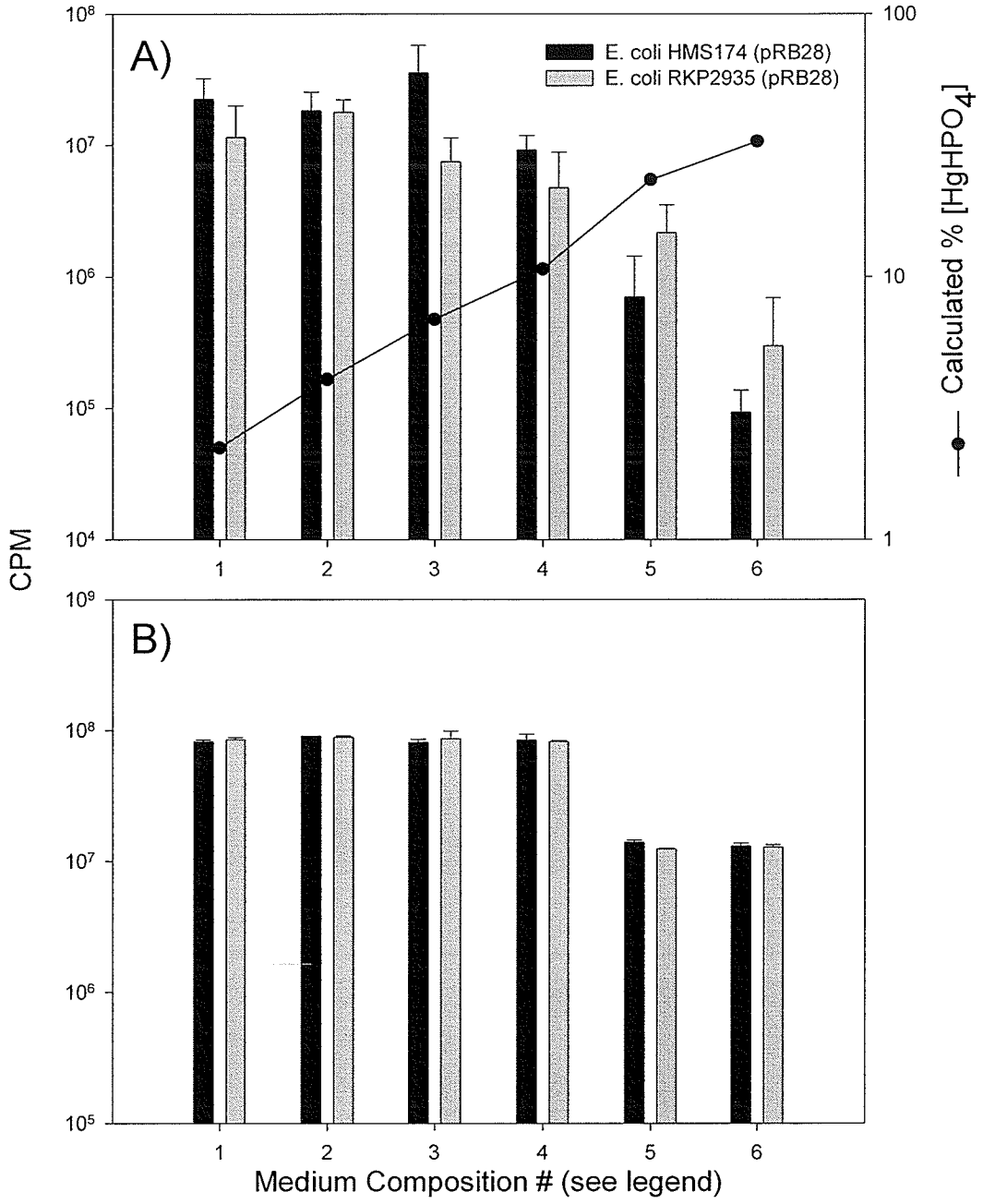

Figure 6.18. Effect of varying [$HgHPO_4$] independently of pH on A) Hg(II) uptake and B) the effect of varying concentration of phosphate and $(NH_4)_2SO_4$ on constitutive light production in *E. coli* HMS174 and *E. coli* RKP2935. Assays were performed in pH 5.9 assay medium containing 50 pM Hg(II). The concentration of phosphate and $(NH_4)_2SO_4$ in the media were 1) 67 mM and 9 mM; 2) 33.5 mM and 9mM; 3) 17.5 mM and 9 mM; 4) 8.75 mM and 9 mM ; 5) 67 mM and 4.5 mM; and 6) 67 mM and 2.25 mM respectively. The % of Hg_{total} as $HgHPO_4$ was calculated using MINEQL+ version 4.5. Samples were incubated 120 minutes prior to measurement. Error bars represent the standard deviation of A) two independent assays of triplicate samples (n=6) and B) two independent assays of duplicate samples (n=4).

Unfortunately, these were the two conditions that yielded the highest proportion of $HgHPO_4$. However, if we consider the pH titration experiments (Figure 6.1), we observed increased Hg(II) uptake in low pH samples that also decreased the constitutive bioluminescence response, thereby further implicating pH, and not Hg(II)-phosphate speciation, for increasing Hg(II) uptake.

6.4 Discussion

6.4.1 pH dependence of Hg(II) uptake In Chapter 3 we showed that the addition of carboxylic acids, which lowered the pH of the assay medium < 6, enhanced the Hg(II)-dependent light response (Table 3.2). In the present chapter we showed that we could achieve the same response by lowering the pH of the assay medium < 6, independent of the presence or absence of carboxylic acids (Table 6.1). Since the genetics and physiology are better understood in *E. coli*, we investigated the effect of pH on Hg(II) uptake under anaerobic and aerobic conditions in this host organism. A pH range of 5 – 8 was chosen because it is well within the growth range of *E. coli* (Ingraham 1987), and it is also representative of many natural environments. Results from this study indicated that the uptake of Hg(II) was substantially enhanced as a result of decreasing pH under both anaerobic and aerobic conditions (Figure 6.1A and B). This was further supported by the increased toxicity of Hg(II) observed at low pH (Figure 6.6A and B).

To ensure the enhanced bioluminescence in our study was Hg(II)-dependent, and not physiologically related to a decrease in pH, a number of control experiments were conducted. First of all, light production from the constitutive control (Figure 6.1C and D) and growth (Table 6.2) was shown to decrease as the pH decreased < 6.

Therefore, even though the cells were physiologically compromised in their light producing capability at pH < 6, the Hg(II)-inducible system still significantly increased. Secondly, lowering the pH of the assay medium might have altered the regulation of the *mer-lux* bioreporter genes independently of Hg(II). For example, regulation of the *mer* operon is sensitive to the supercoiled state of the DNA (Condee and Summers 1992), which can be altered by differences in osmolarity (Higgins et al. 1988). However, we demonstrated that lowering the pH of the assay medium did not play a role as a completely different pH induction profile was observed using Cd(II) (Figure 6.8). Additional evidence that pH specifically enhanced Hg(II) uptake was demonstrated in the acidification experiments. The acidification of cells pre-exposed to Hg(II) resulted in an increased bioreporter response, but not light capability of the cells (Figure 6.5A). The induction profiles from the acidification experiments were similar to assays in which additional Hg(II) was added to samples pre-exposed to Hg(II) (Figure 6.6B), strongly suggesting a Hg(II)-dependence for additional light production. In concordance, the addition of chloramphenicol to the acidified samples resulted in a steady decrease of light production demonstrating that new Hg(II)-dependent protein synthesis was required (Figure 6.5B). Therefore, additional factors that change as a result of pH must also be considered.

6.4.2. Effect of pH on bacterial physiology? One factor that changes as a result of pH is the physiology of the bacteria, which might have contributed to the apparent increase in Hg(II) uptake at low pH. Fluctuations in pH result in differential expression of a number of different genes in *E. coli* (Blankenhorn et al. 1999). One of these gene products might have been responsible for the enhancement of the

Hg(II)-dependent light response at low pH. However, no evidence from this study was provided to support the notion that the enhanced uptake of Hg(II) at low pH was due to the expression of pH stress response protein. Rather, the pH effect observed appeared to be constitutive since preconditioned cells did not respond more quickly than cells that were not preconditioned (Figure 6.4A and B).

6.4.3 Correlation with external Hg(II) speciation? Another factor that changes as a result of varying the pH of the assay medium is the speciation of Hg(II). The calculated speciation of Hg(II) in the bulk solution, as a result of decreasing pH, demonstrated a strong correlation between the formation of $HgHPO_4$ with the increase in toxicity and bioavailability of Hg(II) (Figure 6.15). This led us to believe that $HgHPO_4$ might be the species taken up preferentially by *E. coli.* Since the uptake of inorganic phosphate via PitA requires complexation with a divalent metal, e.g. $MeHPO_4$, we acquired Pit^- strains to determine if PitA was responsible for the non-specific transport of Hg(II). However, regardless of pH there was no difference in Hg(II) uptake using the Pit^- strain *E. coli* RKP2922 or *E. coli* MG1655 (Figure 6.16, 6.17). In addition, when the concentration of $HgHPO_4$ was manipulated independently of pH there was no correlation with Hg(II) uptake (Figure 6.18), which is consistent with the results obtained using *V. anguillarum* (pRB28) (Kelly et al. 2003). Therefore, Hg(II) uptake was dependent on the pH of the assay medium and not the bulk solution speciation of Hg(II).

6.4.4 Effect of pH on Hg(II) binding to bacteria Since uptake could not be correlated with Hg(II) speciation, one also has to consider the effect that pH has on the chemical speciation of the bacterial cell surface. In gram-negative bacteria the

lipopolysaccharide layer has been implicated as the major source of metal binding (Beveridge and Koval 1981). The bacterial surface is negatively charged and contains an abundance of potential metal binding sites, which also could serve as Hg(II)-binding sites. Non-specific binding of Hg(II) to these sites would not be detectable by the *mer-lux* bioreporter, since it only detects Hg(II) that has entered the cytoplasm of the cell. However, changes in external H^+ concentration could alter the speciation of these sites on the bacterial surface. In the Hg(II) binding experiments it appeared that variations in pH from 5.3-7.3 did not significantly affect total Hg(II) adsorption to the cell. However, we could not distinguish intracellular Hg(II) from Hg(II) bound to the cell using this method.

The increased uptake of Hg(II) as a result of increasing H^+ is in contrast to other metals, where increasing H^+ was shown to effectively compete with metal ions at biological uptake sites, hence reducing the toxicity and uptake of these metals (e.g. Hare and Tessier 1996). This could explain the differences observed in Cd(II) and Hg(II) uptake in *E. coli* under varying pH conditions (Figure 6.8), further delineating the FIAM as a predictor of Hg(II) bioavailability. Therefore, the increased Hg(II) bioavailability might be the result of H^+ competing with Hg(II) for "non-specific" negatively charged sites, ultimately resulting in more "free" Hg(II) to enter the cell and induce the bioreporter. Similarly, in the acidification experiments (Figure 6.5), increasing H^+ concentration could potentially displace Hg(II) that was bound at the higher pH from these non-specific sites.

