Synthetic bio-catalytic solutions for mercury pollution

By

Damien Nicholas McCarthy

BSc, MRes

Department of Earth & Environmental Sciences
Faculty of Science and Engineering
Macquarie University, Sydney, Australia

A thesis submitted in partial fulfilment for the degree of

Doctor of Philosophy

MACQUARIE
University

February 2020

Table of Contents

List of Figures

List of Tables

Acronyms and Abbreviations

ADP	adenosine diphosphate
ASGM	artisanal and small-scale gold mining
ATP	adenosine triphosphate
CNS	central nervous system
DFT	density functional theory
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
E. Coli	*Escherichia coli*
EB	endophytic bacteria
EDTA	ethylenediaminetetraacetic acid
ELP	elastin-like polypeptide
ESP	electrostatic precipitator
EK	electrokinetic
FAD	flavin adenine dinucleotide
FBC	fluidized bed combustion
FF	fabric filter
FGD	flue gas desulferization
GEM	gaseous elemental mercury
GM	genetically modified
GOM	gaseous oxidized mercury
Hg	mercury
HgO	mercuric oxides
HgR	mercury resistant microbes
HgS	mercury sulphide
HGT	horizontal gene transfer
LD_{50}	minimum concentration of a substance that kills 50% of a test sample
LDL	lower detection limit
MATS	Mercury and Air Toxics Standards

MBG	micro emulsion based organo-gels
MeHg	various methylmercury compounds
MGDA	methylglycinediacetic acid
Mo	molybdynum
NADPH	dihydronicotinamide adenine dinucleotide phosphate
NAM	US National Academy Medicine
NMerA	N terminal side of MerA polypeptide
OM	organic matter
ORF	open reading frame
PAC	pulverized activated carbon
PAH	polycyclic aromatic hydrocarbon
PC's	phytochelatins
rRNA	ribosomal ribonucleic acid
SBP	solid binding peptide
SCR	selective catalytic reduction
SRB	sulphur reducing bacteria
UL	upper limit
UNEP	United Nations Environmental Program
USEPA	United States Environmental Protection Agency
VDC	variable direct current
WHO	World Health Organization

Acknowledgements

It is difficult to quantify the encouragement, assistance, and guidance I have received from a variety of people and institutions during my PhD program. Safe to say, without that help, it is hard to see how I would have successfully completed the undertaking.

First and foremost I wish to thank the late Dr. Grant C Edwards as my principle supervisor. His depth and breadth of knowledge was a constant inspiration, as was his penchant for hard work, coupled with the handy ability of managing and delivering on many varied projects seemingly all at the one time. Grant was always there when I needed him with solid technical advice, as well as generous material and emotional support, and for that I thank him profusely and sincerely. His contribution is immeasurable. It is with profound sadness to think Grant passed away late during my candidature, and this work is dedicated to his memory.

Next, I thank Dr. Christie Howard for her patience and assistance. Christie's guidance in the laboratory was an invaluable resource for which I owe a great debt of gratitude, as the biomolecular work suffered some non-trivial obstacles and setbacks along the way. Only some words of advice and endless troubleshooting encouragement over the occasional glass of red saw that project completed as planned, and more generally, both her friendship and professional help is hereby saluted.

In recognizing Dr. Howards' contribution, I also acknowledge support from her illustrious husband, Dr. Dave Shintani. Daves' material support and advice is appreciated and noted. A more generous man it would be difficult to imagine, and his kind support and interest in my work cannot be understated. Special thanks to Brandon Purdy, and

thanks to Prof. Glenn Miller and Dr. Dylan Kosta of University of Nevada, Reno, USA for their logistical support, and the staff at Macquarie University, particularly Dr. Louise Brown and Associate Professor Bridgette Mabbutt for their encouragement, and Associate Professor Paul Beggs for his kind assistance, as well as to the staff of the Office of Commercialization and Innovation.

Special thanks also to my daughter Isabella for her love and encouragement. She constantly inspires me. And to the rest of my peers and colleagues, family and friends, particularly my late father, thank you for your support and understanding.

I am also grateful for financial and logistical support provided via Macquarie University Research Training Program (MQRTP) and the Australian Federal Government.

Preface

The basis for this work comes from a deep seated belief in leaving a cleaner environment so future generations of living things have a chance to flourish, those extant and those yet to evolve. I draw great inspiration from the microbial world. Being the oldest among us, over geological time microbes have evolved a myriad of defensive techniques for dealing with environmental pressure, including from toxic heavy metals such as mercury, the subject of this body of work. The challenge is to harness the enormous reservoir of microbial solutions to help us find practical and environmentally friendly answers to the ever-challenging problem of environmental pollutants. It is my hope that the work contained herein will contribute to the existing body of knowledge on mercury pollution remediation, and also inspire others to harness the power of the microbial world in helping solve environmental issues.

Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Damien N McCarthy
13 Feb 2020

.

Abstract

Highly toxic mercury (Hg) causes many adverse biological impacts from exposure to even low concentrations. Mercury is subject to a complex biogeochemical cycle once released into the environment, many aspects of which are driven by living organisms. The extreme toxicity, together with the mobility of Hg once released, has profound impacts on biota, including at least two acute mass poisoning events for human populations in recent history. The transformative nature of the cycle to the chemical and physical characteristics of Hg means that there is no one solution readily amenable to solve the problem.

The main inspiration for this work is derived directly from the bacterial world, whom, having evolved many varied ways of dealing with this toxicant, can now be employed both directly and indirectly to assist with reducing the environmental load of Hg. Some bacterial agents have long been known to possess the ability to enzymatically reduce divalent Hg to elemental Hg^0, whereupon it becomes volatile and passively diffuses as gaseous elemental mercury (GEM). Delivery of bacterial inoculants for remediation purposes to diverse, sometimes remote, and heterogeneous sites has proven logistically very difficult. This work shows that *Pseudomonas veronii* with this $Hg^{2+} \rightarrow Hg^0$ capability can be immobilized on a solid substrate via encapsulation in a biopolymer, and stored for extended periods, while retaining Hg transformation activity after international shipment and subsequent exposure to contaminated soils.

Ideally one wants to capture those induced emissions. Elemental Hg has proven very difficult to capture in this GEM form, notwithstanding the rather complex and sometimes hazardous solutions previously employed. This body of work extends the existing research into capture of GEM in ambient conditions without relying on complex

catalytic and physical separation and capture methods as constitutes the main body of current knowledge in this area. In this work, a modified coir fibre mat was used to show that GEM could be captured in ambient conditions without any prior physico-chemical alteration, by employing a semi-gas permeable silicone based fibre coating, the matrix of which is infused with copper(I) iodide crystals. The coating was applied to coir fibre pre-fabricated as matting. Upon contact with GEM, stable and insoluble copper (I) tetra iodide mercurate is formed and bound stably to the mat. It is envisioned this may be deployed as a geotextile over large terrestrial sites being remediated, or it could be configured for other GEM capture situations including numerous industrial settings.

The final aspect of the work involves development of a biomimetic device for potential remediation of methylmercury in aquatic environments. A synthetic gene was designed, synthesized, and then expressed in a bacterial host, the product of which is a fusion protein consisting of an organo-mercurial lyase and a short tethering polypeptide at the C-terminal with very high affinity for silica. The tethering peptide allows one to directionally immobilize the lyase on a solid silica based substrate, so that the active site is not hampered by steric hindrance or other spatial considerations. The desired product was extracted, purified and tethered via the solid binding peptide (SBP) to synthetic zeolite particles. The enzyme produced is potentially capable of degrading methylmercury while bound.

It is hoped that the combination of these three approaches can assist in reducing the environmental burden of mercury, and adds something valuable by extension to the existing body of knowledge in this area. Due to the distributive and transformational effect of the biogeochemical cycle on Hg, there is no single remedial solution that suits all forms and environmental conditions, but these approaches hopefully add low cost and readily employable solutions to a greater number of problematic contaminated sites.

This page is intentionally left blank

Chapter 1 – Introduction

Despite the ongoing efforts of many professionals and thousands of research hours dedicated to the issue, there has been only incremental progress in finding safe, cost effective, and scalable ways to remediate mercury (Hg) polluted sites. The problem has been compounded historically by ongoing anthropogenic emissions to the environment, and a general nescience and lack of incentive to attack the problem resolutely. To some extent this inaction has been recognized through the United Nations Environmental Programme (UNEP) Minamata Convention on Mercury, agreed to in 2013 by the Intergovernmental Negotiating Committee on Mercury, with 128 signatory nations, of which 85 had ratified the treaty as at December 2018. The convention compels signatories to ban new mercury mines and phase out existing ones, phase out or at least limit the use of Hg, and address legacy pollution problems (United Nations Environment, 2013). Importantly, it provides a global framework for coordinated action on the problem.

Mercury has been used for millennia due to its unique chemical properties. The first known use of Hg was in the production of Neolithic cave paintings, where cinnabar, the fundamental ore of Hg, was crushed to produce vermilion pigments (Çamurcuoğlu, 2015). Other early uses were in decorative work, as a preservative for human remains, and to facilitate silver and gold plating of coins and other objects, and, in recent history, many medicinal formulations (Norn *et al.*, 2008; Ingo *et al.,* 2013). However, by far the most important early use of Hg was as the basis for forming amalgams in gold mining, a practice which continues to this day in artisanal and small-scale gold mining (ASGM) operations.

The widespread use of Hg is interesting, particularly in medicine, given its extreme toxicity was known from very early on. Many Ancients directly associated tremors and severe mental problems with the mining of mercury, with Pliny the Elder postulating

remedies against the toxic effects of the metal in his book *The Natural History*, written as early as circa 79 A.D. (Bostock and Riley, 1855). In fact, Romans only used criminals and slaves in their Hg mining operations because they knew it was essentially a death sentence (Clarkson, 1997). Even so, many 19th and early 20th century medicines, including widely used anti-syphilitic concoctions, included Hg in their formulations. With the assistance of modern science and medicine, the multifarious and severe toxicological effects of Hg on humans are now well established, including injuries to the viscera, skeleton and sensorium (Magos and Clarkson, 2006).

One obvious problem with such long term and widespread use of Hg is the legacy pollution caused by a poor understanding of the impacts of environmental releases of this metal, particularly the complex biogeochemical cycling that takes place in many environmental compartments. The scale of the current problem is extensive, as indicated in Figure 1.1 showing global atmospheric emissions from all sources as at 2016 (Cohen *et.al*., 2016). As Hg is widely distributed atmospherically once emitted, emissions source data as shown should not be confused with actual atmospheric conditions, but highlights global distribution and quantitative emission contribution. An estimated 5000 tons Hg worldwide is atmospherically emitted annually, and perhaps as much as 8000 tons, reflecting a certain ambiguity to the data from poor reporting mechanisms in less developed nations. Nearly 2000 tons of this total is thought to be anthropogenic.

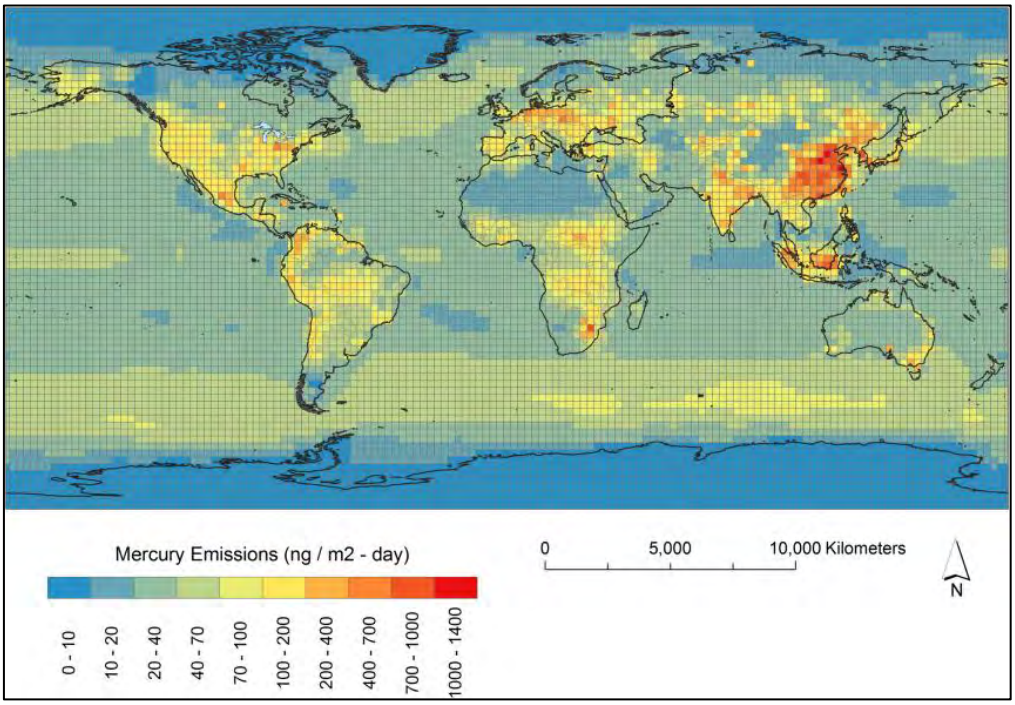


Figure 1.1 Total mercury (Hg^0, Hg^{2+}, $Hg_{(particulates)}$) atmospheric emissions ($m^{-2}\cdot ng\cdot day^{-1}$) from all sources including anthropogenic, biomass combustion, terrestrial and oceanic, re-emissions, and geogenic. (Source courtesy © Cohen *et. al*., 2016)

Figure 1.2 indicates ASGM contributes over 700 tons, or more than one third of total anthropogenic emissions of Hg. In addition, when combined with the one quarter contribution from coal fired power generation, more than half of new anthropogenic emissions of Hg come from these two industries alone (UNEP Global Mercury Assessment, 2013). Natural emissions are mostly geogenic, while re-emissions make up about a third of total emissions, these being largely attributed to anthropogenic sources of previous pollution (Schroeder and Munthe, 1998).