The protonation of amino acids in periplasmic or membrane associated proteins might also play an important role in Hg(II) bioavailability for the same

reasons as described above. Of the amino acids histidine is interesting since the Hg(II)-dependent response under anaerobic conditions mimics the titration of a histidine residue (pKa 6.0). Histidine is an important residue in a number of proteins for the complexation of heavy metal ions. Protonation of histidine residues in such proteins might influence Hg(II)-binding, which in turn would effect the concentration of "free" Hg(II) in the cell. Physiologically, histidine is also an important residue in some proteins as a pH sensor (reviewed in Booth et al. 2002). Protonation of these histidine residues can alter the activity of the protein in a pH-dependent manner, which might indirectly influence Hg(II) uptake. Therefore, in addition to bulk solution chemistry, the speciation of the bacterial surface, periplasmic proteins, and membrane-associated proteins could play a significant role in the bioavailability of Hg(II).

6.4.5 Environmental implications In natural waters it has been estimated that ~ 95% of inorganic Hg(II) is bound to dissolved organic carbon (DOC) (Meili 1997). The effect that DOC has on Hg(II) bioavailability has been examined in both *V. anguillarum* (pRB28) (Kelly et al. 2003) and *E. coli* HMS174 (pRB28) (Barkay et al. 1997). For both of these bacterial species it was determined that Hg(II)-DOC complexes significantly reduced the bioavailability of Hg(II). However, by lowering the pH the bioavailability of Hg(II) was less affected by DOC. It was concluded that in natural waters increasing H^+ could play an important role in enhancing bioavailability by competing with the negatively charged sites on DOC for Hg(II) binding (Kelly et al. 2003, Barkay et al. 1997). Therefore, acidification of natural waters could result in less Hg(II)-DOC complexes and hence more "free" Hg(II) that

would be potentially available for uptake by the Hg(II)-methylating bacteria. Since DOC decreases the bioavailability Hg(II), the role of sulfide in enhancing the bioavailability of Hg(II), which was presumed to be related to the formation of HgS (Benoit et al. 2001, Benoit et al. 1999), might rather be important for competing with DOC for Hg(II) complexation in anoxic sediments.

Once again, the inorganic speciation of Hg(II) in the bulk solution could not be used as an accurate predictor of Hg(II) bioavailability. Therefore, identification of the mechanism(s) of Hg(II) uptake in bacteria, and how differences in physiology affect this process(es), is essential for our understanding and predictability of Hg(II) in natural waters. While the mechanism(s) of H^+ stimulation of Hg(II) uptake in the absence of DOC can not be elucidated from these experiments, the enhanced uptake of Hg(II) as a result of increasing H^+ concentration provides an additional explanation for the enhanced rates of Hg(II)-methylation commonly observed in acidified aquatic ecosystems.

Chapter 7

Search for potential secondary Hg transport systems in *E. coli* via mutagenesis

7.1 Introduction

In Chapter 4 we showed that the protonophore CCCP as well as a number of different metal ions (i.e. Cu(II), Mn(II), Mg(II)) mitigated Hg(II) uptake, as measured using a *mer-lux* bioreporter under aerobic conditions. These results provide evidence against passive diffusion since the diffusion of lipophilic Hg(II) species across the membrane would not be dependent on the H^+ gradient, nor would it be competitive against other metals at the level of uptake. These observations were more consistent with facilitated mechanisms of Hg(II) uptake involving metal transport systems for other metals. However, a clear indication of potential metal transport systems responsible for Hg(II) uptake was not possible. One example of a metal transport system demonstrating similarities, e.g. requirement of a proton motive force for providing the energy and metal competition by a variety of different metals for uptake, that might participate in the accidental uptake of Hg(II) is MntH (Kehres et al. 2000). However, the non-specific uptake of Hg(II) might also involve transport systems that have yet to be identified and/or specific for other essential cellular substrates, as demonstrated in higher organisms (see discussion in Chapter 3).

We hypothesized that the disruption of a gene, whose product is potentially involved in the transport of Hg(II), might result in a Hg(II) resistant phenotype, whereas a gene product involved in the intracellular trafficking of Hg(II) might result in a Hg(II)-sensitive phenotype. The assumptions for this are 1) only one major system would be involved in the uptake of Hg(II) and 2) Hg(II) toxicity is intracellular. Transposon mutagenesis has previously been used to identify a secondary transport system involved in the uptake of Zn(II) and As(III) in *E. coli*

(Beard et al. 2000). Therefore, for the purpose of identifying proteins potentially involved in the uptake/trafficking of Hg, we created random mutations in the genome of *E. coli* via Tn*10* mutagenesis.

7.2 Additional Methods

7.2.1 The Tn10 transposon The mini-Tn*10* derivative105, carried on a hop phage vehicle λNK1324 (λ *b522 c1857* P_{am}80 *nin5*) (Kleckner et al. 1991), was obtained from ATCC. The derivative 105 is marked with a chloramphenicol resistance fragment from Tn*9*. An *ats1 ats2* transposase gene fused to the *Ptac* promoter is located outside of the transposon, and is therefore inducible by IPTG.

7.2.2 Preparation and titering of lysates The preparation of and titering of lysates was performed as previously described (Miller 1992). In small capped test tubes, 0.05 ml of 0.1 M $MgSO_4$ was added to 0.1 ml of ~10^6 phages and 0.2 ml of ~ 3-5 X 10^8 ml^{-1} *E. coli* CSH110 (F^- ara Δ(*gpt-lac*)5 *sup*E *gyr*A *arg*E_{am} *met*B *rpo*B) and mixed gently. The mixture was incubated at 37^{O}C for 10 minutes and then added to diluted R-Top agar (15 ml of LB medium containing 0.005 M $CaCl_2$ per 100 ml of R-Top) and plated on LB agar plates. Plates were incubated face up at 39.5^{O}C overnight. In the morning, the top agar was scraped off and the plate was washed with 1ml of LB medium containing 0.01 M $MgSO_4$. Both were added to centrifuge tubes containing 4-5 drops of chloroform and centrifuged at 5000 rpm for 20 minutes. The supernatant was transferred to a screw-capped test tube and an additional 4-5 drops of chloroform was added. The lysates were stored at 4^{O}C.

For the titering of the lysates, 0.1 ml of *E. coli* CSH110 at 3-5 X 10^8 cells ml^{-1} was mixed with 0.1 ml of 10-fold serial dilutions of the phage lysate. The mixture was incubated at 37^{O}C for 10 minutes then plated on LB plates with 3 ml of R-Top agar (not diluted with LB medium). Plates were incubated overnight at 39.5^{O}C and plaques were counted the following morning. Titres of λNK1324 ranged from 10^{11}-10^{13} cfu ml^{-1} when grown on *E. coli* CSH110.

7.2.3 Tn10 mutagenesis Transposition using λNK1324 was carried out as previously described for λ1098 and λ1105 (Miller 1992). ~ 3 X 10^8 cells ml^{-1} of *E. coli* HMS174 was concentrated 10-fold by centrifuging and resuspending in 1 ml of LB medium containing 0.01 M $MgSO_4$. 0.1 ml of the lysate with a titre of ~ 1-3 X 10^{10} pfu ml^{-1} was added to 1 ml of the resuspended cells and incubated for 15 minutes in a 37^{O}C water bath. 1 ml of LB medium was then added and the cultures were incubated with aeration for 90 minutes at 37^{O}C to allow expression of antibiotic resistance. 0.05 ml of 10^0 –10^{-4} dilutions were then spread plated on LB-Cam plates and incubated at 39.5^{O}C.

7.2.4 Mutant selection Following transposition various methods were attempted to select for Hg-resistant and Hg–sensitive phenotypes, which are described further in the results section. Methods of preparing Hg(II) agar plates involved; 1) spread plating $HgCl_2$ directly onto the plates, 2) $HgCl_2$ was added directly to the cooling agar, or 3) cooled agar was added to sterile petri plates containing $HgCl_2$, which was maintained at an angle to make Hg(II) gradient plates. Selection of As-resistant Tn*10* mutants is also described in the results section and were screened on glycerolphosphate minimal medium (GlyP), which consisted of 15

mM $(NH_4)_2SO_4$, 1 mM KCl, 0.5 mM glycerolphosphate, 0.1 M tris-HCl (pH=6.8), and varying concentrations of Na_2AsO_4.

7.2.5 UV mutagenesis UV mutagenesis was carried out as previously described (Miller 1992). An overnight culture of *E. coli* HMS174 was diluted 1:50 in 30 ml of LB medium and incubated in a 37^{O}C water bath for 2 hours and then placed on a shaker for 2 hours. The culture was centrifuged, resuspended in an equal volume of 0.1 M $MgSO_4$, and placed on ice for 10 minutes. 5ml of the resuspended cells were placed into a sterile petri plate and exposed under the UV lamp for a set duration of time. Samples were titered for viable cells by plating dilutions on LB plates grown overnight at 37^{O}C. 0.25 ml of the mutagenized cells were inoculated into 5 ml of LB medium and grown overnight at 37^{O}C with aeration. Dilutions were then spread plated on M9 minimal medium plates containing varying concentrations of Hg(II) and grown at 37^{O}C.

7.2.6 Chemical mutagenesis N'-methyl-N'-nitro-N-nitrosoguanidine (MNNG) mutagenesis was carried out as previously described (Miller 1992). An overnight culture of *E. coli* HMS174 was diluted 1:50 in 30 ml of LB medium and grown for 2 hours in a 37^{O}C water bath and then placed on a shaker for an additional 2 hours. The culture was centrifuged and washed twice in an equal volume of sodium citrate buffer (9.6 g of citric acid, 4.4 g of NaOH, dH_2O to 500 ml; pH 5.5). The final pellet was resuspended in 15 ml citrate buffer and placed on ice. 0.1 ml of MNNG stock solution (1 mg/ml) was added to 1.9 ml of the resuspended cells and incubated at various times in a 37^{O}C water bath. The cells were spun down, washed twice in phosphate buffer (6.8 g of KH_2PO_4, 1.16 g of NaOH, dH_2O to 500 ml; pH to 7), and

resuspended in 2 ml of phosphate buffer. Samples were titered for viable cells by plating dilutions on LB plates and growing overnight at 37^{O}C. 0.25 ml of the mutagenized cells was inoculated into 5 ml of LB medium and grown overnight at 37^{O}C. Dilutions of the overnight culture were then plated on M9 minimal medium plates containing varying concentrations of Hg(II) and grown at 37^{O}C.

7.2.7 Preparation of $P1_{vir}$ lysates P1 lysates of three putative arsenate resistant donor strains: *E. coli* HMS174 Tn*10*: $As^{R}10$, Tn*10*:$As^{R}18$, and Tn*10*:$As^{R}19$, were prepared as previously described (Miller 1992). An overnight culture was diluted 1:50 in LB-Cam medium containing 5 mM $CaCl_2$ and grown to a density of ~ 1 X 10^8 cells ml^{-1}. Approximately 10^7 phage were added to 1 ml of this subculture and incubated at 37^{O}C for 20 minutes. 2.5 ml of R-top agar was added and then poured onto LB plates. Plates were incubated face up overnight at 37^{O}C. In the morning the R-Top agar was scraped off and placed into a centrifuge tube, along with 1 ml of LB medium used to wash the plate. 5 drops of chloroform was added to the centrifuge tubes. The tubes were centrifuged and the supernatant, containing the P1 lysate, was stored in screw capped test tubes at 4^{O}C.

7.2.8 P1vir transduction transduction into the recipient strain *E. coli* NM522 (*sup*E Δ (*lac-pro*AB) hsd-5 [F' *pro*AB *lac*I^{q} *lac*ZΔ15]) was carried out as previously described (Miller 1992). An overnight culture of *E. coli* NM522 was spun down and resuspended in an equal volume of MC buffer (0.1 M $MgSO_4$, 0.005 M $CaCl_2$). 0.1 ml of the resuspended cells was added to 0.1 ml of 10^0-10^{-2} diluted lysates and incubated in a 37^{O}C water bath for 20 minutes. 0.2 ml of 0.1 M sodium citrate buffer

was then added to inhibit the readsorption of the P1vir phage. 0.1 ml was then plated on LB-Cam plates and grown overnight at 37^{O}C.

7.3 Results

7.3.1 Mutagenesis for the selection of Hg^R and Hg^S phenotypes In an attempt to identify transport system(s) involved in the uptake of Hg(II) we created random mutations in the genome of *E. coli* HMS174 using the mini-Tn*10* derivative 105 carried on the hop phage λNK1324. Transposed cells from 6 independent experiments were spread plated onto LB plates containing 30 μg/ml chloramphenicol (Cam). > 6000 random colonies were then pick plated onto LB-Cam plates to confirm Cam^R. Cam^R colonies were then re-picked onto M9 minimal medium plates containing 0-100 μM Hg(II). These concentrations of Hg(II) allowed for the simultaneous selection of Hg(II)-resistant (Hg^R) and Hg(II)-sensitive (Hg^S) colonies (IC_{50} of HMS174 was ~ 30 μM Hg(II) on these plates). The proportion of cells that were auxotrophic was 2-3 %, which is within the expected range for random Tn insertion (Kleckner et al. 1991). 98 putative Hg^R and 105 Hg^S colonies were grown for 2 successive transfers in LB-Cam broth, spread plated onto LB-Cam plates, and re-picked in triplicate on M9 minimal medium plates of varying Hg(II) concentration. However, this secondary screening could not confirm the Hg^R or Hg^S phenotypes for any of the colonies. In some cases the putative Hg^R phenotypes appeared sensitive and vice versa for Hg^S phenotypes. It is likely that the observed resistance or sensitive phenotypes were dependent on the total amount of bacteria picked, which was not controlled. For example, colonies growing on the higher Hg(II)

concentration plates might of had more bacteria initially on the toothpick, and vice versa for the putative Hg^S colonies.

Since pick plating was unsuccessful we tried to maintain similar bacterial concentration by diluting the transposed colonies. ~ 1000 transposed colonies were spread plated directly onto M9 minimal medium plates containing Cam and varying concentrations of Hg(II). These experiments were repeated numerous times and yielded putative Hg^R and Hg^S phenotypes, but once again these phenotypes could not be confirmed in a variety of different secondary screening methods, which included pick or streak plating onto M9-Cam or LB-Cam plates containing varying Hg(II) concentration, growth in M9-Cam or LB-Cam broth with varying Hg(II) concentration, spread plating onto Hg(II) gradient plates, or transformed with the *mer-lux* bioreporter (pRB28) and assayed for reduced uptake of Hg(II), as measured by reduced light emission (e.g. Figure 7.1).

Unsuccessful with Tn*10* mutagenesis, we then attempted UV (Figure 7.2) and chemical (MNNG) (Figure 7.3) mutagenesis for the selection of Hg-resistant mutants. 4 putative Hg^R colonies were isolated from 3 independent UV experiments (Table 7.1) and 2 putative Hg^R colonies were isolated from 3 independent MNNG chemical mutagenesis experiments (Table 7.2). However, as with Tn*10* mutagenesis, secondary-screening attempts could not confirm the Hg-resistant phenotype.

7.3.2 Tn10 mutagenesis for the selection of As^R Tn*10* mutagenesis was carried out to select for arsenate resistance in *E. coli* HMS174 as a control. For each plate, ~1000 transposed colonies were spread plated onto glycerolphosphate (GlyP) medium containing varying concentrations of arsenate (0-10 mM). From 4

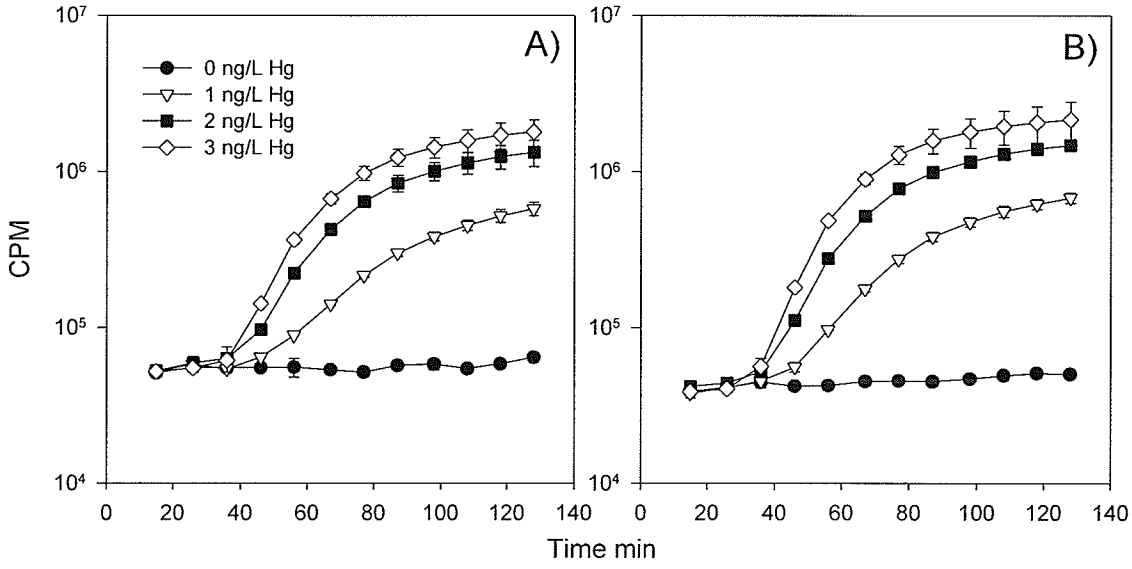

Figure 7.1. Comparison of Hg(II) uptake in A) *E. coli* HMS174 (pRB28) and B) putative Tn*10*:Hg^R *E. coli* HMS174 (pRB28). Error bars represent the standard deviation of triplicate samples.

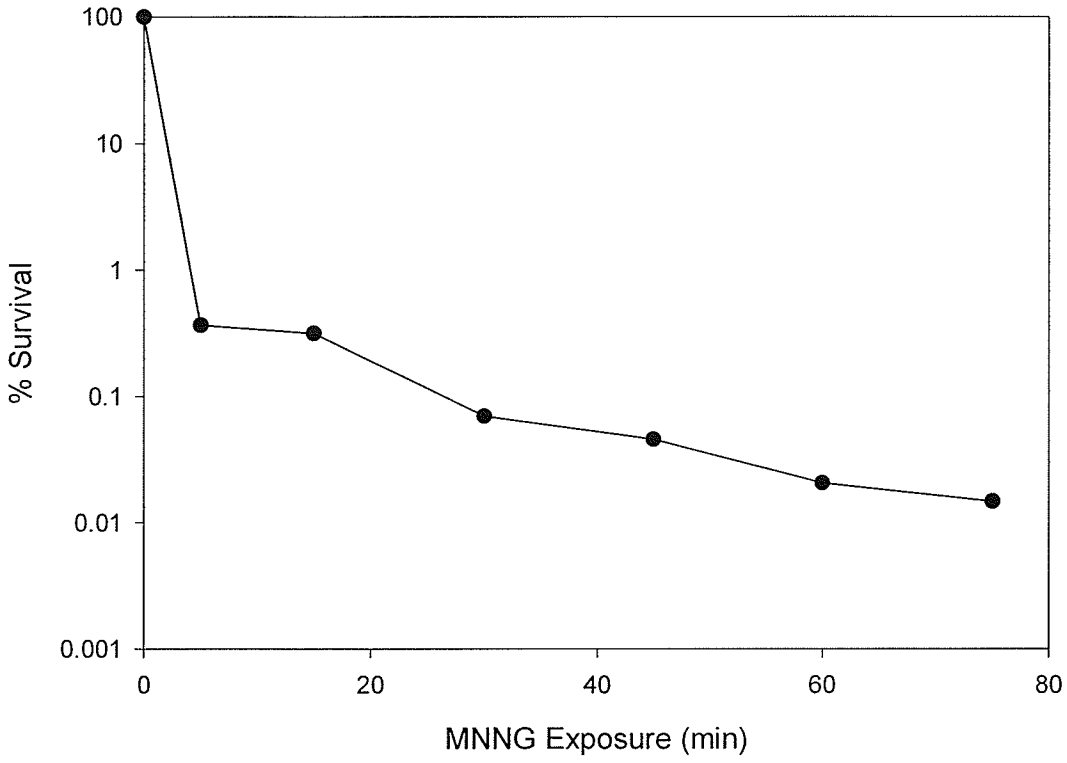

Figure 7.2. Survival % of *E. coli* HMS174 at various times in the presence of 50 μg/ml MNNG.

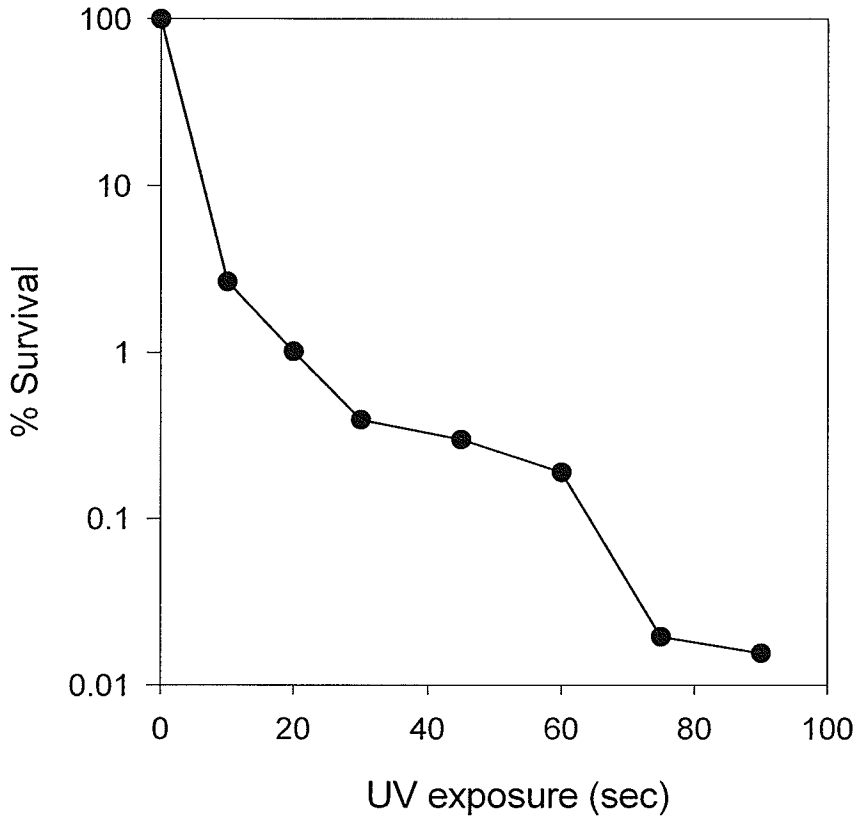

Figure 7.3. Survival % of *E. coli* HMS174 following various UV exposure times.