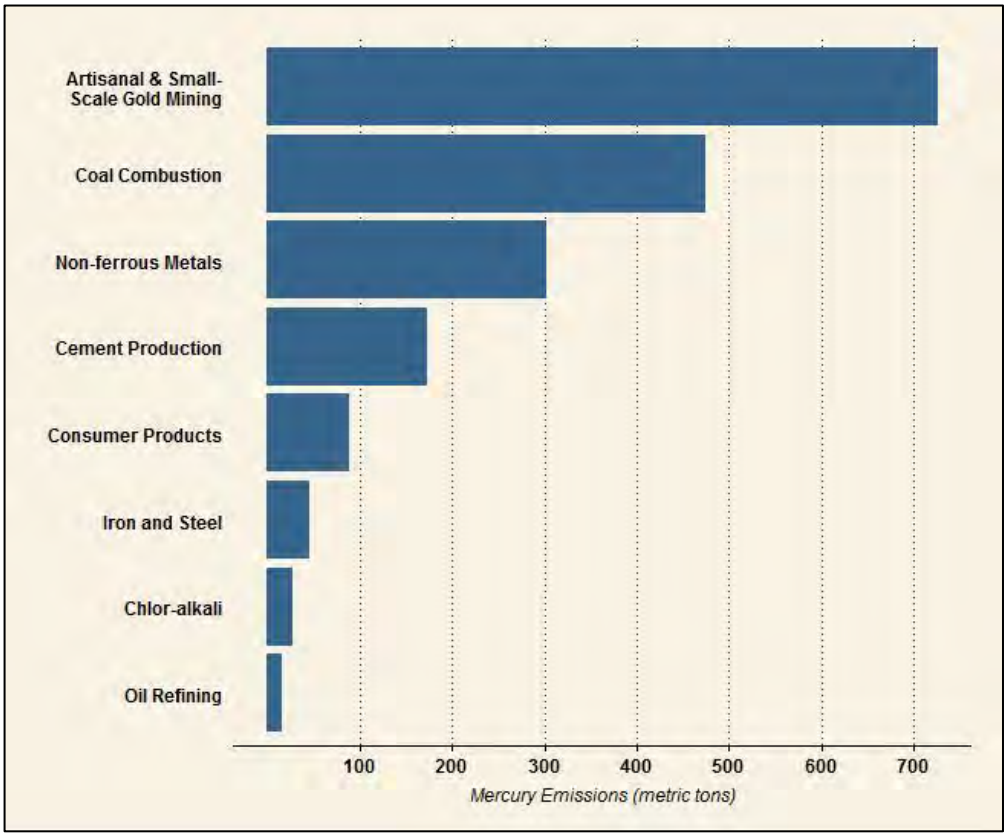


Figure 1.2 Mercury emissions from the eight highest emitting industry sectors as at 2010. Data for 2010 from the 2013 UNEP Global Mercury Assessment. Total estimated global anthropogenic mercury emissions are 1960 metric tons.

Although the toxicity of mercury was well established, it was not until the early 1960s that Japanese researchers correlated increased Hg in urinary samples with the peculiar symptoms of Minamata disease that had affected residents in the local area of Minamata Bay in Japan (Takeushi *et. al.,* 1962). Symptoms included extremity sensory losses, ataxia, constriction of visual fields and hearing loss, as well as neurological disorders (Harada, 1995). Source mercury to the bay was inorganic in this instance, and insights by these researchers facilitated an understanding of the transformative role of bacteria as well as the bio-accumulative nature of the resulting methylmercury (MeHg) compounds. More significantly, the increased exposure risk to human populations was revealed by these findings. Figure 1.3 is a simple stylized representation of this biogeochemical cycle.

.

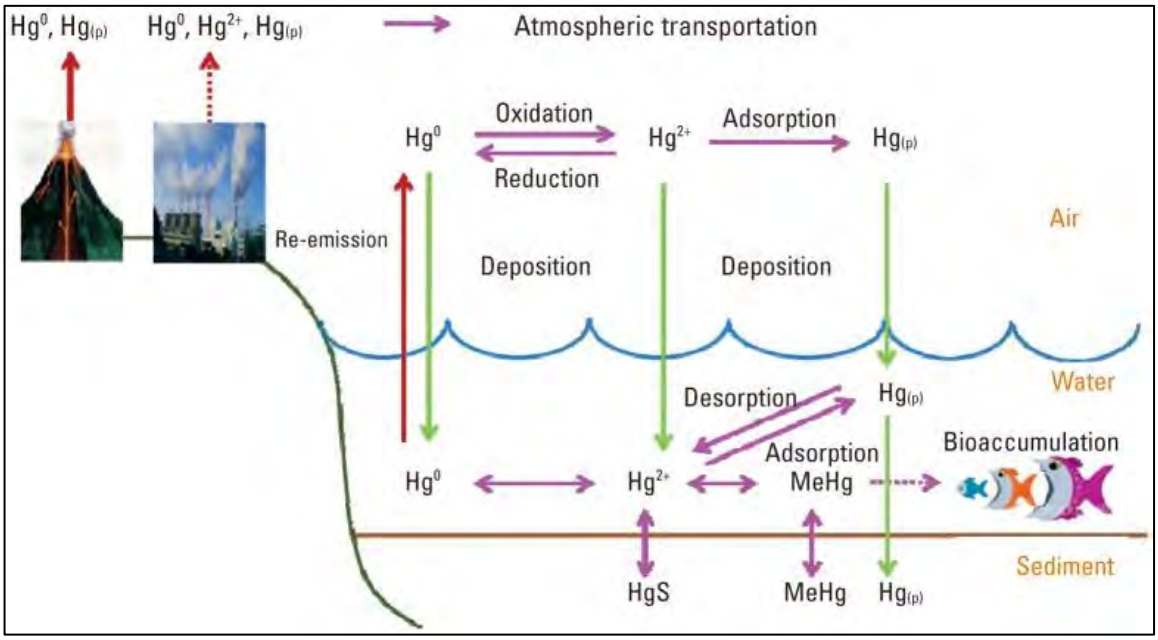


Figure 1.3. Biogeochemical cycle of mercury in a multimedia environment. Hg^0, elemental mercury in atmosphere and dissolved gaseous mercury in water; $Hg_{(p)}$, particulate mercury; Hg^{2+}, reactive gaseous mercury in atmosphere and dissolved reactive mercury in water; MeHg, methylmercury; HgS, mercury sulfide. (Source: Kim and Zoh, 2012)

Mercury poisoning in humans may result from direct vapour inhalation but also from ingestion and absorption through the skin (Gochfeld, 2003). Gaseous elemental mercury (GEM) is lipid soluble and thus readily crosses the alveoli into the bloodstream. Besides vapour, Hg may take the form of metallic elemental mercury, as inorganic salts, or as organic compounds. Methylmercury is particularly toxic as 90% of ingested doses can be absorbed into ones' bloodstream through the gastrointestinal system as compared to less than 10% absorption of elemental metallic form (Olsen, 2018). Natural and anthropogenic emissions are mostly in elemental form, but transformation in the environment converts some fraction of that elemental Hg to MeHg, and it is this form that largely enters and bio-accumulates in the food-chain. The accumulation of MeHg in marine life creates a direct threat for human populations in that seafood consumption is the main exposure risk (WHO, 2016), and fish tissue proteins bind methylmercury

extremely stably and tightly such that it cannot be removed or altered even through rigorous cooking methods.

The most extreme effects are felt by those exposed *in utero*, including lower birth weights with accompanying issues: seizures, developmental delays, sight and hearing problems and neurological atrophy resulting in decreased motor function, language skills and memory (Ha *et al.*, 2017). Effects on exposed individuals of any age are widespread and serious. Specifically, methylmercury causes the most problematic effects on the central nervous system (CNS). Damage is caused through several mechanisms such as binding to sulfhydryl groups incapacitating key enzymes regulating intracellular stress responses, including to oxidative damage, and protein repair (Carvalho *et al.*, 2008). Brain stem signalling can be impaired as can signalling in the occipital cortices that are used to process visual sensory data (Basu *et al.*, 2008). The lipophilic nature of MeHg causes binding to myelin sheaths of axons, disrupting signalling (El-Azab, 2018). Also, energy in cells is supplied by adenosine triphosphates (ATP) via a class of enzymes known as adenosine triphosphatases (ATPase) that break down ATP to adenosine diphosphate (ADP) and free phosphate ions, releasing energy, such as for membrane transport against ionic gradients. Methylmercury can inactivate Na^+ and K^+ APTases resulting in uncontrolled calcium entry and eventual cell death (Huang *et al.*, 2008).

Additional disruptions to biomolecular pathways may result from, for example, attenuation of selenium dependent enzymes as MeHg can sequester Se, confirmed through studies that show a dampening of this effect where increased Se is supplied (Ralston and Raymond, 2010). Isolated enzymatic effects can interrupt whole metabolic cycles, such as the nitric oxide system, leading to downstream effects such as ischemia – the insufficient blood supply to organs and muscle tissue (Yamashita *et al.*, 1997). The picture is further complicated through genetic polymorphisms, for example in heme biosynthesis (Echeverria *et al.*, 2006), serotonin transporters (Echeverria *et al.*, 2010),

and heat shock proteins (Chernyak *et al.*, 2012), where populations will have varying allele dependent dose responses from similar MeHg exposures. Ingestion of elemental metallic mercury is not as toxic, as it is not readily absorbed and is eventually excreted through faeces. Figure 1.4 on the left shows accumulated mercury beads in the intestinal tract. However, even ingested metallic mercury can be widely distributed throughout the body impairing things such as lung function, as can be seen in Figure 1.4 on the right, showing ingested metallic mercury settled in the lung compartment.

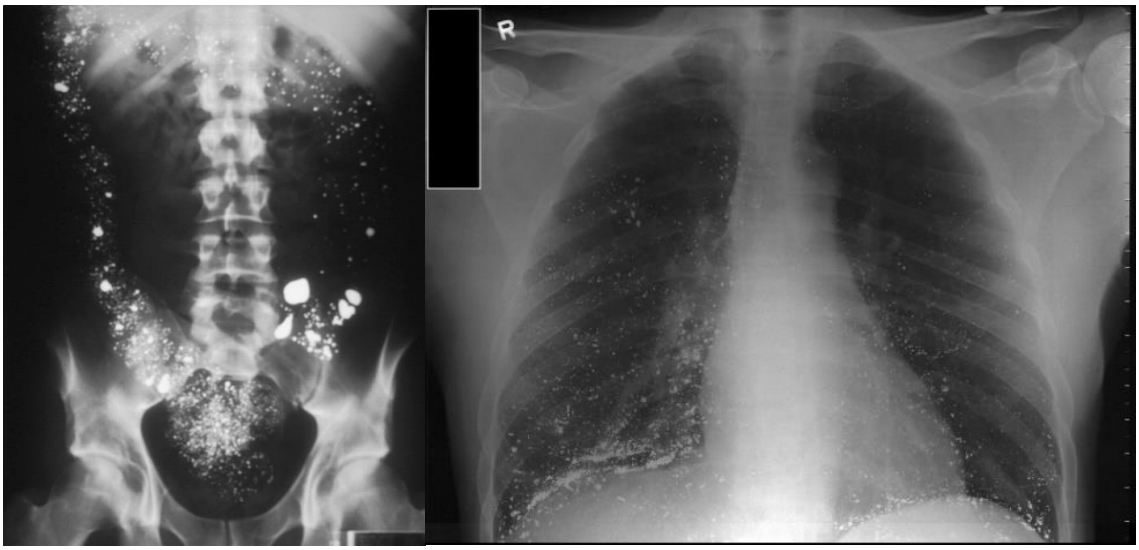


Figure 1.4 (L) Radiograph in a male patient who intentionally ingested 8 ounces of elemental mercury. Image courtesy of Fred P. Harchelroad and Ferdinando L. Mirarchi. **(R)** Radiograph of subject who intentionally ingested liquid metallic mercury from a blood pressure instrument. Note how mercury beads can be seen deposited in lung fields. (Image courtesy of Shuchi Vyas).

Methylmercury includes any compound where mercury is bonded to carbon atoms (methyl, ethyl, phenyl, or similar groups). The pharmacokinetics and clinical significance varies between compounds, and the problem is compounded by *in vivo* inter-conversion between mercury species (Bernhoft, 2012). For example, inhaled gaseous elemental mercury is readily transported across lung membranes, but then is rapidly oxidized to

other forms, although not efficiently enough to stop considerable deposition of elemental mercury in brain tissue (Vahter *et al*., 1995). On the other hand, methylmercury is readily absorbed through the gut, but does not efficiently cross the blood-brain barrier. However, when it does cross this barrier, it gets progressively demethylated (Vahter *et al*., 1995). In contrast, salts of mercury are often insoluble, normally stable, and readily excreted. Toxicity is dependant on form, dose and exposure rates. Internal partitioning is species dependent; vapour targets brain tissue, salts target the gut lining and kidneys, while methylmercury is widely distributed (Clarkson *et al.*, 2007).

Environmental formation of methylmercury is largely biogenic, with sulphur and iron reducing bacteria dominating production (Parks, *et al*., 2013). Genetically, a two gene cluster (*hgcA* and *hgcB*) was identified by Oak Ridge National Laboratory in the US as being essential to the process. Gene knockouts of one or both genes from the cluster resulted in loss of methylating functionality, while restoration of both genes revived this capability. Additionally, the researchers found gene orthologs present in methylators but absent in non-methylators suggesting a common biochemical pathway among methylating species. (Parks *et al*., 2013). In terms of abiotic factors, high anionic content is important, but redox and pH are not, except for their roles in dissolved organic carbon and other ion concentrations which play sequestering roles (Frohne, *et al*., 2012).