Table 7.1. Putative *E. coli* HMS174 Hg^R colonies following UV mutagenesis.

	[Hg] μM						
	70	80	90	100	120	140	160
UV Exposure (Sec)							
0	-	-	-	-	-	-	-
60	-	-	-	-	-	-	-
75	-	1 *	-	-	-	-	-
90	-	1	1	1	-	-	-

* Number of colonies observed

Table 7.2. Putative *E. coli* HMS174 Hg^R colonies following MNNG mutagenesis.

	[Hg] μM						
	70	80	90	100	120	140	160
50 μg/ml MNNG Exposure (sec)							
0	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-
45	-	1*	-	-	-	-	-
60	-	-	-	-	-	-	1
75	-	-	-	-	-	-	-

* Number of colonies observed

independent experiments, 22 putative resistant colonies were isolated. These colonies were grown in LB-Cam broth for 2 successive transfers, diluted to an OD of 0.4, and streak plated on GlyP medium containing Cam and varying concentration of arsenate (0-10 mM). Unlike the Hg(II) experiments, secondary screening on GlyP agar plates confirmed the resistance phenotype for all 22 colonies (Table 7.3 and Figure 7.4). Arsenate resistance was also confirmed by measuring the OD of the cells growing in GlyP broth culture of varying arsenate concentration (Figure 7.5)

7.3.3 P1vir transduction of As^R Resistance to arsenate, as a result of Tn*10* mutagenesis in *E. coli* HMS174 was further confirmed by P1 transduction. P1 Lysates of 3 putative arsenate resistant *E. coli* HMS174 cultures (Tn10: As^R 10, Tn10: As^R 18, and Tn10: As^R 19) were used to transduce the recipient strain *E. coli* NM522. Following P1 transduction, Cam^R *E. coli* NM522 colonies were isolated and grown for two successive transfers in LB-Cam. $\sim 10^5$ cells were inoculated into GlyP-Cam broth containing varying concentration of arsenate (0-10 mM). The transduced *E. coli* NM522 Cam^R cells were resistant to arsenate concentrations up to 10 mM, thereby confirming that the disrupted gene in *E. coli* HMS174 was responsible for the arsenate resistance phenotype (Figure 7.6).

Table 7.3. Growth of *E. coli* HMS174 Tn*10*: putative arsenate resistant mutants on GlyP-Cam plates containing varying concentration of arsenate. Growth is indicated on a +/- scale with (+++) representing the highest and (-) representing no growth.

	Concentration of arsenate (mM)						
	0	**1**	**2**	**4**	**6**	**8**	**10**
1	+++	++	++	+	+	+	-
2	+++	+++	+++	++	+	+	+
3	+++	+++	+++	++	+	+	+
4	+++	+++	+++	+++	++	++	+
5	+++	++	++	+	+	+	-
6	+++	+++	+++	+++	++	++	+
7	+++	+++	+++	+++	+++	++	+
8	+++	++	++	+	+	+	-
9	+++	+++	+++	++	+	+	+
10	+++	+++	+++	+++	+++	++	++
11	+++	+++	+++	+++	+++	++	+
12	+++	+++	+++	++	+	+	+
13	+++	+++	+++	+++	+++	++	++
14	+++	+++	+++	++	+	+	+
15	+++	+++	+++	+++	++	+	+
16	+++	+++	+++	+++	++	+	+
17	+++	+++	+++	++	+	+	+
18	+++	+++	+++	+++	+++	++	++
19	+++	+++	+++	+++	+++	++	++
20	+++	+++	+++	+++	++	++	+
21	+++	+++	+++	+++	+++	++	++
22	+++	+++	+++	++	++	+	+
HMS174	+++	++	-	-	-	-	-

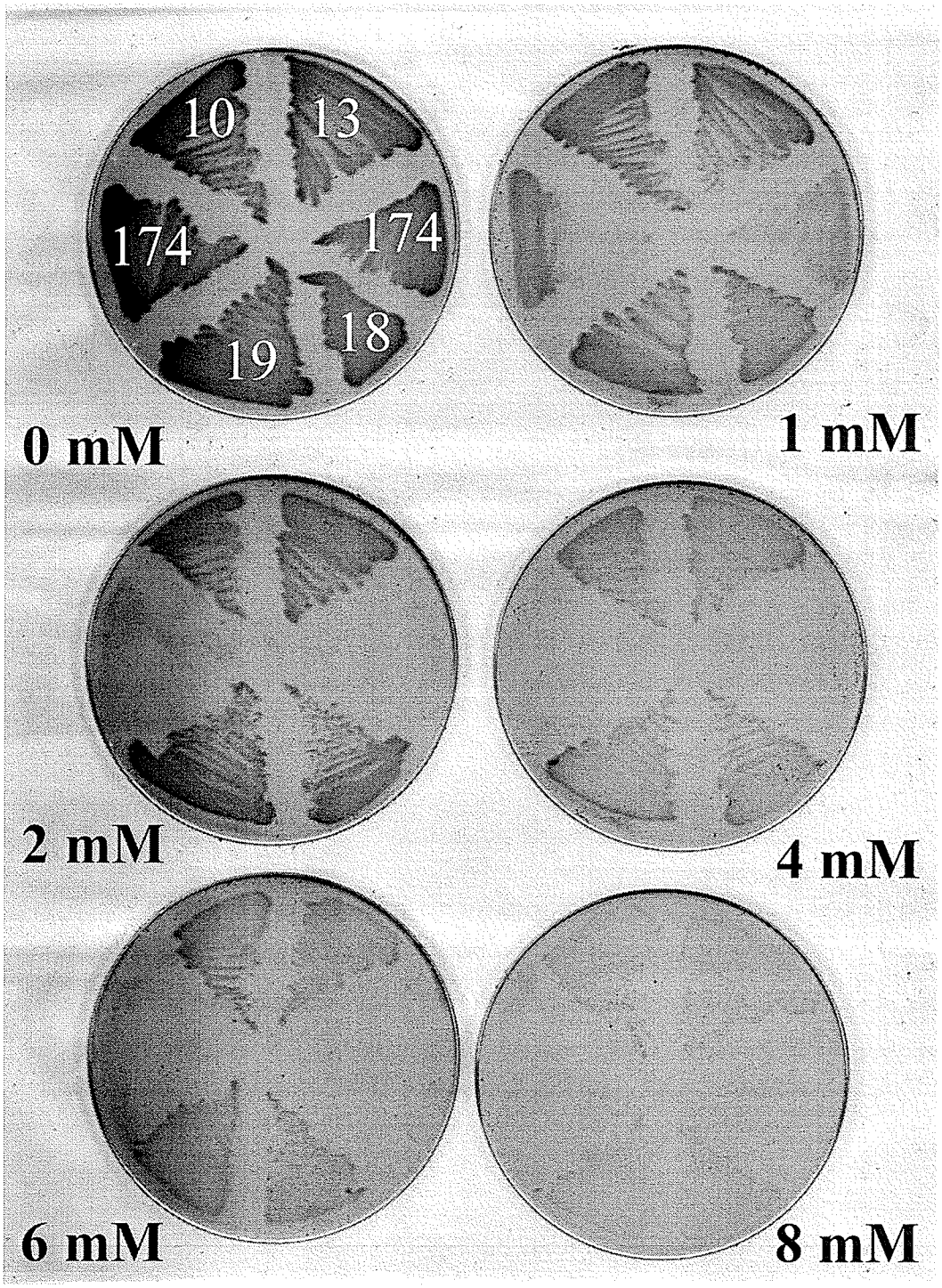

Figure 7.4. Growth of *E. coli* HMS174 (labeled 174) and *E. coli* HMS174 Tn*10*: putative arsenate resistant mutants (labeled 10, 13, 18, and 19) on glycerolphosphate plates containing varying concentrations of arsenate. Cultures were grown overnight at 37^0 C in LB-Cam broth and diluted to an OD_{600} of 0.4 before being streaked. Cultures were grown for 48 hours at 37^0 C.

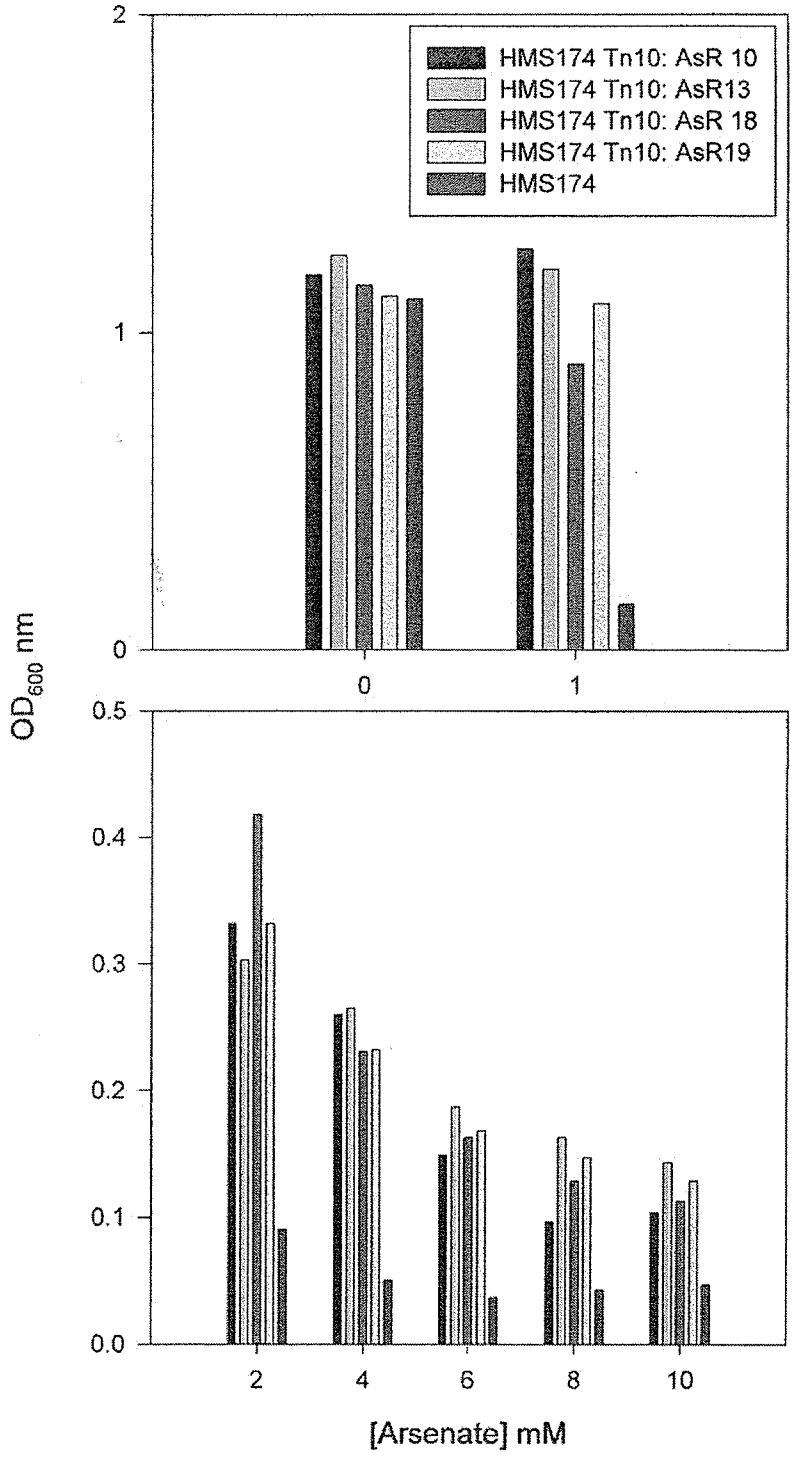

Figure 7.5. Final cell concentration of Tn*10*: putative As^R *E. coli* HMS174 strains in glycerolphosphate medium containing varying concentrations of As. Cultures were first incubated overnight at 37^0C in LB medium and then diluted. ~ 10^5 cells were used for the final inoculum and the cultures were grown at 37^0C for 48 hours prior to OD_{600} measurement.

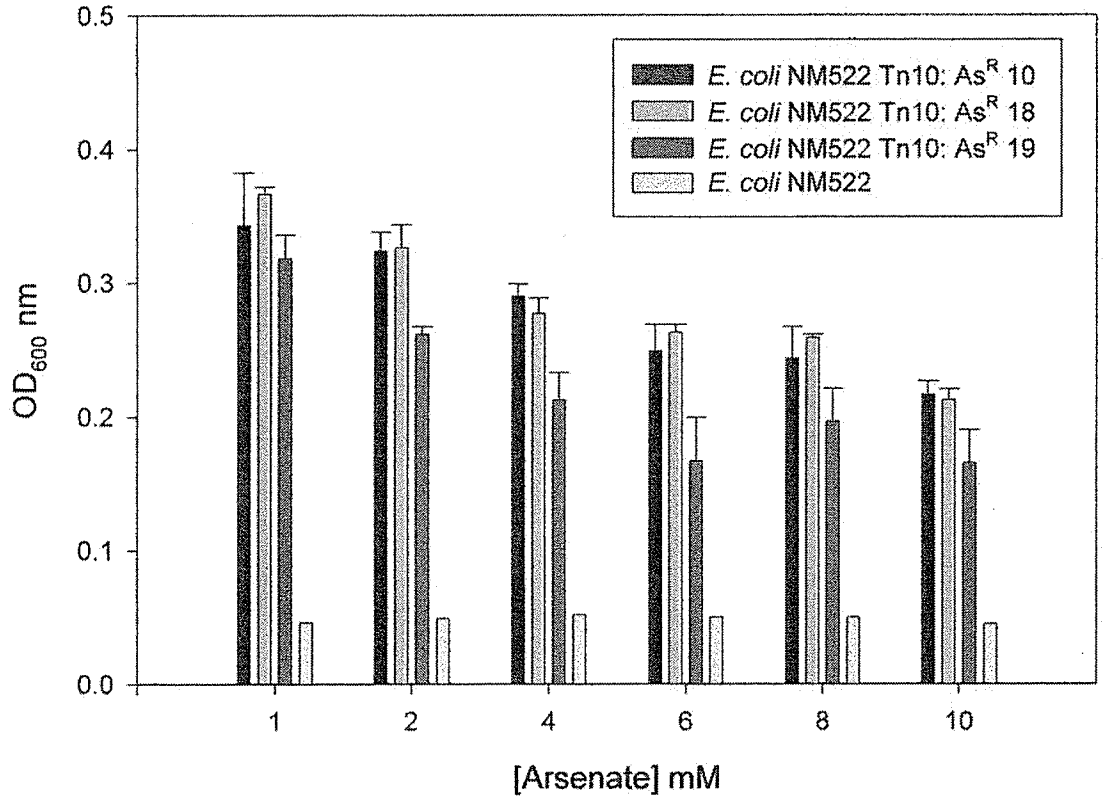

Figure 7.6. Growth of the P1*vir* transduction recipient Cam^R strains of *E. coli* NM522 in glycerolphosphate medium containing varying concentrations of As. Cultures were first incubated overnight at 37^0C in LB medium and then diluted. ~ 10^5 cells were used for the final inoculum and the cultures were grown at 37^0C for 48 hours prior to OD_{600} measurement.

7.4 Discussion

Tn*10* mutagenesis was a valid approach to identify genes involved in arsenate resistance, however we had no success with Hg(II). One possibility is that the uptake of Hg(II) involves an essential gene product. The "knockout" of an essential gene under the growth condition tested would not enable growth and therefore, cannot be tested using these methods. A second possibility is that multiple transport systems are responsible for the uptake of Hg(II), as demonstrated with the toxic non-essential metal Cd(II) (see discussion in Chapter 5). Evidence for this was provided in the differences of Hg(II) uptake observed under aerobic and anaerobic conditions (Chapter 3, 5, and 6). Therefore, the "knockout" of only one of these transport systems might not hinder Hg(II) uptake or toxicity. A third possibility is that the uptake of Hg(II) into the cytoplasm is not required for toxicity. For example, Hg(II) interactions with the phospholipid membrane or with periplasmic or membrane bound proteins would likely present the first site of toxicity for Hg(II). Whereas arsenate (AsO_4^{3-}) toxicity stems from its molecular homology with inorganic phosphate (PO_4^{3-}), thereby interfering with phosphate homeostasis inside the cell (Bennet and Malamy 1970).

Hg(II) is a "soft" metal that has a very high affinity for sulfhydryl and nitrogen donor ligands (Meyer and Nockemann 2003) and hence, bacterial cells would present many sites for Hg(II) binding before it even reaches the cytoplasmic membrane. Therefore, even with a membrane transport system for Hg(II) disrupted, toxicity would still arise from binding and disrupting essential cellular functions in the outermembrane, within the periplasm, or at the cytoplasmic membrane (e.g.

DeSilva et al. 2002, Myshkin and Konyaeva 2000, Delnomdedieu 1992, Delnomdedieu et al. 1989, Vallee and Ulmer 1972). The role of the periplasmic metallochaperone, MerP, for the delivery of Hg(II) to the membrane transport protein, MerT, strongly suggests that cellular ligands present in the periplasm, and on the cytoplasmic membrane, are a "sink" for Hg(II) toxicity. The binding of Hg(II) within and on the cell could explain the cell concentration dependence of our screening procedures, which coincides with increasing sensitivity of *mer-lux* bioreporters in the presence of decreasing cell concentration (Rasmussen et al. 1997).

The potential difficulty in identifying uptake systems for "sulfhydryl loving" metals might also explain why specific Cu(II) transport systems in *E. coli* have yet to be identified (Rensing and Grass 2003). Since Hg(II) has such a high affinity for cellular ligands, we conclude that the identification of possible secondary transport systems should not involve traditional toxicity screening methods.

Chapter 8

Thesis General Conclusions

8.1 Limitations of toxicity assays for predicting Hg(II) uptake

Results from toxicity assays were the first to demonstrate that total metal concentrations were relatively poor indicators of toxicity. This ultimately led to the discovery that differences in metal speciation have a significant effect on metal toxicity. It has been commonly stated that in order for a metal to exert a toxic effect it must be in a form that is accessible for the organism to take up into the cell (i.e. bioavailable) (e.g. Najera et al. 2005). In bacterial cells however, numerous sites for Hg(II) toxicity could potentially exist outside of the cytoplasm, which could include Hg(II) interactions with the outermembrane, within the periplasm, and at the cytoplasmic membrane. Hg(II) bound to any of these sites outside of the cytoplasm would not be available to the enzymes responsible for Hg(II) transformations, including the *mer-lux* bioreporter. Therefore, predictions regarding Hg(II) uptake on the basis of toxicity data alone might not be applicable for our purpose of predicting Hg(II) uptake into the cell.

A commonly used method that has correlated toxicity with bioavailability has involved bioluminescent organisms, where the loss of light in the presence of a toxic substance is used as an indicator of toxicity, and hence uptake (e.g. Ritchie et al. 2001, Vulkan et al. 2000, Stumm and Morgan 1996, Hughes and Poole 1991). However, when toxicity assays, using constitutive light production from (pRB27), were compared with uptake, using the *mer-lux* bioreporter (pRB28) in the same bacterial host, it was shown that toxicity assays were not an accurate predictor of Hg(II) uptake into the cell. For example, the addition of 1 mM histidine alleviated toxicity (Figure 4.6), but had no effect on or significantly enhanced Hg(II) uptake

under aerobic and anaerobic conditions respectively (Figure 4.5). Therefore, toxicity assays could not accurately predict what forms of Hg(II) were actually taken up into the cytoplasm of the cell. It is likely that the formation of Hg(II)-histidine complexes prevented Hg(II) ligand exchange with weaker cellular ligands within or on the cell, thereby alleviating toxicity. Whereas the weaker Hg(II) complexes, such as $HgCl_2$, $Hg(OH)_2$, and $Hg(NH_3)_2^{2+}$ would be more likely to participate in ligand exchange with cellular ligands, resulting in the observed toxicity. With all of the potential sites of toxicity in Gram-negative bacteria, it becomes evident why the results obtained using toxicity assays would be generally poor indicators of Hg(II) uptake into the cell.

8.2 Evidence not supporting passive diffusion of Hg(II)

At the start of the work on this thesis it was assumed that the mechanism of Hg(II) uptake into bacterial cells, not containing or expressing the *mer* operon, was via passive diffusion. This assumption was primarily based on two earlier studies, which demonstrated the passive diffusion of mM concentrations of the $HgCl_2$ species across eukaryotic- based lipid bilayer membranes (Bienvenue et al. 1984, Gutknecht 1981). Aquatic Hg(II) research has therefore focused predominantly on the chemical speciation of Hg(II) in the bulk solution, correlating the formation of neutrally charged Hg(II)-species in the bulk solution with Hg(II) uptake in microorganisms (e.g. Jay et al. 2002, Benoit et al. 2001, Benoit et al. 1999, Morel et al. 1998, Barkay et al. 1997). However, many results obtained throughout this thesis using the *mer-lux* bioreporter conflicted with the passive diffusion model.

First of all, if passive diffusion was the predominant mechanism of Hg(II) uptake in bacteria no differences in the uptake of Hg(II) under anaerobic and aerobic conditions should have been observed. However, ~ 100-fold higher concentration of Hg(II) was required for a detectable response under anaerobic conditions (Figure 4.1). Secondly, the addition of amino acids, which would form charged hydrophilic complexes with Hg(II), should mitigate passive Hg(II) uptake. However, hydrophilic Hg(II)-amino acid complexes enhanced Hg(II) uptake under anaerobic conditions (e.g. Figure 3.11) and did not decrease Hg(II) uptake under aerobic conditions (e.g. Figure 3.13). Thirdly, assay media promoting the formation of $HgCl_2$ in the bulk solution would be expected to result in faster and higher rates of Hg(II) uptake, in comparison to assay media where charged Hg(II) complexes predominated. However, Hg(II) uptake was dependent on the total Hg(II) concentration in the bulk solution, regardless of the inorganic speciation in the bulk solution under anaerobic (e.g. Figures 4.2A and 6.18) and aerobic conditions (e.g. Figures 4.3). This was clearly illustrated when there was still no significant change in response despite an 8 order of magnitude difference in the proportion of the neutrally charged $HgCl_2$ species (Figure 4.2C). There was also no detectable difference when higher concentrations of Hg(II) were used to investigate the effect of varying inorganic species on Hg(II) toxicity under aerobic and anaerobic conditions (Figure 4.4). These results were not surprising considering the relatively low lipophilicity of $HgCl_2$ and $Hg(OH)_2$ in comparison to other molecules known to diffuse passively across

membranes (Table 1.1). Furthermore, taking into consideration the high affinity of Hg(II) for cellular ligands, in comparison to the inorganic speciation of Hg(II) in the bulk solution, it was not surprising that the model of passive diffusion of Hg(II) was not supported in the test bacteria.