Wetlands conducive to methylmercury formation show increased formation during flooding events, with concomitant increases seen in vegetation, peat and multi-cellular aquatic life (Frohne *et al.*, 2012). Abiotic and biotic demethylation also occurs and can be reductive or oxidative. Oxidative demethylation is mediated mainly through UV exposure, and also by bacterial co-metabolism, while reductive processes are bacterially driven (Zheng (W), *et al.*, 2012). The concept is stylized in Figure 1.5.

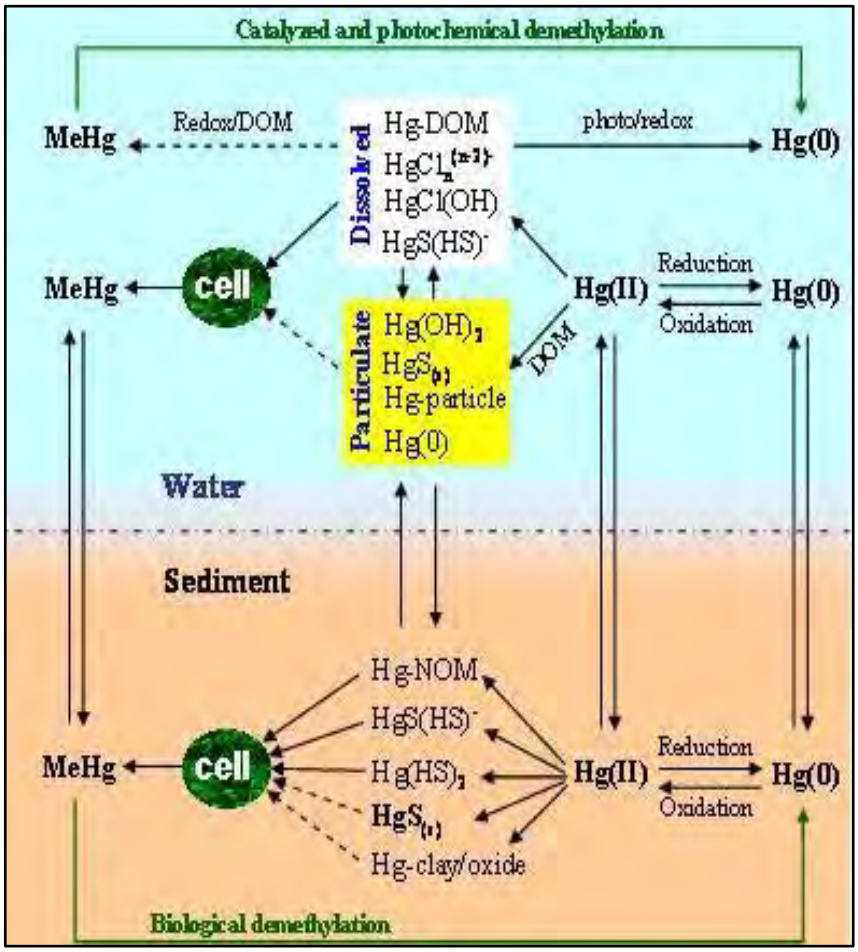


Figure 1.5. Abiotic and microbial transformation pathways between inorganic mercury and methylmercury (Image: Oak Ridge National Laboratory, Tennessee USA)

In summary, mercury has no biological function, and in many cases has severe toxicological effects, rendering this a very important pollutant, as recognized by the United Nations Environment Programme where it is designated a priority pollutant. While the Minamata Convention is a sound first step in addressing the problem, there remain significant challenges, particularly in that not every nation is a signatory. For example, the initial restrictions on mercury trade being implemented through this mechanism caused a fall in supply that led to increased market prices. This resulted in new mines being developed in non-signatory Mexico and Indonesia, with estimated new production at about 1000 tons annually (UNEP, 2017). Management and implementation of the goals of the treaty will take careful ongoing efforts. In the interim, it is critical that legacy pollution is addressed such that the environmental load can be reduced.

Thesis Structure

Given that broad overview, it is now necessary to review in detail the literature surrounding the key aspects of the work contained in this thesis. As such, the following chapter initially explores terrestrial mercury remediation techniques, using both traditional physico-chemical processes and bioremediation using various microbial strains. This is followed by an examination of the biomolecular basis for microbial mercury resistance mechanisms, leading into a review of biomimetic tools that may be constructed and used, based on those mechanisms. Lastly, a detailed examination of the challenges in capturing gaseous elemental mercury is presented. The review provides background and trends in the various areas relevant to this thesis and clarifies the focal points of this particular work. This situates the enclosed research within the broader ongoing work undertaken by the scientific community in this space.

Having reviewed the present issues in detail to expose knowledge gaps, the rationale behind the thesis work is then explained, and the aims of the experimental work are articulated in Chapter 3. This is followed by chapters (4,5 and 6) presenting the experimental work as peer reviewed journal articles and includes a draft manuscript that has not been published as yet due to commercial in confidence issues. The body of work is then brought together in a discussion (Chapter 7) that examines the results in some depth, but also reflects on issues of experimental design and opportunities for improvement. Finally, a future research roadmap is presented, given the results achieved and any experimental shortcomings exposed, to address further questions posed by the results.

Chapter 2 - Literature Review

2.0 Terrestrial mercury remediation – non microbial

Diffuse contamination from atmospheric mercury deposition is obviously difficult to remediate, but concentrated terrestrial pollution offers an opportunity for direct intervention. The global mercury budget in Figure 2.1 shows why point source solutions are critical as once released, mercury is difficult to contain. It is estimated that Hg cycling occurs for millennia before sequestration to ocean sediments (Selin, 2009). Source and sink data are quite difficult to determine for a lack of data sets (Agnan *et al*., 2005), however estimates can be seen in Figure 2.1, where it quite clearly shows the increased cycling due to anthropogenic activity since the Industrial Revolution. Importantly, estimates show humans act as sinks for an estimated 10^{-7} of deposited atmospheric Hg (Selin, 2009), highlighting the human health impact.

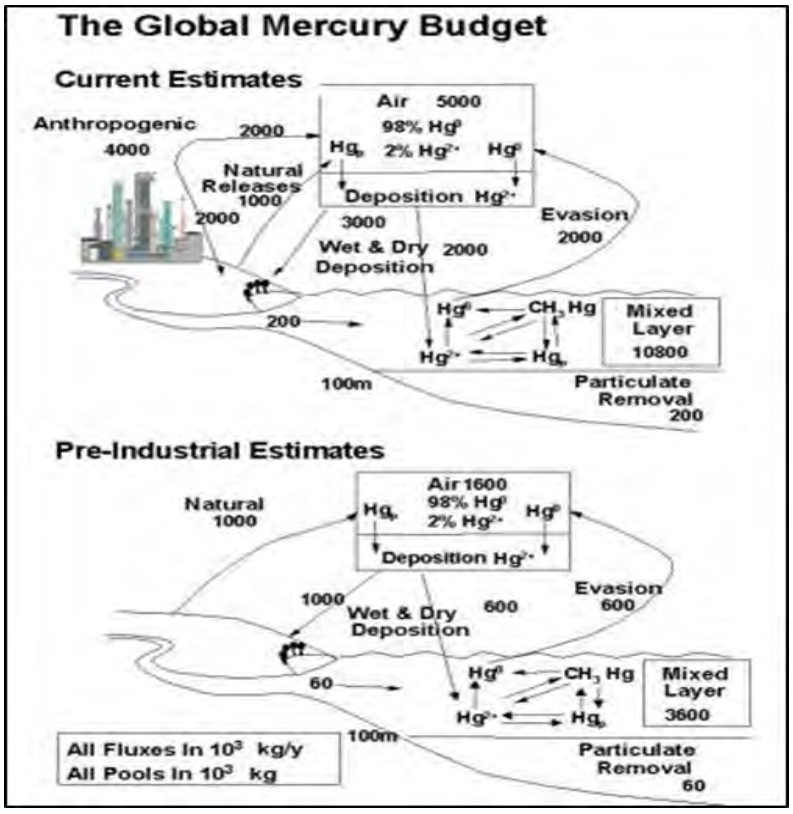


Figure 2.1 The global mercury budget. Current ambient air Hg levels are approximately 1.6 m^{-3}·ng, versus pre-industrial estimates of 0.5 to 0.8 m^{-3}·ng (Image: Canadian Government @ www.ec.gc.ca/mercure-mercury).

In polluted sites, such as decommissioned chlor-alkali plants, large gold extraction operations, ASGM and other industrial settings, Hg mobility is often an issue. Besides volatility, Hg may mobilize terrestrially, for example through becoming soluble, so site specific hydrology and soil compositional issues are relevant. Terrestrial Hg interacts with complexing agents, with anions of chlorides, disulphides, hydroxyls and organic matter ligands that contain sulphur (Liao *et al*., 2009). Mobility is dependent on many factors. Organic matter (OM) in acidic soils and sediments, for example, attenuates Hg mobility due to high affinity with hydroxyl, carboxyl and thiol groups associated with that OM. On the other hand, mineral content dominates Hg immobility in neutral or alkaline conditions (Frohne *et al.*, 2012).

This picture is complicated somewhat by some complexes becoming readily soluble. For example, Cattani *et al*. (2009) confirmed that increased Hg mobility could be seen when fulvic acids were shown to impart solubility to Hg-OM complexes. Regardless of solubility, OM breaks down via microbial action over time through a multi stage complex cycle and predicting long term release of Hg is very difficult. As such, leaving Hg *in situ* is not a good long term solution, even if the problem is currently deemed stable.

In terms of soil and sediment composition mediating mobility, minerals tend to have high surface area to volume ratios, allowing for greater interaction with Hg, as do smaller sediment sizes. It is known that mercury accumulates in finer grain sediments probably due to higher surface area availability (Kelly and Rudd, 2018). Sorbents of Hg are therefore dominated by clays, particularly those with oxides and hydroxides or iron sulphides (Liao *et al*., 2009). It is not always clear what the actual molecular basis of this sorbtion is, although efforts continue in this area through compositional studies of bound fractions using X-ray absorption technologies, such as by Serrano *et al*. (2012). What is known is that chloride ions outcompete hydroxyls for Hg, which can have the effect of

mobilizing Hg through formation of $HgCl_2$ particularly in alkaline conditions, because mercuric chloride is readily soluble (Xu *et al.*, 2015).

The types of terrestrial sites most often encountered when dealing with mercury contamination are detailed in Table 2.1. Also noted per type is characteristic speciated Hg form for these situations. Sampling is usually undertaken either directly from soils or adjacent water bodies. Site information largely comes from a review by Wang *et al.* (2012) on past remedial attempts. Several well established physico-chemical strategies have previously been developed to remediate soils with varying degrees of success and applicability. These are summarized in Table 2.2 and a brief explanatory summary is provided detailing the characteristics of each. Many of these activities are undoubtedly not subject to publication. In terms of clarifying cost, much of this is opaque due to lack of publication or where published, data are buried in broad chart of accounts line items, especially with publicly traded entities and governmental reports.

Table 2.1 Typical mercury polluted site types, mercury source in that industry and typical residual mercury species per site**.** (where x = Cl^-, Br^-, I^- or ClO_4^-) Source: Wang *et al*., 2012.

Site type	Hg use or source	Hg pollutant species
Mercury mining	N/A – mercury is the target	HgS, Hg^{2+}, Hg^0
Gold mining	Mercury is contained in associated ores, or added as an amalgam in ASGM activities	HgS, Hg^{2+}, Hg^0
Zinc and lead smelters	Mercury is contained in associated ores	HgS, Hg^{2+}, Hg^0, $Hg^{2+}X$
Chemical production facilities	As cathode in chlor-alkali production, various other forms in Hg compound manufacture dependant on chemistry	Hg^{2+}, Hg_2^{2+},Hg^0, Hg^+,$Hg^{2+}X$
Landfills	Various, often liquid elemental Hg from thermometers, mercury lamps etc.	Hg^{2+}, Hg^0, HgO, $HgCl_2$
Military installations	Weapons manufacture	Hg^{2+}, Hg^0, HgO
Forestry and timber	$HgCl_2$ impregnation as preservative	$HgCl_2$, Hg^{2+}, Hg^0
Others	Coal fired power stations, manufacture of batteries, lamps, thermometers, electrical equipment	Hg^{2+}, Hg^0, $Hg^{2+}X$, HgO,

Bacterial bioremediation will be treated separately as it directly relates to the focus of this thesis and deserves a more detailed examination. Table 2.2 highlights both physico-chemical strategies and phyto-remedial strategies for mercury contamination remediation.

Previous strategies have benefits and drawbacks as noted below, and it is a trade-off for remediation specialists in determining the correct strategy to follow. For example, vitrification certainly works in long-term binding of mercury, however the process is very destructive to soils. Analysts must be multidimensional in their approach (Khan *et al.*, 2004). This is true for any remedial strategy, not just mercury, but is a salient issue often

overlooked by primary researchers. Also, the proclivity of Hg to undergo environmentally driven physical and chemical transformation and distribution mean that carefully thought out management plans must be employed during both *ex situ* and *in situ* remediation, and might include extensive engineering and other physical containment strategies, regardless of the actual technique employed (Sims, 1990).

Table 2.2 Non-microbial mercury remediation strategies for terrestrial contamination proposing benefits and drawbacks of each method.