8.2.1 Bacterial limitations for passive diffusion Chemical modelers, attempting to correlate neutral Hg(II)-complexes formed in the bulk solution with bioavailability, need to also consider ligand exchange reactions of Hg(II) with cellular ligands outside, on, and within a bacterial cell (see Figure 1.7 for schematic illustration of the cell). For example, Hg(II) would first encounter bacterial exudates in the boundary layer, including organics and metal chelating sideophores, which could alter the speciation of Hg(II) before even reaching the cell. Assuming Hg(II) diffuses unaltered through the boundary layer, it then has to cross the outermembrane, which is negatively charged and is a major site in Gram-negative bacteria for metal binding (Beveridge and Koval 1981). The diffusion of Hg(II) through the LPS and across the asymmetric bilayer is unlikely because of their chemical characteristics (see discussion in Chapter 1, section 1.5.1). Therefore, the uptake of Hg(II) across the outermembrane likely involves diffusion through the water filled channels of porin proteins embedded in the outer membrane (Brown et al. 2002), but awaits experimental verification. However, one also has to consider that Hg(II) interactions with accessible amino acid side chains in these porin proteins present an additional site for ligand exchange and potential sites for Hg(II) toxicity.

The outermembrane presents a major barrier that Hg(II) species formed in the bulk solution must encounter. For speciation models to accurately predict Hg(II) bioavailability, such species would have to penetrate through this outermembrane unaltered. However, the biggest challenge is to understand the bioinorganic chemistry of Hg in the periplasm of Gram-negative bacteria. The periplasm, which accounts for up to 20% of cell volume (Stock et al. 1977), has never been considered in chemical speciation models that correlate bulk solution chemistry to the uptake of the metal. However, the microenvironment of the periplasm should be considered separate from the surrounding environment and would likely have a significant affect on Hg(II) speciation before it even reaches the cytoplasmic membrane (Williams and Frausto da Silva 2000). This has important implications not only for passive diffusion models, but also for models such as the FIAM discussed in Chapter 4.

The fate of Hg(II) in the periplasm would likely involve Hg complexation reactions with thiolates of cysteine and imidazolium nitrogens of histidine residues in proteins, including metallochaperones, and thiol-containing molecules such as glutathione and cysteine. Therefore, "labile" hydrophobic or hydrophilic Hg(II) species formed in the bulk solution are likely to be negligible in the periplasm. Assuming that Hg(II) formed in the bulk solution has diffused unaltered across the outermembrane and through the periplasm it is now faced with the polar head groups of phospholipids and accessible amino acid side chains of the proteins embedded in the cytoplasmic membrane. The major phospholipid in *E. coli* is phosphatidylethanolamine (Neidhart et al. 1990), which contains an accessible amine group that was shown via NMR to bind specifically with $HgCl_2$ (Delnomdedieu et al.

1989), presenting yet another potential site of toxicity and limitation for passive diffusion. Proteins embedded in the cytoplasmic membrane present additional sites for Hg(II) ligand exchange, which could result in toxicity, or possibly transport across the membrane. To what extent the speciation of Hg(II) is altered by the cellular ligands remains to be determined, but could provide insight into specific sites of Hg(II) toxicity and/or possible mechanism(s) of uptake across the cytoplasmic membrane. Within the cytoplasm Hg(II) would interact with a different subset of cellular ligands. Therefore an understanding of the fate of Hg(II) within the cytoplasm could increase our knowledge of the enzymatic processes involved in the bacterial mediated transformations of Hg(II).

8.3 Potential mechanism(s) of Hg(II) uptake

The indiscriminate uptake and toxicity of inorganic Hg(II) species, such as $HgCl_2$, $Hg(OH)_2$, and $Hg(NH_3)_2^{2+}$, did not support the passive diffusion model, but was consistent with ligand exchange (Hudson 1998). In the simplest case, Hg(II) ligand exchange with cellular ligand molecules in the boundary layer or the periplasm might result in a lipophilic species that diffuses passively across the cytoplasmic membrane. The passive diffusion of such molecules across the membrane would be driven by the concentration gradient, and would therefore not require additional energy provided by the cell. However, under aerobic conditions the addition of CCCP significantly mitigated Hg(II) uptake (Figure 5.1), suggesting that Hg(II) uptake was not only facilitated, but driven in part by the H^+ gradient. In addition to

the H^+ gradient, the Na^+ gradient is also believed to be involved in the uptake of Hg(II) in *E. coli* (Selifonova and Barkay 1994), which suggests that multiple facilitated transport systems could exist for the accidental transport of Hg(II). Additional facilitated mechanisms of Hg(II) uptake into bacterial cells could also potentially be via facilitated diffusion or ATP-dependent transport systems, neither of which have been investigated.

8.3.1 Role of cellular organics in Hg(II) co-transport? One possibility is that Hg(II) complexes formed outside the cytoplasmic membrane mimic an essential substrate that is subsequently transported across the cytoplasmic membrane. Examples of which might include Hg(II) complexation with glutathione or amino acids, which are subsequently transported into the cell. The enhanced uptake of Hg(II) observed in the presence of varying amino acids (Figure 3.11) and the potential role of the Na^+ gradient (Selifonova et al. 1994) support this hypothesis, but requires further experimental verification. Such a mechanism for Hg(II) uptake in bacteria would be similar to mammalian cells, in which the transport of Hg(II) across the membrane via Na^+ dependent amino acid transporters or organic anion exchangers is dependent on the formation of Hg(II)-glutathione and Hg(II)-cysteine complexes (Bridges et al. 2004, Zalups and Ahmad 2004, Zalups et al. 2004, Zalups 2000). Since the concentration of glutathione in *E. coli* is ~ 6-7 mM (Apontoweil and Berends 1975), its potential importance in Hg(II) sequestration and transport requires further investigation.

8.3.2 Role of non-specific metal transporters in Hg(II) uptake? The non-specificity metal transport systems provides additional possible mechanisms for the accidental uptake of Hg(II) in bacteria. These systems are responsible for the accumulation of other non-essential metals such as Cd(II), Pb(II) and Ag(I) (reviewed in Nies 1999), however, Hg(II) was never considered or examined in any of these studies. Results obtained with the bioreporter were compared with characteristics of known metal transport systems, and the inorganic phosphate transporter, PitA, appeared a likely candidate for the accidental uptake of Hg(II). For example, PitA is driven by the H^+ gradient and is capable of transporting a variety of different divalent cation metals as a neutral $MeHPO_4$ complex (reviewed in van Veen 1997). In comparison, the bioreporter demonstrated a strong correlation, based on external speciation models, between the formation of $HgHPO_4$ and uptake (Figure 6.15). There was also mitigation of Hg(II) uptake in the presence of CCCP (Figure 5.1) and in the presence of a number of different competing metal ions (see results in Chapter 5). While PitA seemed a likely candidate, *E.coli* strains devoid of PitA demonstrated that it was not likely involved in the uptake of Hg(II) (Figures 6.16-6.18). Hence, we too "fell victim" to the enticing correlations observed using Hg(II) speciation models.

Since a number of different metals mitigated Hg(II) uptake (Chapter 5) a clear indication of what metal transport system(s) might be involved could not be determined. However, another possible transport system that displays similar characteristics with the results obtained using the bioreporter is MntH. This transporter is specific for Mn^{2+}, but is also capable of transporting additional non-essential metals, e.g. Cd(II), is driven by the H^+ gradient, and is also regulated by

OxyR (Kehres et al. 2000). Since the bioreporter displayed increased sensitivity under aerobic conditions, and Mn(II) (Figure 5.9) and CCCP (Figure 5.1) mitigated Hg(II) uptake, MntH is a potential candidate to be further investigated in future studies for the uptake of Hg(II).

The high affinity of Hg(II) for ligands in the periplasm and the cytoplasmic membrane is likely a major reason why Hg(II) is so toxic to bacterial cells. Considering all the sites of potential Hg(II) interactions throughout the cell it was surprising that the bioreporter, which is devoid of MerP and specific Hg(II) transport proteins, was capable of responding to such low concentrations of Hg(II) without the aid of metallochaperones. For example, each cell would only be exposed to ~ 30,000 atoms of Hg(II) in the bioreporter assays performed in assay medium containing 5 pM Hg(II), which is comparable to Hg(II) concentrations in pristine environments. This would explain the role of the metallochaperone MerP, which sequesters Hg(II) in the periplasm and transfers it to MerT (Wilson et al. 2000). However, periplasmic metallochaperones for other metals, e.g. Cu(II) and Ni(II), might present additional assistance for Hg(II) uptake in bacterial cells devoid of MerP. For example, the conserved GMXCXXC metal binding motif found in MerP is also found in Ni(II) and Cu(II) metallochaperones. Hg(II) binding to these metallochaperones might provide a possible mode of Hg(II) sequestration in the periplasm, which could subsequently deliver Hg(II) to a transport system embedded in the cytoplasmic membrane. Evidence for this was demonstrated in yeast cells, where the Cu(II) metallochaperone Atx1 was shown to bind and specifically transfer Hg(II) to Ccc2, which is the membrane transporter for Cu(II) (Rosenzweig et al. 1999). The fact that Cu(II)

mitigated Hg(II) uptake under aerobic (Figure 5.12) and anaerobic conditions (Figure 5.16), further supports a potential role of Hg(II) uptake via transport systems specific for Cu(II).

As with the uptake of other non-essential heavy metals, e.g. Cd(II), it is likely that multiple routes of Hg(II) uptake exist. The expression of many bacterial transport systems is dependent on the metabolic requirements of the cell (reviewed in Nies 2003). If such transporters are also involved in the accidental uptake of Hg(II) then increased uptake rates might be observed under conditions that increase the expression of these systems. For example, the *nik* operon of *E. coli* is induced under anaerobic conditions to supply the cell with Ni(II), which is required for the hydrogenase 1 enzyme (reviewed in Mulrooney and Hausinger 2003). This implies that in addition to studying the chemical speciation of Hg(II), the physiological conditions of the microorganisms inhabiting that ecosystem would also need to be considered. Therefore, in order to increase our predictive ability of Hg(II) in aquatic ecosystems the identification of these transport mechanisms is crucial. Many of these transport systems are found over a wide range of genomes and therefore, identification of mechanisms of Hg(II) uptake in a well characterized model organism could also be applicable in other natural organisms. Since the genetics and physiology of *E. coli* have been well characterized, it serves as an excellent model organism to do these initial studies.

8.4 Potential limitations of using bioreporters for the detection of Hg(II) in natural samples

In numerous environmental studies, the proportion of Hg_{Total} measured in aerobic natural samples that is "bioavailable" has quantitatively been reported using Hg(II)-bioreporters (e.g. Hintelman et al. 2002, Scott 2001, St. Louis et al. 2001, Rasmussen et al. 2000). Using the *mer-lux* fusion (pRB28), it was demonstrated that *lux* is also a suitable reporter for the detection of trace concentrations of Hg(II) under anaerobic conditions in *Escherichia coli* and *Vibrio anguillarum*. While bioreporters are currently the only method available to accomplish this, there are still many problems that need to be addressed in order to more accurately quantify bioavailable Hg(II).