Strategy	Benefit	Drawback
Soil washing	Plume is contained by total removal. Can be effective in removing 99% of Hg contaminant.	Requires excavation for *ex situ* processing. Destruction of matrix profile. Scale.
Mobilizing	Increased accessibility to tightly bound fractions for secondary removal processes	Containment problems within area being managed. Leaching to water table. Residual waste.
Thermal desorption	Proven technology, efficient and effective.	Cost and logistics, requires capture of emissions, scale.
Stabilization	Proven method. Local containment of problem.	Destruction of matrix.
Vitrification	Proven method. Permanent solution.	Destruction of matrix, requires significant energy inputs.
Electrokinetic (EK)	*In situ* treatment.	Often requires chelating agents and other modifying additions, high expertise required.
Excavation	Simplicity. Permanently effective.	Cost, loss of matrix. Scale issues.
Phyto-extraction	Simplicity and cost effective.	Low efficiency & secondary processing required. Geographical constraints. Containment.
Phyto-stabilization	Simplicity and cost effective.	Brevity of solution, low efficiency, geographical constraints.
Phyto-volatilization	Simplicity and cost effective.	Low efficiency, simply moving problem to atmosphere, geographical constraints.

Physical techniques include gravity concentration, froth flotation, magnetic and electric separation, thermal desorption and scrubbing (Cyr, 1993; Cox *et al*., 1996;

Navarro *et al*., 2009; Wang *et al*., 2012). Other techniques such as that proposed by Dr. John Rudd for remediation of the Grassy Narrows First Nation site in Canada, which among other things proposes diverting ground waters for treatment prior to re-establishing flows, are less well established. The strategies noted in Table 2.2 are summarized below.

Soil washing involves excavation of the contaminated matrix and separation of Hg via physical and chemical separation techniques. As mercury tends to bind to finer clays and silts, water is used to create a particle size gradient. However, even though Hg is concentrated in smaller fractions, it is widely distributed among all fractions as noted by Sierra *et al*. (2011) meaning secondary processing is required. Chemical extraction techniques are used for concentrates, with an iterative and differing chemical approach required for each due to metal fractionation from differing binding characteristics of the original matrix. The drawback arises as solubilizing Hg generates a large volume of contaminated sludge, and increases Hg mobility, requiring careful management of any leakages. It is more commonly used in Europe, and then mostly to clean sediments rather than excavated soils, as those sediments tend to have low clay content reducing reagent requirements. (Dermont *et al*., 2008; Xu *et al*., 2015).

Mobilizing techniques often accompany soil washing, and also often involve solubilizing Hg. *Hydrometallurgy* involves the addition of reagents in fluid form that may incorporate acids or bases, surfactants, chelators, salts, and redox reagents to enhance metal mobility to aid extraction. Acids and bases are used to both solubilize and precipitate. Surfactants target desorption from mineral surfaces by altering hydrophobicity, which also aids in froth floatation as the contaminant adheres to bubble surfaces. Chelating agents isolate Hg through formation of co-ordination complexes that can be precipitated. Salts containing chloride ions are often used to form Hg-chloro complexes that are readily soluble (Schuster, 1991), while redox agents facilitate these reactions through valence manipulation. Efficiency is impacted by the geochemistry

including granular characteristics, texture, cation exchange and buffering capacities, OM content, mineral species, and clay content. Remediation specialists need to be cognisant of Hg concentration and speciation, as well as the chemistry of removal reagents and residency time of those reagents (Dermont *et al*., 2008).

Stabilization techniques can be used for *in situ* and *ex situ* treatment to limit mobility of Hg in soils. Binding agents create stable insoluble Hg compounds that are less mobile and labile over wide ranging pH and redox conditions (Zhang and Bishop, 2002). It is often accompanied by *solidification* that encapsulates the compounds in a durable solid matrix. This stops release into surface and ground waters, and uptake by microbes, and can be used over a wide range of substrates and soil matrices (Svensson, 2006). Roy *et al.* (1991) found they were able to stabilize several toxic heavy metals including mercury using Portland cement, and it has been widely used since for this purpose. Donatello *et al.* (2012) reported up to 99% of Hg was bound using alkali-activated fly ash, although this was not tested in soils but rather tested after doping the material with 5000 mg kg^{-1} Hg. Fly ash mercury capture is described in the literature in section 2.5 on gaseous mercury capture.

Thermal desorption is a popular alternative and is achieved because Hg has a tendency to volatilize with increased temperature (Lodenius and Braunschweiler, 1986). Excavated material is processed in simple roasting kilns and the exhaust treated to isolate the mercury. Kunkel *et al*. (2006) removed over 99% of mercury in a soil column using thermal desorption from Ottawa sand followed by vacuum assisted extraction, although it is not clear how cost effective this would be in the field. Chang and Yen (2006) found soil needed to be heated to above 700 °C for several hours to ensure post treatment levels of 2 mg kg^{-1} Hg from a starting concentration of up to 140 mg kg^{-1}. This paper shows the methods utility but also highlights the high cost, estimated at $US^{(2006)}$ \$834 m^3 while generating waste of 1.2 m^{-3}·kg. This cost seems representative given similar studies

(Mulligan *et al.*, 2001; Chang and Yen, 2006). Many field studies show high effectiveness with variable efficiency, largely due to matrix geochemistry. As mercury is often encountered as HgS and HgO, temperatures often need to be in the range of 650 – 800 °C. Solar powered extraction has also been demonstrated although efficiency is lower and markedly variable (Navarro *et al*., 2009). Mercury can be captured from the exhaust stream by activated charcoals or other scrubbing devices, or via exhaust cooled retorting. Although thermal treatments are widely used and largely effective, there are several major drawbacks. High energy consumption, altered matrix chemistry, and repartitioning of coexistent metal contaminants remain challenges. Lower temperatures and longer thermal exposure times have been employed to counteract these problems however efficiency goes down dramatically (Qu *et al*., 2004; Kucharski *et al*., 2005).

Vitrification converts mercury to a much less soluble form by entrapment in inert glass with the addition of sufficient SiO_2 and alkali concentrations (>1.4 wt %) for example Na and K, to the soil (USEPA, 1997b). Mixed contaminants are often treated in this way due to its universal applicability. For example, mixed waste that also contained mercury from a nuclear fuel generation trial facility was vitrified at the Parson Chemical ETM Enterprises site in Michigan USA, and soil concentration of mercury went from 34 mg kg^{-1} to 40 µg kg^{-1}, where residual leachability was as low as 0.2 µg L^{-1}, (USEPA, 1995). While soils containing OM can reduce energy costs as it is itself combustible, concentrations above 10% become problematic from unwanted off-gassing (USEPA, 2007a). Drawbacks also include clays and moisture content effecting efficiency, while matrix content may pose hazards from unwanted off gasses, for example from furans and dioxins, (USEPA, 1997b), and this method is very energy intensive.

Electrokinetic (EK) remediation uses direct current to mobilize ions, placing electrodes and ion exchange membranes within the matrix and collecting ions at the cathode. Contaminants can be removed by precipitation. Poor efficiency due to Hg's low

solubility means mobilizing agents must be introduced. Reddy *et al.* (2003) removed up to 97% of mercury from 500 mg kg^{-1} Hg spiked clay soils using 0.5M KI as the lixiviant with 1.5 VDC cm^{-1} voltage gradient, noting higher OM content reduced efficiency. Similar results were achieved by Shen *et al*. (2009) although they used a different cathode configuration to conserve energy, noting for each 1% increase in matrix OM a 2.63% decrease in efficiency was found. More traditional environmentally friendly soil conditioners such as ethylenediaminetetraacetic acid (EDTA) are not quite as efficient but much safer. The effectiveness may be more than doubled compared to EDTA by use of methylglycinediacetic acid (MGDA) and Tween® 80 (Falciglia *et al*., 2017), although PAH co-contaminants confounds direct comparisons in this case.

Phytoremediation relates to the use of plants to decontaminate soils and was developed in the 1980s as a cost effective strategy that was relatively straightforward to implement with little environmental impact. There are three main strategies employed, those being translocation to biomass, biomass assisted stabilization and also volatilization (Tangahu *et al*., 2011). *Phytoextraction* translocates contaminants from soil to above surface biomass where it can be harvested (phytomined). However, what was once envisioned as a panacean solution has not lived up to the research efforts, as there have been thousands of articles published with few field trial successes noted. Bioaccumulation coefficients of >10 as calculated are required for 50% soil metal reduction over 25 years (Robinson *et al*., 2015), suggesting this is not viable, particularly as this calculation assumes ideal phytoextraction conditions that are rarely encountered in practice. The addition of endophytic bacteria (EB) can aid extractability by plants. Accumulation of Hg was 70% lower in the absence of EB (De Souza *et al*., 1999). Selected indigenous isolated EBs were used in association with Hg tolerant beans for soil with a Hg concentration of 25 mg kg^{-1}, reducing the mercury content by 96% in 24 h which was a remarkable result (Chacko *et al*., 2015). Problems may include low efficiency,

although this may be assisted with EBs, and geographic constraints apply for growing suitable biomass. Further, the biomass needs harvesting and post treatment, and can be voluminous.

Immobilizing contaminants using plants through either adsorbtion or precipitation within the root zone is known as *phytostabilization*. He *et al*. (2001) found the tobacco plants root zone was better than foliage in immobilizing Hg, suggesting translocation to leaf material was not the best solution here. Genetically modified (GM) tobacco was used by Ruiz *et al*. (2003), where certain genes (discussed in section 2.3) were cloned into the chloroplast genome, increasing levels of immobilized Hg compared to wild type. In this case, as pollen does not contain chloroplast genes, there was little chance of cross contamination from GM plants. Aquatic macrophytes are often used for wetland remediation, and this research generally confirms earlier observations about high root zone activity (Chattopadhyay *et al*., 2010). These studies reveal the most appropriate plants are from the *Leguminosae* family. In addition to effective stabilization of Hg, they are soil restorative and play a pivotal role in successional colonization by species, important for total site remediation (De Andrés *et al*., 2007; Villar-Salvador *et al*., 2008). Besides legumes, GM flowering plants, poplar trees, rices cultivars, grasses and other shrubs have all been tried with variable success rates. (Rugh *et al.*, 1998; Bizily *et al*., 1999; Bizily *et al*., 2000; Heaton *et al.*, 2009).

Phytovolatilization involves plant mediated intracellular redox reactions that render compounds volatile, and normal transpiration releases the contaminant to the atmosphere (Meagher, 2000). Selenium, arsenic and mercury are examples of metals that can be remediated in this manner, at least in theory. The process seems very slow, and efforts to speed up remediation using transgenic plants have been attempted. However, bench scale research using GM plants has shown mixed results. As many bacteria are known to contain the *mer* operon, a cluster of genes conferring Hg resistance

via volatilization, these have been cloned into *Nicotiana* and *Brassica* species, however results were not conclusive (Heaton, 2009). The process seems to be too slow to be viable, as evidenced by Liu *et al*. (2010) who used the *arsM* gene to catalyse volatilization of arsenic, and although a tenfold increase in removal rate compared to wild type is noted, only 2-4 % of arsenic was removed after 30 days. Phyto-volatilisation has not been shown to be viable for mercury.

2.1 Terrestrial mercury remediation – microbial

Bioremediation using mercury resistant microbial (HgR) strains seems more promising in that soils may be remediated *in situ*, and this approach also seems much less intrusive. The general method is separated into two main categories. *Stimulation* involves altering abiotic conditions to induce overpopulations of the desired microbes, an approach often taken for vadose zone contaminants. *Augmentation*, on the other hand, requires the addition of bacterial inoculants, and often also involves soil preconditioning to confer competitive advantages to the added microbial populations, and is used where target microbes are non-endemic (Adams *et al*., 2005). Microbial interaction with soil bound metals often alters their physico-chemical state, and that can aid immobilization or mobilization depending on the metabolic processes of the organism (Gadd, 2010). This aspect can be utilized for soil remediation, including where appropriate, for mercury. There are several bioremedial strategies, including bioleaching, bioprecipitation, biosorption, and biovolatilization.

In *bioleaching*, metals are solubilized by microbes. This has proved ineffective for Hg, as highlighted by Dronen *et al*. (2004) who compared acid washing to bioleaching of coal, and found acid washing far more effective. US Patent US 7998724 B2 (Barole and Hamilton, 2011) proposes a slightly different approach, that being acidification of coal to mobilize Hg followed by exposure of the aqueous phase to microbial agents. The authors cite unpublished work making assessment difficult but of note is that microbial selection is limited to acidophiles. Particle size is a determinant of efficiency as noted by Guven and Akinci (2013), likely due to increased surface area interaction for finer grains, but this work did not include Hg. In fact the method has rarely been shown to work for Hg as yet, and is reserved mostly for precious metal extraction rather than bioremediation, although there is some movement in this direction of late (Nguyen, 2015; Yang *et al*., 2018).

Bioprecipitation can be effective in isolating metals from the biosphere, but is generally limited to anaerobic metabolism, constraining its utility. It is based on the concept that both sulphides and phosphates are released by microbes, causing metal bound precipitates to form on cell surfaces. Precipitates are stable and more readily recoverable, and the technique has been widely demonstrated for many metals, including mercury (Boswell *et al*., 1999; Renninger *et al*., 2001). It may be useful in wetlands, and also has the advantage of removing unwanted sulphates. The technique is widely used in wastewater treatment.