8.4.1 pH limitations? Under anaerobic conditions the detection limit of the *mer-lux* bioreporter was highly dependent on a number of different factors. In pH 7 assay medium Hg(II) concentrations of > 250 pM was required for a detectable response (Figure 4.1), which could be reduced significantly in the presence of specific amino acids (Figures 3.11) or by lowering the pH of the assay medium below 6 (e.g. Figure 6.1 and Table 3.2). This means that results obtained through the use of this bioreporter to measure bioavailable Hg(II) in natural anaerobic sediment pore waters becomes quite difficult to interpret. For example, in anaerobic circumneutral waters it might only be applicable to use in Hg(II)-contaminated sites. In less-Hg(II)-contaminated sites the assays become complicated because of the requirement of amino acids or low pH in the standards, which unfortunately could have a significant effect on the bioavailability of Hg(II) in the natural samples (Figures 3.29 and 3.30). Since the sensitivity of the bioreporter is significantly increased at low pH (Chapter

6), independently of organic acids (Table 6.1), the potential of using the bioreporters to study the bioavailability of trace Hg(II) in acidified environments is more plausible.

8.4.2 Standardization limitations? An additional problem is with the standardization of the bioreporter assays. In many previous studies, standard Hg(II)-induced light curves were first prepared in chemically defined assay medium. Quantification of bioavailable Hg(II) was then determined by comparing the light response in the natural samples with the standard curve and extrapolating the concentration of Hg(II) that was equivalent in light response. This method is based on the assumption that the standard is 100% bioavailable (Barkay et al. 1998). However, in defined assay medium we determined that at least 20% of the standard Hg(II) addition remains in the bulk solution (Figures 6.12 and 6.13). Therefore, the response at best would be to 80% of the total Hg(II) adsorbed by the cell at that specific time. However, of this 80% adsorbed, the actual amount that induces light production can be speculated to be quite small because of the high binding capacity of the bacterial cell (i.e. Hg(II) bound to the outermembrane, within the periplasm, or on the cytoplasmic membrane would not be detectable by the bioreporter). While it's true that a lot of the Hg(II) probably adsorbs to the outside of the cells, this is "normal" behavior for Hg. The idea is to standardize the number of cells used, and to compare what happens when these new cells are put into standards, and when they are put into natural waters. In both cases, a similar portion of available Hg(II) should stick to the cells, and a similar portion would be taken up.

8.4.3 Physiological and cellular considerations Differences in bacterial physiology resulting from the chemistry of the varying natural water samples would provide an additional problem to be considered in future bioreporter studies. While constitutively expressed *lux* systems have been used to address how these differences in water chemistry affect the light producing capability of the cell, it tells us nothing about potential differences in Hg(II) uptake. Differences in water chemistry would alter the proteome of the host bioreporter cell, which might result in differences in Hg(II) sequestration, intracellular trafficking, and/or transport into a specific bacterial species.

Another consideration, which complicates matters even further, is that the mechanisms of Hg(II) uptake and/or binding capacity of cells for Hg(II) could be species specific. For example, differences in the LPS and the presence or absence of S-layers in Gram-negative bacteria might have substantial effects on the binding capacity of the cell. The cell wall of Gram-positive bacteria, which consists predominantly of techoic acids and peptidoglycan, has a very high affinity for metal binding (Beveridge and Murray 1980). Since the bioreporter only responds to intracellular Hg(II), these potential differences in transport and binding capacities need to be carefully examined. Therefore, results obtained using one or two different bacterial species might not be applicable for all bacteria in general. Evidence for this was provided in earlier work, not pertaining to my thesis, where the *mer-lux* bioreporter plasmid (pRB28) was transformed into a few different bacterial species (Table 8.1). Even though the *mer-lux* fusion plasmid was stable in these bacterial hosts, the responses to trace concentrations of Hg(II) (< 15 pM) varied substantially

Table 8.1. Transformation of the bioreporter plasmid (pRB28) into various bacteria and their subsequent responses to trace concentrations of Hg(II) under aerobic conditions.

Bacterial host	Response to trace Hg(II) concentrations (< 15 pM)
Pseudomonas stutzeri	Ultrasensitive**
Vibrio natriegens	Non-inducible
NR31*	Non-inducible

* A Gram-negative natural isolate obtained by K. Scott from the ELA.

** Induced to very high levels even in the absence of standard Hg(II) additions

in comparison to *E. coli* or *V. anguillarum*. The reason for these differences were not investigated, but might be explained by differences in cellular binding capacities and/or species specific differences in Hg(II) uptake mechanisms available. For example, while *mnt*H might be responsible for the accidental uptake of Hg(II) in *E. coli* it does not appear to be present in the two sequenced *Desulfovibrio* species.

8.5 Environmental implications

While the work presented in this thesis was predominantly laboratory based, the results obtained have important implications for increasing our knowledge and predictability of the bacterial mediated cycling of Hg(II) in natural environments.

8.5.1 Role of the mer operon in pristine environments The initial reported in vitro threshold for induction of the *mer* operon in the presence of mM thiols was ~ 3 nM (Ralston and O'Halloran 1990) and therefore, the importance of bacteria reducing Hg(II) was never considered significant in non-polluted environments. However, Hg(II) concentrations ranging from fM-pM are capable of inducing the *mer* operon, as indicated using *mer-luc* (Virta et al. 1995) and *mer-lux* (Kelly et al. 2003 and e.g. Figure 3.1) bioreporters. This implies that the role of bacteria containing the *mer* operon in non-polluted sites might be more important than previously believed.

8.5.2 Role of bacteria in the biomobilization of Hg(II)? In natural environments Hg(II) is tightly bound to a variety of different particles and therefore, the water-extractable fraction has often been related to the bioavailability (e.g. Rasmussen et al. 2000). Using the bioreporter in natural sediment pore water *V. anguillarum* was capable of detecting Hg(II) concentrations as low as 50 pM (Figure

3.29 and 3.30), which was much lower than the 300-500 pM of Hg(II) required in defined assay medium (e.g. Figure 4.1 and 6.3A). Since the sediment pore water samples would contain a variety of different high affinity Hg(II) ligands the opposite effect would have been expected. However, the binding affinity of Hg(II) for bacterial cells (Figures 6.12 and 6.13) might be important for releasing particle-bound Hg(II) complexes, which would not be present in the water-extractable fraction. For example, the release of Hg(II) bound to clay particles was demonstrated using a *mer-luc* bioreporter in *Pseudomonas fluorescens* (Petanen and Romantschuk 2003). This release and subsequent uptake of Hg(II) by bacteria could then potentially enter the soluble phase. This is especially relevant considering that concentrations of bacteria in natural environments can range from thousands to millions of cells/ml. Therefore, bacterial contributions in the biomobilization of Hg(II) could be quite significant. This could also imply that bacteria might be important sources of Hg(II) to the sediments of aquatic ecosystems. For example, bacteria residing in oxic surface waters would be the first to encounter Hg(II) introduced to aquatic ecosystems from e.g. atmospheric deposition. Over their relatively short life spans, the Hg(II) accumulated by these bacteria could be re-released to other bacterial species, including the bacteria capable of methylating Hg(II), following the sedimentation and subsequent degradation of these dead cells.

8.5.3. Role of bacteria in the biomagnification of MeHg? An additional possible role of bacteria, which might also have been overlooked, is in the MeHg biomagnification paradigm. The biomagnification of MeHg in aquatic food webs is of great environmental concern and ultimately begins with the uptake of Hg(II) into

specific bacterial species residing in anoxic sediments, which is still unknown. Once in the cell Hg(II) can then be methylated, which is then proposed to be "released" from the cell and accumulated into lower trophic organisms, such as phytoplankton, via passive diffusion (e.g. Mason et al. 1996). The consumption of these organisms by higher trophic organisms results in the biomagnification of MeHg up the aquatic food chain, which ultimately results in higher concentrations of MeHg in top predatory fish. However, the role of bacteria in this biomagnification process has not been considered, despite their high cellular concentrations in natural waters and relatively high affinity for binding Hg. Therefore, the biomagnification of Hg in food chain might start with the bacteria. One also needs to consider that organisms such as phytoplankton, which are the same lower trophic organisms that are proposed to begin this biomagnification process, graze upon bacteria. Since the release of MeHg from Hg(II)-methylating bacteria has never been directly demonstrated, they could potentially be the first step/food-source in this biomagnification process.

While remediation strategies are limited for the biomagnification of MeHg in the aquatic food chains, alternative preventative strategies should focus on the uptake of Hg(II) into the Hg(II)-methylating bacteria. In light of the complex cell structure of gram-negative bacteria, the facilitated uptake of Hg(II) in higher organisms, the non-specific uptake of other heavy metals in bacteria, and the results presented in this thesis using the *mer-lux* bioreporter, secondary systems for Hg(II) uptake likely exist in bacteria. The identification of alternative routes for Hg uptake in bacteria might lead to explain why different environmental conditions favor some Hg

transformations over others, which would greatly increase our predictive capabilities of Hg in aquatic ecosystems.

8.6 Future work

Despite Hg(II) being one of the best studied heavy metals, it was apparent throughout this thesis that there are still a lot of unanswered questions that need to be addressed.

8.6.1 Identification of the mechanisms of Hg(II) uptake in bacteria not containing or expressing the mer operon. Further attempts to identify the transport systems involved in the accidental uptake of Hg(II) could involve collecting or creating specific "knock out" mutants for an array of different transport systems. Based on the *mer-lux* bioreporter assays and the uptake of Hg(II) in higher organisms this should include a variety of different transport systems (e.g. metal transport systems, organic anion transporters, oligopeptide and amino acid transporters, outermembrane proteins, and transporters involved in the uptake of cytsine and glutathione). Since toxicity screening was not successful, it might be useful to transform the bioreporter into these acquired/created strains and assayed for their ability to accumulate trace concentrations of Hg(II). For creating random mutations via mutagenesis, I would suggest transforming pools of randomly transposed colonies with the *mer-lux* bioreporter plasmid (pRB28) and scaling down the *mer-lux* assays to enable a photometer to measure bioluminescence in microtitre plates. "Non-

inducing" or "hypersensitive" cells identified using such a method could then be further examined for its potential role in Hg(II) transport.

8.6.2 Hg(II) interactions within the cell. To what extent the speciation of Hg(II) is altered by the cell, both in the periplasm and in the cytoplasm, needs further investigation. Knowledge of this could provide valuable insight into specific sites of toxicity and/or possible mechanisms of intracellular trafficking and/or transport. This would also have important implications for chemical modelers attempting to make predictions of Hg(II) uptake on the basis of external speciation only.

8.6.3 Reevaluation of the significance of bacteria in the Hg(II) cycle of aquatic ecosystems. Despite the high affinity of bacterial cells for Hg(II), and their relatively high concentrations in natural waters, they have been largely neglected in some important aspects of the Hg(II) cycle in aquatic ecosystems. Bench scale experiments could be designed to address some basic questions regarding the potential significance of bacteria in e.g. the biomobilization of Hg or in the biomagnification of MeHg.

8.6.4 Standardization of bioreporter assays. There are many problems to be addressed with the standardization of the bioreporter assays before qualitative analysis of bioavailable Hg(II) concentrations are made. For example, differences in water chemistry might alter the binding capacity and/or the proteome of the host bioreporter cell, which could result in differences in Hg(II) sequestration, intracellular

trafficking, and/or transport into a specific bacterial species. While constitutively expressed *lux* systems have been used to address how these differences in water chemistry affect the light producing capability of the cell, it tells us nothing about potential differences in Hg(II) binding or uptake. However, many of these problems would likely be addressed with a better understanding of the bioinorganic chemistry of Hg(II) within the cell, and with the identification of the mechanisms of Hg(II) uptake.

8.6.5 Creation of a SRB bioreporter for the detection of Hg(II). While neither *E. coli* or *V. anguillarum* are capable of methylating Hg(II) (Wei MSc. thesis University of Manitoba) they do serve as model organisms for the study of Hg(II) uptake under aerobic and anaerobic conditions in gram-negative bacteria. The transformation of the bioreporter plasmid into an array of different bacterial species, including Gram-positive bacteria, could address some general questions regarding species specific mechanisms of uptake and/or binding capacities for Hg(II). However, the next phase of bioreporter development should obviously include the sulfate reducing bacteria (SRB), which are the principal methylators of Hg(II) in aquatic ecosystems. Attempts of this were previously made using a *mer-lac* fusion contained on a shuttle vector. However, the uptake of Hg(II) in SRB was not detectable using this reporter system (Barkay pers. commun.). Because of the much higher sensitivity of bacterial and firefly luciferase, it might present a better reporter alternative. The development of *mer-lux* or *mer-luc* fusions to study Hg(II) uptake in SRB would provide an invaluable tool to increase our knowledge and predictability of

Hg(II) in natural waters. For example, it could be used to determine what effect sulfide speciation and varying environmental conditions has on the bioavailability of Hg(II). It could also be used to compare Hg(II) uptake in methylating and non-methylating SRB, which could address some basic questions regarding why only some strains of SRB are capable of methylating Hg(II).

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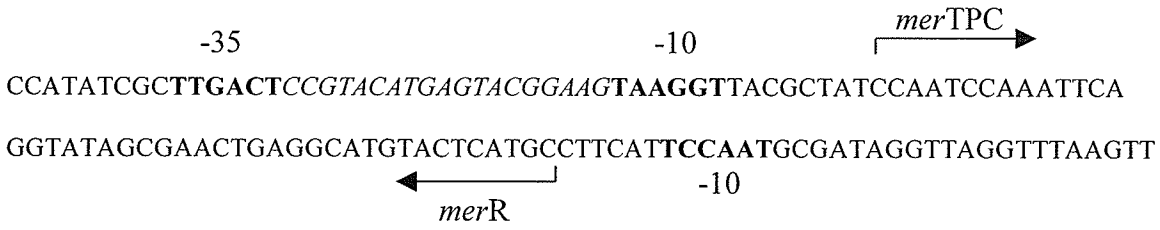
Appendix 1

DNA Sequencing of the (pRB28) and (pRB27) plasmids

Mutations of one or two bp in the non-optimal 19 bp interhexamer spacer region can have a significant effect on the expression of the *mer* operon (Parkhill and Brown 1990). Therefore, the sequence of the regulatory region of the *mer-lux* plasmid (pRB28) was required to ensure that the lower threshold for induction was not a result of a mutation(s) in the *mer* regulatory region. For sequencing purposes, a QIAGEN Mini Prep Kit was used to isolate the plasmids (pRB28) and (pRB27) from *E. coli* HMS174. Regions of the (pRB28) plasmid to be sequenced were amplified by arbitrary PCR (Caetano-Annoles 1993) using the Tn5-5' primer (5'GGGCTAAATCT GTGTTCT CTTCGG 3') and the ARB1 primer (5'GGCCACGCGTCGACTAGTA CNNNNNNNNNNGATAT 3'). The PCR products generated by these two primers were subjected to a secondary amplification step with the Tn5-5' primer and the ARB2 primer (5' GGCCACGCGTCGACTAGTAC 3'). PCR products from the second amplification step were sent to the University of Calgary for sequencing. Comparison of the sequence obtained from the *mer-lux* bioreporter (pRB28) with the known sequence of regulatory region of the Tn*21 mer* operon (Park et al. 1992) confirmed that the regulatory region of the *mer-lux* bioreporter was not subjected to mutations, which might have otherwise enhanced its sensitivity (Figure A1).

The plasmid (pRB27) was also sequenced to determine the nature of this constitutive phenotype, which was believed to be due to a deletion of the *mer* regulatory region (Barkay et al. 1998). Primer lux271 (5' TCACCACGTGGAA GCCATTCATCT 3') was designed from the sequence of *lux*C from *V. fischeri* and used as the initial primer for "sequence-walking" of the (pRB27) plasmid.

A)



B)

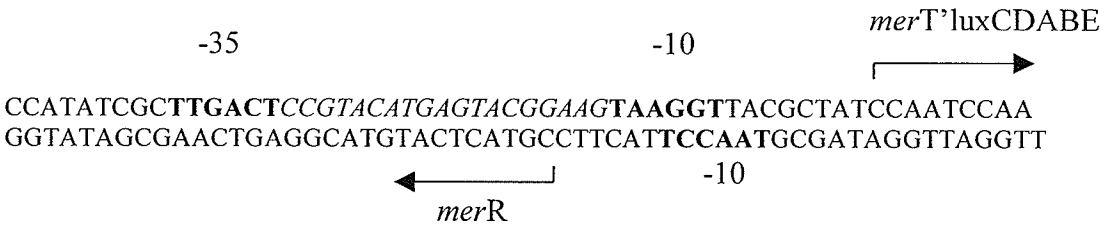
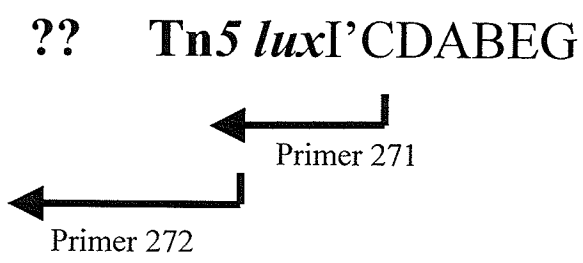


Figure A1. DNA sequence of A) the regulatory region of the Tn*21 mer* operon (Park et al. 1992) and B) the regulatory region of the *mer-lux* plasmid (pRB28). The –10 and –35 hexamers of the structural gene transcript are indicated on the top strand in bold and the 19 bp interhexamer spacer region is indicated in italics. On the bottom strand the –10 hexamer of the regulatory gene, *mer*R, is indicated in bold.

From this sequence the primer 272-5' (5' TAGGAAGCCAGTTCATCCATCCAT CGCT 3') was designed and used to extend the sequencing through the region where the regulatory region of the *mer* operon was located in the (pRB28) plasmid. As predicted from restriction digests, the *mer*Ro/p region of (pRB27) was deleted (Figure A2). From the sequence it appears that expression of *lux* is dependent on the *kan*R cassette promoter, but requires experimental verification.

A) Primers for sequencing (pRB27)



B) (pRB27) DNA sequence

TCACGCAACTGGTCCAGNACTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTC

ATGGCTTGTTATGACTGTTTTTTTGTACAGTCTATGCCTCGGGCATCCAAGCAGCAAGCGCGTT

-35 -10

ACGCCGTGGGTCGATGT***TTGATG***TTATGGAGCAGCAACGA***TGTTACGCA***GCAGGGCAGTCGCC

CTAAAACAAAGTTAGGCATCACAAAGTACAGCATCGTGACCAACAGCACCGATTCCGTCACA

→ **aminoglycoside (6') acetyltransferase (Kan)**

CTGCGCCTCATGACTGAGCATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCAT

ATCGTCGAGTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGGAAC

AGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATACATTGCAATGCTGAATG

GAGAGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTGGAAGCGGGGACGGATGGTG

GGAAGAAGAAACCGATCCAGGAGTACGCGGAATAGACCAGTCACTGGCGAATGCATCAC

AACTGGGCAAAGGCTTGGGAACCAAGCTGGTTCGAGCTCTGGTTGAGTTGCTGTTCAAT

GATCCCGAGGTCACCAAGATCCAAACGGACCCGTCGCCGAGCAACTTGCGAGCGATCCG

ATGCTACGAGAAAGCGGGGTTTGAGAGGCAAGGTACCGTAACCACCCCAGATGGTCCAG

CCGTGTACATGGTTCAAACACGCCAGGCATTCGAGCGAACACGCAGTGATGCCTAACCC

TTCCATCGAGGGGGACGTCCAAGGGCTGGCGCCCTTGGCCGCCCCTCATGTCAAACGTTAGAC

ATCATGAGGGTAGCGGTGACCGTCAGTGCCGATAAGTTCAAAGTTAAACCTGGTGTTGATACC

AACATTGAAACGTTGATCGAAAACGCGCTGAAAAACGCTGCTGAATGTGCGGCGNNGGANGT

CACAAAGCAAATGGCAGCAGACAAGAAAGCGATGGATGAACTGGCTTCCTATGTCCGCAC

GATGAAGAGCAGAAGTTATCATGAACGTTACCATGTTAGGAGGTCACATGGAAGATCAGATC

→*lux*I'

CTGGAAAACGGGAAAGGTTCCGTTCAGGACGCTACTTGTGTATAAGAGTCAGG TATAAAGGT

ATTCTAAGTCTTCGTTATCAAGTGTTTAAGCAAAGACTTGAGTGGGACTTAGTTGTAGAAAATAA

CCTTGAATCAGATGAGTATGATAACTCAAATGCAGAATATATTTATGCTTGTGATGATACTGAAA

ATGTAAGTGGATGCTGGCGTTTATTACCTACAACAGGTGATTATATGCTGAAAAGTGTTTTTCCT

GAATTGCTTGGTCAACAGAGTGCTCCCAAAGATCCTAATATAGTCGAATTAAGTCGTTTTGCTGT

AGGTAAAAATAGCTCAAAGATAAATAACTCTGCTAGTGAAATTACAATGAAACTATTTGAAGCTA

TATATAAACACGCTGTTAGTCAAGGTATTACAGAATATGTAACAGTAACATCAACAGCAATAGAG

CGATTTTTAAAGCGTATTAAAGTTCCTTGTCATCGTATTGGAGACAAAGAAATTCATGTATTAGG

TGATACTAAATCGGTTGTATTGTCTATGCCTATTAATGAACAGTTTAAAAAAGCAGTCTTAAATT

AATGTTGTTAAATCATTAATTTATTTTAAATACTAAGTATATTATAGGGAAAATAATGAATAA

→ *luxC*

ATGTATTCCAATGATAATTAATGGAATGATTCAAGATTTTGATAATTATGCATATAAAGA

AGTTAAACTAAATAATGATAATAGAGTAAAATTATCTGTCATTACTGAAAGTTCAGTTTC

AAAAACATTAAATATCAAAGATAGAATTAATCTAAATTTAAATCAGATTGTGAATTTTTTA

TATACCGTNGGTCAACG

Figure A2. A) Schematic of the primers used for sequencing the "regulatory" region of (pRB27) and B) the DNA sequence. Coding regions are indicated by the arrows and the gene name. The Tn*5* insertion in *lux*I' is underlined.