Biosorption techniques employ either whole cells or isolated proteins which are immobilized on a suitable substrate. Sewage and activated sludge plants often use biofilters that utilize microorganisms or their derived proteins to break down OM and bind pollutants. While adding complexity, these techniques could potentially be employed for *ex situ* batch processed soils. There is a lack of published field data in regards to soils and Hg, but there are a few noteworthy studies. For example, modified biomass was used to effectively adsorb Hg (Bae *et al*., 2003). A metallo-regulatory protein with high Hg affinity was isolated and simply applied to biomass. Further work by the same team overexpressed this protein in GM *E .coli*, and biosorption capacity increased six-fold compared to wild type. Phytochelatins (PCs) normally expressed in plants under metal stress have been used where GM micro-organisms over-expressing PCs have shown up to 50-fold increased cellular metal concentration, which is remarkable (Sauge-Merle *et al*., 2003). However, it hasn't yet been demonstrated for Hg, and it is unclear whether this would be suitable as intracellular accumulation of mercury is not a strategy employed naturally to any high degree, due to extreme toxicity. Sorbents using fungal strains incorporated into clays have some advantages of high surface area, mechanical strength and sorption efficiency, though these have been more broadly configured as opposed to the high Hg affinity approach by Bae *et al.* (Ruta *et al*., 2010; Fomina and Gadd, 2003).

Biovolatilization is a strategy employed by microbes for toxic metals amenable to this physico-chemical transformation, including mercury. The resultant gas escapes to the atmosphere, permanently removing the contaminant from their surrounds. The basic remedial concept utilizes microbes harbouring the *mer* operon that can increase the rate of natural Hg removal through the intracellular reduction of Hg^{2+} which is then emitted passively as GEM. Rather than introducing new strains, it often seems the best method is to utilize indigenous HgR microbes. Saouter *et al.* (1995) first showed this utility over twenty years ago. Highly Hg contaminated pond sediment was inoculated with 10^5 cells mL^{-1} of HgR bacteria that had been isolated from the sediment. Increased volatilization was noted after enrichment by comparing the aqueous and headspace Hg concentrations. A combined bacterial-leach–reduction method was trialled at scale on Minamata Bay sediments (Nakamura *et al*., 1999) however, more successful was acid leaching followed by inoculation with indigenous isolated HgR (Iohara *et al*., 2001) which resulted in rapid reduction of Hg^{2+}. Extremophiles have also been shown to maintain this mercury volatilizing capacity which may be of great benefit in cleaning more challenging sites. For example, *Deinococcus spp*, a radiation-resistant strain carrying the *mer* operon has been tested in subsurfaces containing Hg and radionuclides. *D. radiodurans* (Brim *et al*., 2000) and *D. geothermalis* (Brim *et al*., 2003) both reduced Hg^{2+} to Hg^0 during exposure to a gamma radiation dose of 50 Gy.

The fact elemental mercury is virtually insoluble has been exploited in aqueous waste streams using a bacterial consortium immobilized in a fixed bed reactor by having the reaction occur under a stable layer of water, initiating precipitation, which can be collected as metallic mercury beads. Partitioning to the atmosphere was negligible in this instance, and this technology has been demonstrated at scale (Brunke, *et al*., 1993)

This remedial method (bio-mediated volatilization) is low cost and non-destructive to soils, relatively easy to implement and manage, and uses natural organisms that can

be environmentally safe, particularly endemic HgR. This technology can be readily implemented at scale. The method is often implemented *in situ*, greatly reducing costs and logistical complexity. The drawbacks are that inoculating remote locations can be challenging because getting viable organisms to site can be difficult. This can be overcome through the use of immobilized cell technologies. Immobilizing cells creates a portable bio-substrate available for transport to site in a readily manageable format. The challenge is maintaining the inoculant and media once deployed, maximizing contaminant interaction and minimizing environmental pressures. Cells must volatilize Hg at sufficient rates and for sufficient time to reduce the contaminant to appropriate levels within acceptable temporal and economic parameters. The emitted GEM also requires capture if a permanent disruption to the biogeochemical cycle is to be implemented.

As the work presented in this thesis aims to overcome inoculant delivery and GEM capture problems, literature on the bio-molecular basis for this physico-chemical transformation of mercury via microbial volatilization will now be explored in some depth in the following sections, as will techniques for whole cell immobilization used in to facilitate transport of living bio-inoculants. GEM capture is detailed in section 2.5.

2.2 Biomolecular basis for microbial mercury resistance

Transforming oxidized metallic mercury to GEM by microbes is mediated by the DNA sequence containing the *mer* operon, a system widely distributed taxonomically. It can be located on transposons, plasmids or on chromosomal DNA (Osborn *et al*., 1997). Distribution is facilitated by highly efficient Horizontal Gene Transfer (HGT), so the occurrence of *mer* is geographically widespread as well as occurring across a wide selection of both Gram-positive and Gram-negative strains (Bogdanova *et al*., 1998). The appearance of *mer* systems in deep sediments suggests the evolution of an ancient system to deal with geogenic mercury (Ramond *et al*., 2009). This longevity has allowed for the evolution of many versions of the operon, including those with methylated mercury resistance. It has genes coding for proteins involved in recognition, transport and transformation, and for many components of associated biochemical pathways (Mathema *et al*., 2011).

Many HgR strains have been characterized, as have varying *mer* operons. The two basic categories of *mer* can be characterized as *narrow spectrum* resistant for those only reducing Hg^{2+}, and *broad spectrum* for those also having methylmercury transformation capability, or more specifically carrying the *mer*B gene. Functionally, the basic *mer* processes are regulatory and Hg transport, and reduction of Hg^{2+} by *mer*A. Ancillary genes are encountered in *mer* variants, including genes to transport and degrade methylmercury, and these are mostly located proximally to *mer*A. This operon is modular in nature and highly mobile (Liebert *et al*., 1997; Barkay *et al*., 2003), and these factors have allowed for such wide distribution. This mobility is highlighted by the research of Bogdanova *et al*. (1998) who found incongruent results between the rRNA phylogeny of HgR and their associated *mer* genes. Further, exactly replicated *mer* loci were found to be globally distributed (Kholodii *et al*., 2002), suggesting this is not random but rather

a function of the residence of the operon on mobile DNA elements (Barkay *et al*., 2003). The linear structure of narrow spectrum (A) and broad spectrum (B) *mer* operons is depicted in Figure 2.2.

(A) <*mer***R**> (promoter) *mer***T**>*mer***P**>*mer***A**>*mer***D**>

(B) <*mer***R**> (promoter) *mer***T**>*mer***P**>*mer***F**>*mer***C**>*mer***A**>*mer***G**>*mer***B**>*mer***D**>*mer***E**>

Fig 2.2 Basic structure of the narrow spectrum *mer* operon (A) and, (B) the broad spectrum operon. (A) represents the linear organization of genes responsible for core functions of the mercury resistance operon, while (B) represents the operon with accessory genes (*mer*F,C,G,B and E) at their common place of insertion. *mer*R and D are regulatory genes, and *mer*A and B are the mercury transformative genes. All other genes code for transport related proteins. <> represents transcription direction.

The core regulatory gene is *mer*R, and for Gram-negative bacteria, the additional down regulatory gene *mer*D. The function of *mer*D was deduced by Nucifora *et al*. (1989) by creating a *mer*A-*lac*Z fusion protein in GM *E. coli*, and observing reduced β-galactosidase activity in the presence of the *mer*D protein product. *mer*R also acts as a repressor. It functions mainly as a mercury responsive transcription regulator, and undergoes conformational change upon interaction with mercury that allows polycystronic transcription of the operon. It continuously represses its own transcription, and represses transcription of the genes downstream of the promotor region in the absence of mercury (Summers, 1992).

Hamlett *et al*. (1992) showed through point mutation frameshifts that transport functions of *mer*T and *mer*P were associated with Hg^{2+} resistance, and that *mer*T was critical while *mer*C was not required. Further, Kiyono *et al*. (1995) showed via gene knockout experiments that these transport genes (*mer*T and P) were limited to Hg^{2+} and not required for methylmercury resistance, and by inference, other transport genes for MeHg species were required. Sone *et al*. (2017[a]) determined the role *mer*C plays in MeHg uptake when they expressed this in *E. coli*, resulting in hypersensitivity to increased

intracellular MeHg. *mer*F is only found in Gram-negative bacterial strains and is a trans-membrane Hg^{2+} transporter (Amin and Latiff, 2017).

Signal processing inhibition of *mer*G by sodium azide showed the likely residence for the protein was the periplasm, as this compound strongly inhibits protein export. Deletion of the gene resulted in no effect on inorganic mercury resistance, but increased sensitivity to phenyl mercury. Reinsertion of the gene restored resistance. These results revealed *mer*G is responsible for resistance to phenyl-mercury and is likely due to reduced cell permeability rather than transformation of it (Kiyono and Pan-Hou, 1999). Sone *et al*. (2017[b]) confirmed the role of *mer*E in mercurial uptake by the cell, with a cysteine pair in the first transmembrane domain of the polypeptide being the critical residues. The histidine residue proximal to this cysteine pairing is responsible for Hg^{2+}, while the histidine residue on the periplasmic side is responsible for MeHg transport, in particular CH_3Hg^+. The mercury transformative roles of *mer*A and *mer*B will now be reviewed in depth to complete the basic operon functionality.

*mer*A codes for a cytosolic flavoprotein, mercuric reductase, that catalyses a double electron transfer to mercuric ions, forming elemental mercury using dihydronicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor. The enzyme was initially purified from *Pseudomonas aeruginosa* by Fox and Walsh (1982) who deduced this reduction pathway. Structurally, the enzyme works as a homodimer, with cysteine residue pairs on each subunit playing critical roles in the reaction, namely Cys_{136} and Cys_{141} (redox-active) on one subunit, and Cys_{558} and Cys_{559} (C-terminal pair) on the other subunit (NB. Tn501 numbering). The active site is at the interface of these subunits, with the relevant cysteine residues coming from both separate polypeptide chains. The co-enzyme flavin adenine dinucleotide (FAD) group is located on the C-terminal side of the disulphide bonds, and helps facilitate redox (Distefano *et al*., 1989).

The arrangement creates a double active site at the dimeric interface, stylized in Figure 2.3.

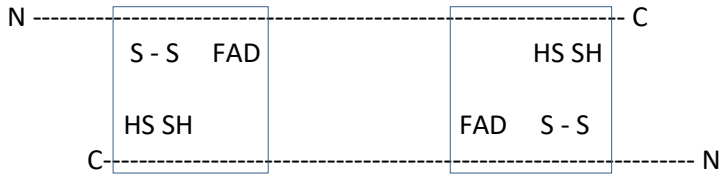


Figure 2.3 Polypeptide alignment of *mer*A subunits showing relative positions of relevant cysteine pairs and FAD location. Boxed sections are the active sites.

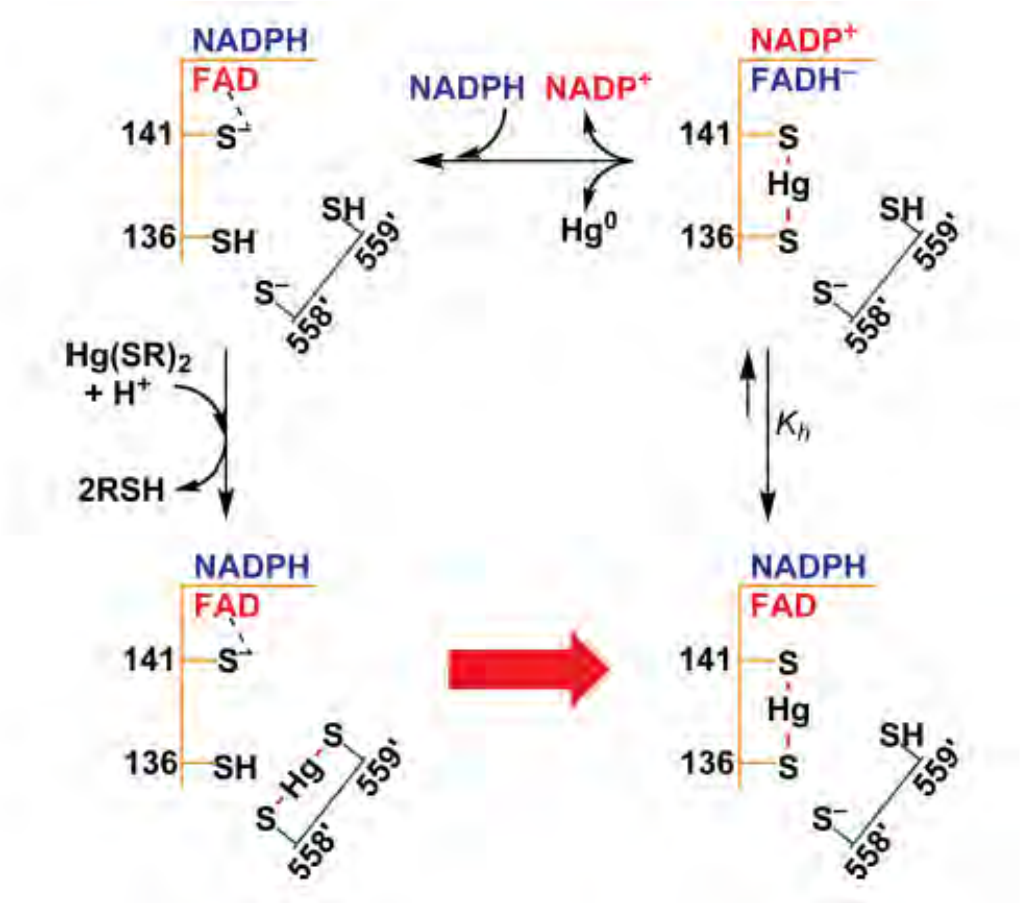


Figure 2.4 Enzymatic reduction of Hg^{2+} by *mer*A. The oxidized cofactors are shown in blue while the reduced are shown in red. The polypeptide chains from each subunit at the dimer interface are depicted in orange and green. The dashed line represents charge transfer. K_h is the equilibrium constant between the NADPH/FAD and $NADP^+/FADH^-$ redox states. The red arrow shows the Hg^{2+} transfer studied in work by Lian *et al*. (2014). (Image courtesy: Lian *et al*., 2014)

Quantum and molecular mechanical studies propose the metallochaperone-like domain at the N-terminal of *mer*A (N*mer*A) binds divalent mercury and transfers it to the C-terminal cysteine pair of the other polypeptide chain at the interface, Cys_{558} and Cys_{559} (Ledwidge *et al*., 2005; Lian *et al*., 2014). A conformational change moves the complex into the interior buried active site where Hg^{2+} is passed to Cys_{136} and Cys_{141} (Miller *et al*., 1986). NADPH then transfers hydride to FAD that results in a two electron reduction of FAD to $FADH^-$ and oxidized $NADP^+$. $FADH^-$ then reduces the bound Hg complex at Cys_{136} and C_{141} (S-Hg-S) to yield Hg^0 (Lian *et al*., 2014). The stoichiometric equation for the reduction reaction is:

$$\mathbf{Hg^{2+}(RS)_2 + NADPH + H^+ \longrightarrow NADP^+ + Hg^0 + 2RSH}$$

In terms of demethylating mercury, the relevant enzyme is coded for by *mer*B. Mechanistically, broad spectrum HgR first transport MeHg into the cytoplasm as previously discussed, where it encounters the organo-mercurial lyase produced by *mer*B. This enzyme cleaves the C-Hg bond in a protonolysis reaction producing Hg^{2+} and CH_4. The divalent ion is then passed to *mer*A for reduction as above (Parks *et al.*, 2009). It seems that the reductase enzyme facilitates release of the lyase bound mercury (Silva and Rodriguez, 2015). Although the reaction has not been fully elucidated, it would appear that it functions as proposed by Parks *et al*. (2009). In particular, it is unclear from where the proton is derived that drives catalysis.

The monomeric enzyme has two cysteine residues in the active site that are known to be critical, namely Cys_{96} and Cys_{159}, both strictly conserved across *mer*B variants. However, Parks *et al.* (2009), using density functional theory (DFT) calculations and three representative MeHg substrates, showed that these cysteine residues cannot be the

proton source, but rather act through trigonal coordination to activate the Hg-C bond, and suggest Asp_{99} protonates the carbanion leaving group, cleaving the Hg-C bond and releasing the hydrocarbon. Later X-ray crystallography work by Wahba *et al*. (2017) showed that this aspartic acid residue, and the corresponding occasionally substituted serine residue, are responsible for not only catalytic activity but also initial binding of the substrate to set up the coordination geometry required for the protonolysis reaction. The basic catalytic reaction is:

$$\mathbf{R\text{-}Hg^{2+} \xrightarrow{merB} Hg^{2+} + R\text{-}H}$$

Homologous open reading frames (ORFs) of *mer* have been found in archaeal lineages, and at least one extremophilic archaeon, *Sulfolubus sulfataricus*, has been shown to reduce Hg^{2+} (Schelert *et al*., 2004). Further, the *mer* operon has been found in deep sediment cores (Osborn *et al*., 1997), and both these pieces of evidence strongly suggest an ancient bacterial lineage for this resistance to Hg. Diversity is widespread but notably, *mer*B diversity is much more limited, suggesting a more recent evolution, which Barkay and Wagner-Dobler (2005) proposed may be in response to anthropogenic production of MeHg, although methylation of mercury occurs naturally as well.

The global distribution of the operon was demonstrated around the turn of the century by analysis of *mer* restriction fragments from various geospatially differentiated environmental samples (Hart *et al*., 1998; Bogdanova *et al*., 2001; Narita *et al*., 2003). In terms of remediation strategies, this global distribution is important because it allows for indigenous isolates at the contaminated site to be amplified numerically and employed in that location without the problems associated with introducing foreign microbial strains, the impact of which may be difficult to both model and manage.

Boyd and Barkay (2012) reviewed several hundred characterized *mer*A homologs using bioinformatics to assess phylogenetic relationships, and further, parsed specific gene information for those lineages. One of their phylograms is presented below, the details of which are not important to this work, but is included to indicate not only the variety between operons but also to indicate the bacterial distribution of the operon. A thorough examination of the phylogeny is beyond the scope of this work, but suffice to say the ability to degrade mercury is widespread throughout the bacterial world, and it is this fact that can be employed to assist in endeavours to remediate a variety of soil matrices in wide ranging geographic locations over various environmental niches.

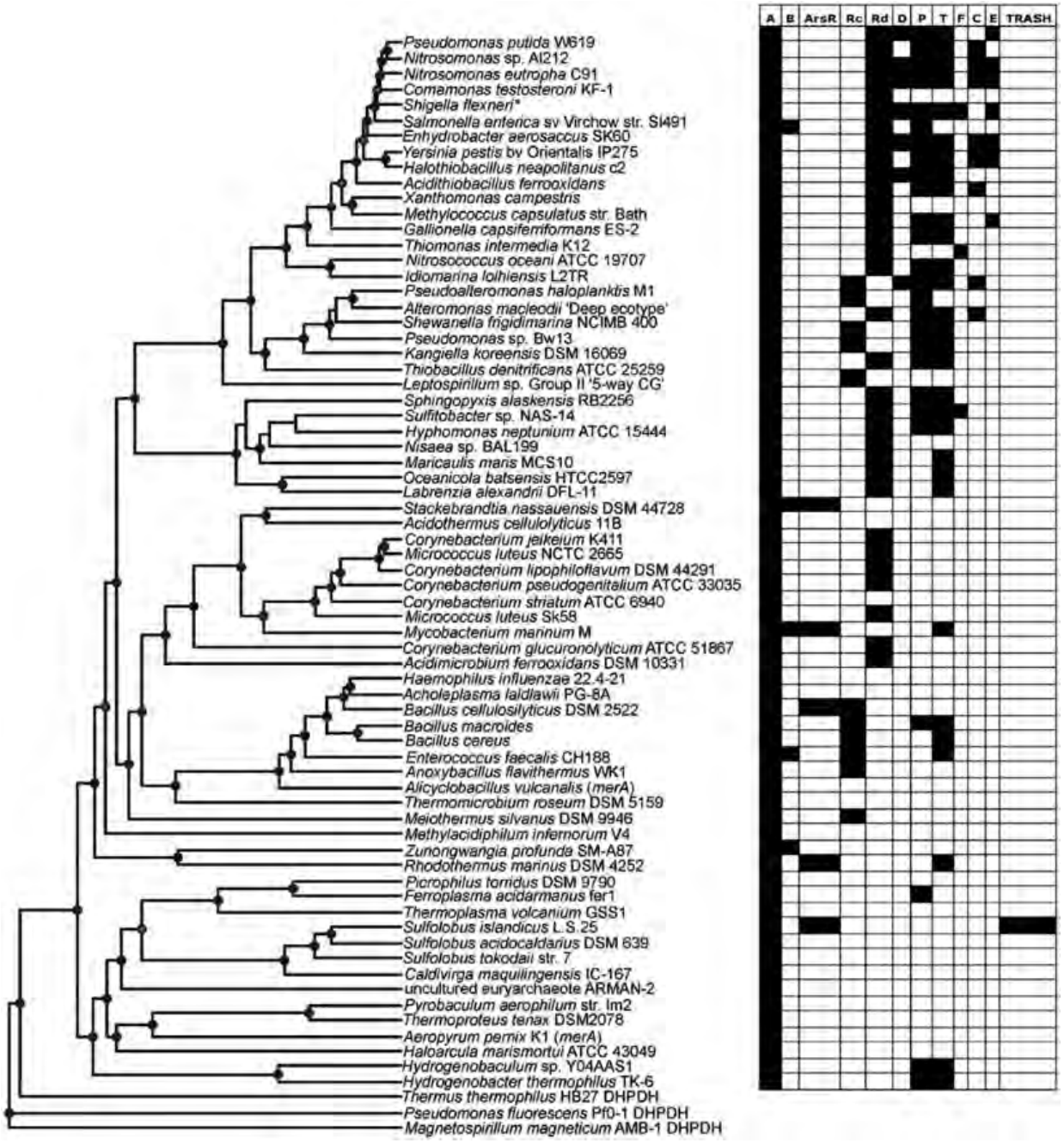


Figure 2.5. Rate smoothed phylogenetic tree of representative *mer*A and the distribution of individual *mer* functions encoded in the associated operon. TRASH represents the TRASH domain consisting of conserved histidine residues. (Source: Boyd and Barkay, 2012).

The *mer* operon is frequently associated with Tn21-like transposons, and that may pose a problem because they are genetically linked to antibiotic resistance (Liebert *et al*., 1999), an attribute that could spread through HGT to native populations in the vicinity being remediated. In fact, bacterial resistance to mercury was first noted when analysing

clinical samples for plasmid mediated antibiotic resistance back in the 1960s (Smith, 1967). Hg volatilization as the resistance mechanism was shown by Komura and Izaki (1971), and confirmed by plasmid curing after which time resistance was reduced. Wireman *et al*. (1997) found HgR coupled with antibiotic resistance was not random. No conclusions were made initially over which selective pressure it was, antibiotics or mercury (Skurnik *et al*., 2010) or possibly some other mode. It is known gene transfer occurs through *mer*s proclivity for association with highly mobile transposons and conjugative plasmids (Bogdanova *et al*., 1998), and it seems which particular operon is likely selected for by mercury species. It is known plasmid curing occurs on environmental scales (Spengler *et al*., 2006), and so the risks of antibiotic resistance spreading could potentially be managed by induced environmental scale curing if this was possible. This adds cost and complexity, and timing would be critical because the operon would need to remain environmentally functional until remediation was completed, and implies the need to physically quarantine the area during this period. The issue is complicated by the fact environmental samples are more likely residing on transposon type Tn5041 (Kholodii *et al*., 2002) that differs structurally from Tn21 type transposons, and it is unclear whether antibiotic resistance transfer is of similar scope without empirical evidence, as these environmental isolates have not been characterized well as yet in terms of antibiotic resistance capacity. Of course *ex situ* treatment is also an option that mitigates against this unwanted gene transfer.

2.3 Biomimetic tools for bioremediation – construction and applications

Traditionally, inoculation with microbes in liquid cultures is used for bioremediation, biocontrol, and other soil conditioning of terrestrial environments. However, there are many problems with this method in terms of delivery and also viability (Blumenroth and Wagner-Döbler, 1998). Further, this method is particularly difficult in aquatic settings. Biomimetic tools have been used as a way of overcoming these issues in many soil applications for things such as N_2 fixation and other biologically inspired uses (Van Elsas and Heijnen, 1990). The main method of construction is to immobilize cells or enzymes with the desired metabolic function on a solid substrate. These can be applied in various capacities in all environmental compartments. More sophisticated approaches may be achieved through circumventing living cells and directly employing the cellular *machinery.* This is done by isolating the relevant microbial protein(s) and either using them directly, or after first binding them to a substrate that can readily interact with the contaminant. Immobilization can be achieved at the enzyme and even the organelle level (Kierstan and Bucke, 2000; Arechederra and Minteer, 2008). Binding to substrates is achieved by entrapment or other attachment such as adsorbtion, covalent bonding or crosslinking, and flocculation can also be used (Mattiasson, 2018; Zou *et al*., 2018).

Biocatalytic and biosensoric tools of this kind require sound immobilization technology that meets the technical requirements while ensuring viability and maintenance of the desired bioactivity. Biofunctionalized solid substrates, particularly those using enzymes in a cell free environment, have advantages over free catalysis, for example substrate specificity, enhanced catalytic power, and the harsher and variable conditions amenable to such applications. Free cells, on the other hand, often require very specific conditions for activity (Gianfreda and Rao, 2004). Additionally, intracellular considerations such as transport, metabolism, toxicity and community competition may

be negated with the use of enzymes. Research in this area is largely confined to human health sciences, but biotechnology applications of this sort are gaining popularity in environmental considerations. Immobilized enzymes are widely used industrially, and often require stabilization to remain functional, however that subject is outside the scope of this work. This is not to discount the important role of bound whole cells, as they can often be an inexpensive and facile method to treat pollutants, and are often preferred to enzymes, as more complex biocatalysis can be achieved.

For bioremediation purposes, immobilized microbes can be an efficient way of creating a ready store of sufficient quantity that can be deployed to difficult contaminated sites, for example the ocean (Nunal *et al*., 2014). In terrestrial settings, comparing cell survival rates against free cells in field trials is confounded by differing soil matrices, OM ratios, clay and mineral content, and microbial community dynamics among other factors (Cassidy *et al*., 1996). Most assays of this kind are done in aqueous media, but it is generally understood immobilizing cells produces greater viability. For example, Murugesan *et al.* (2008) used immobilized algae where survival rates were in the order of 98% as compared to free cells where rates were less than 50%. In this case, there was little difference in adsorption of cadmium at concentrations of 1.6 mg L^{-1}, with immobilized cells achieving adsorption of 48 mg g^{-1} cells as compared to 45 mg g^{-1} for free cells.

Biocatalytic efficiency can be increased using immobilized cells in some cases, as demonstrated by Sati *et al*. (2014) where contaminant sorption capacity of immobilized cells as compared to free cells was 25% higher on average over a range of contaminant concentrations and various contaminants, although mercury was not part of that study. Immobilized cells used for volatilization often retain high rates of bioactivity, as shown by Okino *et al*. (2000) where values between 92% and 98% of 40 mg Hg L^{-1} recovery within 24 h were not uncommon. The benefit of using concentrated loads of immobilized cells for mercury can be seen in McCarthy *et al*. (2017) where Hg

volatility rates were 10^4 m^{-2}·ng·h^{-1} above background levels when cells were applied to mercury enriched gold mine tailings.

Genetically modified strains can also be used in this manner. For example, *mer* transport genes *merT* and *merP* were expressed in an *E. coli* strain, as they have high selectivity for mercury and are responsible for transport into the cytoplasm (Chen and Wilson, 1997). The cells also had cytoplasmic metallothioneins, and were immobilized in a hollow fibre bioreactor, removing 99% of 2.6 mg L^{-1} Hg from wastewater, but overall survival rates were relatively low, likely due to Hg's cumulative toxicity. Adsorption does not seem to be a good strategy for mercury regardless of the efficiency, as this will rapidly decrease as cells die. Even so, using *Bacillus cereus* immobilized in a polysaccharide matrix, Sinhaa *et al*. (2012) found these cells had Hg biosorption capacity of 104.1 mg g^{-1}, and although this trial was done *ex situ* in tightly controlled continuous batch mode conditions, this method removed 10 mg L^{-1} of Hg, a significant result.

Direct immobilization can be achieved by adsorbtion and electrostatic binding but these bonds are weak and allow leakage of the cells and enzymes into the surrounding environment, which is often not desired. Covalent bonds are stronger, but require binding chemistry that may be toxic to organisms and so viability is often lower (Mohamad *et al*., 2015). However, covalent bonding techniques are often used for enzyme immobilization. They can include cyanogen bromide activation, where material containing glycol groups is activated and allows binding to enzymes, and for those support materials containing amino groups, diazotation allows for binding on tyrosine and histidine radicals formed after treatment with $NaNO_2$ (Datta *et al.*, 2013). Peptide bonds can be formed between carboxyl or amino groups of enzymes after activation of reciprocal groups on the support matrix. For materials such as rubber which doesn't need additional solid supports, crosslinking is a common form of immobilization by reagents that react with the enzyme and form bridges within the polymer, effectively stably capturing the enzyme.

Glutaraldehyde is a common reagent used for this purpose as bonds are robust over wide ranging physico-chemical conditions and the reaction is reversible (Datta *et al.,* 2013).

Alternatively, cells may be entrapped in a solid porous matrix where pore sizes need to be smaller than cell diameters to ensure the cells do not leak, one benefit of which is less downstream processing (Juntawang *et al*., 2017). This method offers some protection against environmental pressures, but can also limit interaction with the contaminant. Entrapment matrices can include polyacrylamide gel, starches, celluloses, gelatin, silicones and rubber (Andreani *et al*., 2015; Borin *et al*., 2018), and may take the form of suspensions, emulsions and colloidal gels. Microencapsulation is one type of common entrapment where cells or enzymes are enclosed during spherical droplet formation which is then itself enclosed within semi-permeable membranes (Rathore *et al*., 2013).

Where leakage is not of concern, particularly for indigenous isolates, encapsulated cells in biodegradable matrices can be beneficial, such as water-soluble biopolymers. This has been widely used on industrial scales (Moo-Young *et al*., 1992; Wang et al., 2009). In regards to soil contamination, it is sometimes preferable that mobility not be impeded once inoculation has occurred. These biopolymers can themselves be reversibly attached to solid substrates for delivery to contaminated sites, where they degrade upon contact with water, releasing the inoculant and additionally, a nutrient supply.

A wide choice of immobilizing substrate is available to the practitioner, including but not limited to mineral surfaces (e.g., alumina, silica, calcium and glass) and organics (e.g., starch, gums, and celluloses). Minerals as scaffold materials require little modification if any and may be used over wide ranging physico-chemical conditions. They also offer some choice over binding methods, and with large surface area to volume ratios offer high biocatalytic activity per unit (Torres-Salas *et al*., 2011). They are also readily stored and transported. Non aqueous situations are sometimes problematic in that

mercury often needs to be in the mobile phase, requiring solvents. Bench scale studies demonstrate immobilized enzyme tolerance to solvents, opening up the way for their application under harsher conditions (Patel *et al*., 2014).

Bulk natural substrates are ideal because of availability and low cost. Studies on Australian zeolite characteristics by Stelting *et al*. (2012), show they play roles in cell survival, surmised due to specific water retention capacities, an important factor for long term storage. A more recent study by the same authors showed significantly higher survival of immobilized *Pseudomonas sp*. strain ADP in sterile soil, and full retention of its atrazine degrading functionality after a ten week period using a xanthan gum based biopolymer coated on zeolite (Stelting *et al*., 2014). McCarthy and Edwards (2018) bound an organo-mercurial lyase on zeolite particles using a co-expressed solid binding peptide with high affinity for silica to tether the enzyme. Similarly, ceramic-like matrices or *biocers* are simple to construct and have been used to immobilize cells and can be freeze dried, with good cell viability for surviving cells, and retention of biology activity. Biocers offer production advantages because mild temperatures and no solvents are required (Bottcher *et al*., 2004), however the process of freeze-drying results in high mortality (Soltmann *et al*., 2003).

Various combinatorial approaches have been developed and adapted, for example, encapsulation of both cells and enzymes in microemulsion based organogels (MBGs). An example is a biosorbent approach for remediating mine wastewater where a zeolite/bentonite mixture was functionalised with enzymes, sorbitol and mannitol, and a zeolite/bentonite substrate functionalised with *Penicillium simplicissimum.* (Nsimba, 2013). Nsimba (2013) also used a zeolite-alginate complex that was generated by impregnating natural zeolite into alginate gel beads. These resulted in a ten percent increase in Hg adsorption capacity over pH 2-7 with no loss of activity compared to non-immobilized.

In one notable study twenty years ago, the binding substrate was a living cell. Parathion and paraoxon were detoxified with enzymes anchored and displayed on the cell surface of *E. coli*, using recombinant self-generated surface loop proteins tethering the relevant enzyme (Richins *et al*., 1997). *mer* inspired systems have high selective affinity for mercury, and are ideal Hg isolators from co-contaminants, as other ions do not impede activity (Deng and Wilson, 2001). A novel design application was the creation of an adsorption and release mechanism based on temperature changes. An efficient and recyclable Hg^{2+} sorbent was created by synthesizing *mer*R with elastin-like polypeptides (ELPs) (Kostal *et al*., 2003). ELPs contain a number of repeating pentapeptide (valine-proline-glycine-valine-glycine) sequences that facilitate aggregation at elevated temperature, but solubilize in ambient temperatures. This allows for Hg^{2+} to be sorbed by MerR at low temperature, and then the ELP-Merr*R*-Hg^{2+} complex is precipitated at elevated temperatures. The precipitate is then removed and Hg chemically separated.

Within technical boundaries, there seems to be limitless applications and designs for biomimetic tools that can be applied to various environmental issues, including for remediation of mercury. As previously noted, the research undertaken in this thesis makes use of two biomimetic approaches, one using immobilized cells, and one using immobilized enzymes.

2.4 Gaseous mercury capture

Atmospheric pollution is problematic in the sense that it readily crosses international boundaries, eroding the emitters will to counteract the problem. Gaseous Hg is estimated to circle the globe three times before deposition (UNEP, 2013). For this reason emitters must be pressured, for example through litigation, or compelled legislatively, to counteract point source issues. This is particularly important for mercury due to its volatile nature and extreme toxicity, as recognized by the UN, the World Health Organization (WHO), and more formerly through the Minamatta Convention. In the US, the Mercury and Air Toxics Standards (MATS) were initially introduced to reduce mercury (and other) atmospheric pollutants from power plants (USEPA, 2011), while in Europe, the 2010/75/EU Industrial Emissions Directive sets average mercury emission limits at 50 $m^{-3} \cdot \mu g$ (European Commission, 2010). Australia currently is a signatory but has not ratified the Minamatta Convention, has piecemeal limits on Hg emissions, and has only nebulous terminology in its environmental legislation, such as "*likely impact on the environment*" as per Protection of the Environment Operations Act 1997 (NSW) s 128. Australia is bound by international laws such as the Basel Convention and the Rotterdam Convention, but they only provide guidance on safe handling, storage, and transportation of mercury (Bramwell *et al*., 2018).

Much research has gone into atmospheric emissions, cycling, depletion events and geographical distribution of mercury, culminating in emission-deposition models that are iteratively refined as fresh data comes to hand (Slemr *et al*., 2011; Song *et al*., 2015). While that work is critically important to better inform decision makers and researchers, those aspects are outside the scope of this work. Rather, this review concentrates on emission capture challenges and technologies. Much of the literature relates to coal fired power station emissions abatement, as coal combustion is a major contributing factor in

anthropogenic atmospheric mercury. The cement industry is another major emitter of mercury, and the different challenges in that industry as compared to coal are also well canvassed in the literature as will be seen in the following section. Little research on fugitive Hg emissions, let alone abatement technologies, has gone into fracking coal and shale seams, or standard natural gas extraction, although these operations potentially have fugitive emission problems. ASGM mercury emission abatement is under-represented in the literature, even though this industry is the major source of anthropogenic emissions. This is likely due to the under-regulated and decentralized nature of ASGM operations. These and associated issues are explored in detail in this section.

Coal fired power stations

In combustion processes, any form of Hg is decomposed to Hg^0, without exception (Pavlish *et al*., 2003). As gaseous elemental mercury has low solubility and reactivity, removal from flue gas streams can be challenging. For coal fired power stations, temperatures of flue gases exiting the furnace are typically 1200°C, dropping during heat transfer processes before entering the stack at around 150°C (Park *et al*., 2004). When cooled to below 400°C, Hg^0 is partially oxidized in reactions involving oxygen and halogen species, largely chlorine, and upon further cooling, can form particulates upon contact with other material, largely oxygen and high sulphur content carbonaceous ashes. Mercury then exists primarily as gaseous elemental mercury, solid inorganic mercuric compounds, and as $Hg^{2+}X$ [where X is Cl_2(g), SO_4(s), O(s,g), and S(s)] (Galbreath and Zygarlicke, 2000). This is represented below in Figure 2.6. Concentrations of Hg in flue gas normally range from 5 to 10 m^{-3}·μg, with variable fractions representing Hg^0(g), $Hg^{2+}X$(g), and Hg(p) depending on the decomposition environment and source combustion material.

Scrubbers generally require Hg as water soluble oxidized species to facilitate capture (Dranga *et al*., 2012). The issue is complicated by the heterogeneous nature of flue gases, thermal gradients, and particulates that substantially affect the physicochemical nature of Hg, catalysts and sorbents. These factors render efficient capture of mercury difficult.

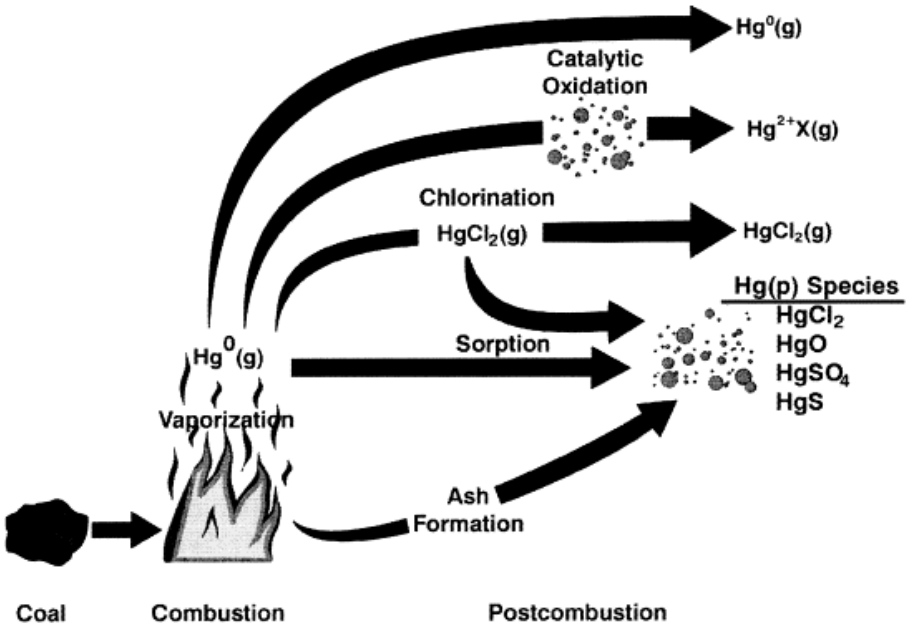


Figure 2.6 Basic representation of mercury transformations in coal combustion from initial vaporisation to subsequent flue gas speciation. (Image courtesy: Galbreath and Zygarlicke, 2000)

Post combustion, Hg^0 can remain monatomic or partially react, forming Hg^{2+} and Hg_2^{2+} compounds, with Hg^{2+} dominating oxidized forms due to the relative instability of Hg_2^{2+} at low concentrations (Aylett, 1975). Chlorination of Hg^0 occurs, as HCl evolves naturally during combustion. HCl occurs in coal at concentrations ranging from 25-150 mg m^{-3}, and post-combustion, reacts as Cl_2 or HCl with Hg to form $HgCl_2$ (Meij, 1991). While Cl_2 only represents about 1% of flue gas total HCl species, it is the most important

chlorinator, and at these concentrations would be in excess as a reactant gas compared to mercury (Hall *et al*., 1991; Laudal *et al*., 1996). The reaction requires metal catalytic activity, supplied by fly ash, and can be inhibited by the presence of SO_2 which can deplete available Cl_2 (Pan *et al*., 1994), unfortunately including through formation of toxic sulphryl dichloride, a lachrymator, otherwise known as tear gas (Wakefield and Maynard, 2012). Additionally, elemental sulphur can be partially chlorinated, yielding disulphide dichloride, which reacts with water forming what is infamously known as mustard gas, as listed in Schedule 3 Part B – Precursor Chemicals of the Chemical Weapons Convention (OPCW, 2019). Chlorinated Hg species either remain as gas in the flue stream, or are adsorbed onto carbonaceous ash particles, with porosity and surface area dominating adsorption characteristics (Senior *et al*., 1998). Other sorbtion mechanisms of note are acid-base reactions with $HgCl_2$ and alkaline particles, which can result in ash enriched with Hg precipitates (Ghorishi and Gullett, 1998).

Besides chlorination, O_2 and NO_2 potentially react with Hg species, although kinetics are unfavourable for direct reactions. Rather, in the presence of carbon rich ash, O_2 and NO_2 play important roles in Hg adsorbtion onto particles, largely as solid HgO (Hall *et al*., 1991). NO_2 seems to promote formation of $Hg^{2+}X$ species. Sulphur rich ash particles are also reactants and adsorbants. Also, SO_2 is released during combustion, and a fraction of this is oxidized to SO_3, which can react with H_2O to form H_2SO_4. This condenses on particle surfaces, where it can either react with Hg directly, or create sulphur rich active sites for mercury sorption on particles (Zhao and Rochelle, 1998). Relative to Hg^0, particulates and $Hg^{2+}X$ species are amenable to capture by conventional methods such as wet scrubbers and fabric filters. Where they evade capture, these two forms are more likely to deposit locally, whereas Hg^0 is distributed much more widely (Galbreath. and Zygarlicke, 1996).

With some understanding of the mercury chemistry in flue gas, it is possible to better design catalysts and sorbents, and improve Hg removal efficiency. However, there are some preliminary steps that can reduce the mercury load prior to combustion, the most salient being mining ores with low intrinsic mercury (Goodarzi and Goodarzi, 2004), and separation pre-processing. Low Hg lithologies can be mined where practicable, and beneficiation to remove gangue minerals aids reduction of mercury in feed coal (Finkelman, 1994). Similarly, rejects (or *pyrites*) of denser material discarded by pulverisers often account for 1% of total feed coal volume, but may contain 10% of the total mercury (Hower *et al*., 2005).

Mercury sorbtion to fly ash is enhanced if the unburned carbon fraction is higher, however this negatively impacts secondary use of fly ashes, for example as an additive in cement manufacture (Külaots *et al*., 2004). When fly ash is used as a pozzolonic additive, unburned fractions of carbon always have negative impacts on the desired characteristics. For this reason, many combustion facilities aim to fully burn out fly ash (Hower *et al*., 2010), indicating the necessity for strict Hg emission regulations. Further complicating matters regarding carbon is the evolving nature of post-combustion conditions. Changing chemistry and temperatures in turn affects bulk and pore size and therefore surface area and chemi-sorption characteristics of particulates. When temperatures drop to below 300°C, Hg sorbtion on particles increases while carbon combustion is dampened (Külaots *et al*., 2004). Source coal and pulverizing characteristics also play critical roles in determining the nature of evolved particles from coal combustion. The factors effecting mercury adsorption on ashes can be summarized as the amount and distribution of available carbon in fly ash, the temperature of flue gas, and feed coal characteristics.

In terms of removing unwanted materials from flue gasses emanating from coal combustion, the earliest implemented were flue-gas desulphurization (FGD) technologies

when successful legal action in England first forced the issue in 1929. This had the unintended benefit of aiding removal of mercury. SO_2 is removed by FGD with an efficiency between 80-90% depending on method (Kairies *et al*., 2006). Most systems use wet scrubbers, and require a two stage process where fly ash is first reduced via electrostatic precipitators (ESPs). This partitions much of the mercury that is sorbed to the fly ash. The acidic SO_2 gas continues through alkaline atomized sorbent slurries composed of limestone, hydrated lime or magnesium hydroxide or similar (Kairies *et al*., 2006.

The design details are outside the scope of this work, suffice to say there are many, such as venturi-rods, packed bed scrubbers and spray towers among others, all designed to maximize reactant surface area and residence times, such that sufficient SO_2-reactant interaction takes place. Whatever method, this still leaves sulphuric acid (as described previously) downstream of FDGs. Mercury in fly ash captured by ESP's or fabric filters (FFs) has been empirically correlated to amount and type of carbon in fly ash (Hower *et al*., 2010). Partitioning of trace elements including mercury within ESPs and FF arrays is also a function of temperature at the collection point and the spatial configuration of the array. Predicting overall mercury capture is very difficult because of the evolving physico-chemical characteristics of the fly ash as the temperature drops (Hower *et al*., 2010), most importantly effecting surface area morphologies. Predictive models and capture designs must also include desorption factors, particularly at higher temperatures.

The other relevant pollutant (CO_2 is irrelevant to mercury in this context) that warrants attention is NO_x abatement technology. Fluidized bed combustion (FBC) results in less NO_x and is achieved by suspended combustion over jets of air between 800-900°C, and may be designed as bubbling bed or circulating (Basu, 1999). Alternatively, or in addition to FBC, selective catalytic reduction (SCR) reduces NO_x through injection of ammonia to the gas stream, converting NOx to N_2 and H_2O. Problems result from

unconsumed ammonia which can adsorb to fly ash and impede mercury sorbtion. The system may be installed on the *hot* or *cold* side of FGDs, upstream and downstream respectively. Unfortunately, reducing NO_x may have the unwanted effect of reducing mercury sorbtion on fly ash, by reducing NO_2 and hence $Hg^{2+}X$ particulate formation as per the mechanisms previously discussed. These indirect measures only remove a portion of mercury in any case, and much remains as the gaseous species Hg^0 and $HgCl_2$, together with unbound and fly-ash bound particulates that have evaded capture by ESPs and FFs.

Direct intervention for Hg reduction is relatively more recent and involves injection of pulverized activated carbon (PAC) fine particulates, generally at lower temperatures, which seems to aid sorbtion (Presto and Granite, 2006), either directly into existing FGD systems or as a powderised spray upstream of FGDs. Other important pollutants such as polycyclic aromatic hydrocarbons (PAH), dioxins, and furans can also be captured in this way. Important PAC efficiency parameters are mercury speciation and concentration, carbon particle size distribution and surface characteristics, pore structure, flue gas temperature and chemistry, among much else (Yang *et al*., 2007). Further, surface chemistry is determined using techniques requiring complex mathematical modelling and many assumptions (Zheng *et al*., 2012). Thus, it is difficult to compare PAC-Hg removal efficiency between reports in the literature. It is self-evidently important that the PAC is captured efficiently, as it can transport important environmental toxins, escaping in a more concentrated form bound to particulates. The generic overall design scheme for coal fired power stations including scrubbing devices is pictured in Figure 2.7.

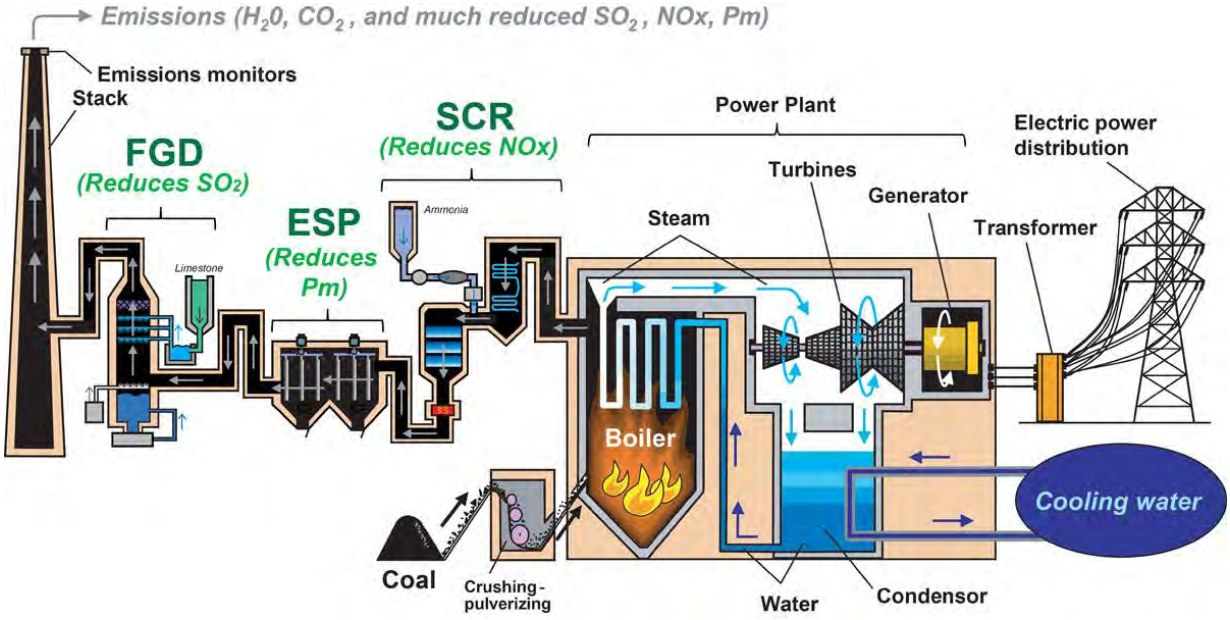


Figure 2.7 Typical coal fired power station schematic including flue gas scrubbing devices (FGD, ESP, and SCR). Individual set ups differ both in sequence and design. PAC technology for removing mercury is not shown. Note: The exiting flue gas composition is more complex than as described. (Source courtesy: Kentucky Geological Survey, Kentucky University 2019 ©).

Feed coal type is important to Hg chemistry, including the fact pulverized coal is often blended (including to non-coal sources) to stabilize combustion operating parameters around optimized settings. Comparisons are confounded because coals are heterogeneous, and graded, characterized, and blended in numerous ways, resulting in differing flue gas chemistry. There are two main categories for coal; bituminous and sub-bituminous. Sub-bituminous has lower calorific capacity so more is required for the same energy output (Vassileva and Vassilev, 2006). Bituminous coal mercury is generally associated with pyrite (FeS_2) and cinnabar (HgS), whereas in sub-bituminous coals it is bound within the organic fraction (Zheng *et al*., 2012). Bituminous coals see more mercury capture with wet FGD devices compared to sub-bituminous coals (av. 50% to 30% respectively) for similar starting mercury concentrations. SCR in combination with FGD

can increase rates to 65-90% total removal in bituminous coals. This compares to FF where Hg capture ranges from 70-85% (Zheng *et al*., 2012).

A major limiting factor and one of the main challenges, particularly in wet FGD processing, is the evasive nature of Hg^0 such that much research has gone into oxidative catalysis. Catalytic oxidation of mercury process development can be categorized into three main areas; SCR, carbon-based, and metal oxide catalysts (Presto and Granite, 2006). The developments are largely trial and error, as both the catalytic and mercury adsorbance mechanisms remain speculative in many areas. It is difficult to design optimum catalysts while these fundamental aspects remain uncertain. The related speculative chemistry is outside the scope of this work.

During previously mentioned SCR processes, the oxidation catalyst is typically vanadium pentoxide (V_2O_5)-tungsten trioxide (WO_3) over titanium dioxide (TiO_2). NO is reduced by injected NH_3, upstream of the SCR, and at temperatures above 300°C. NH_3 adsorbs to V_2O_5 sites. NO reacts either as gas or as a weakly adsorbed species (Niksa and Fujiwara, 2005). Mercury oxidation incidentally accompanies this reaction. With addition of HCl and/or H_2SO_4, mercury oxidation rates up to 95% have been shown, but only in laboratory scale tests. Typical operational results range from 30-70% (Lee *et al*., 2003). Besides vanadium oxides, iron oxides with HCl has been shown experimentally to oxidize Hg^0 at 250°C to a level of 90%, however actual operational rates are closer to 10-60% (Dunham *et al*., 2003). Other metals including copper, gold, silver and palladium have all shown promising results at lower temperatures but operational data is lacking and the economics are questionable given the cost of these metals.

For carbon catalysts, PAC and fly ash is known to sorb some portion of Hg^0, and redox mechanisms involving gaseous acids can result in oxidation, catalysed by trace metal inclusions. Quantification is difficult due to mercury desorbtion processes that occur

concurrently, the details of which are not well understood. The most common elements associated with PAC surfaces are oxygen, nitrogen, phosphor, hydrogen, chlorine and sulphur. While in ambient conditions, oxygenated functional groups form, and can be neutral, basic or acidic in nature (Salame and Bandosz, 2001). Additional functional groups can emerge through impregnation with nitrogen- or sulphur-containing reactants (Lahaye, 1998). Although effective to high degrees in some instances, the problem arises due to the requirement for carbon-to-mercury ratios greater than $3000:1^{v/v}$, and while it has more scrubbing utility than just for mercury, this creates voluminous material requiring post processing (Pavlish *et al*., 2003). Where fossil fuels and other mercury-containing organics are gasified, the temperatures required during processing are usually too high to allow for Hg oxidation (Zheng *et al*., 2012), requiring downstream removal by precipitation during processing into commercial syngas.

Cement manufacture

During cement manufacture, the processes and environment produce quite different mercury chemistry compared to coal fired power stations. Importantly, mercury input from raw materials and fossil fuels is variable, and operational phase changes and internal re-cycling events hinder equilibrium states being achieved, making detailed chemical analysis difficult (Zheng, *et al*., 2012). A cement manufacture overview schematic is provided in Figure 2.8. It is important to understand the additives, processing cycles and related Hg chemistry to reveal mercury partitioning opportunities. Similar technologies that can remove mercury such as FGD are employed where appropriate, but can have negative impacts on cement quality. The average emissions from cement manufacture are around $28\mu g.m^{-3}$ Hg, higher than for coal fired power stations at 5 to $20\mu g.m^{-3}$ (Johansen and Hawkins, 2003).

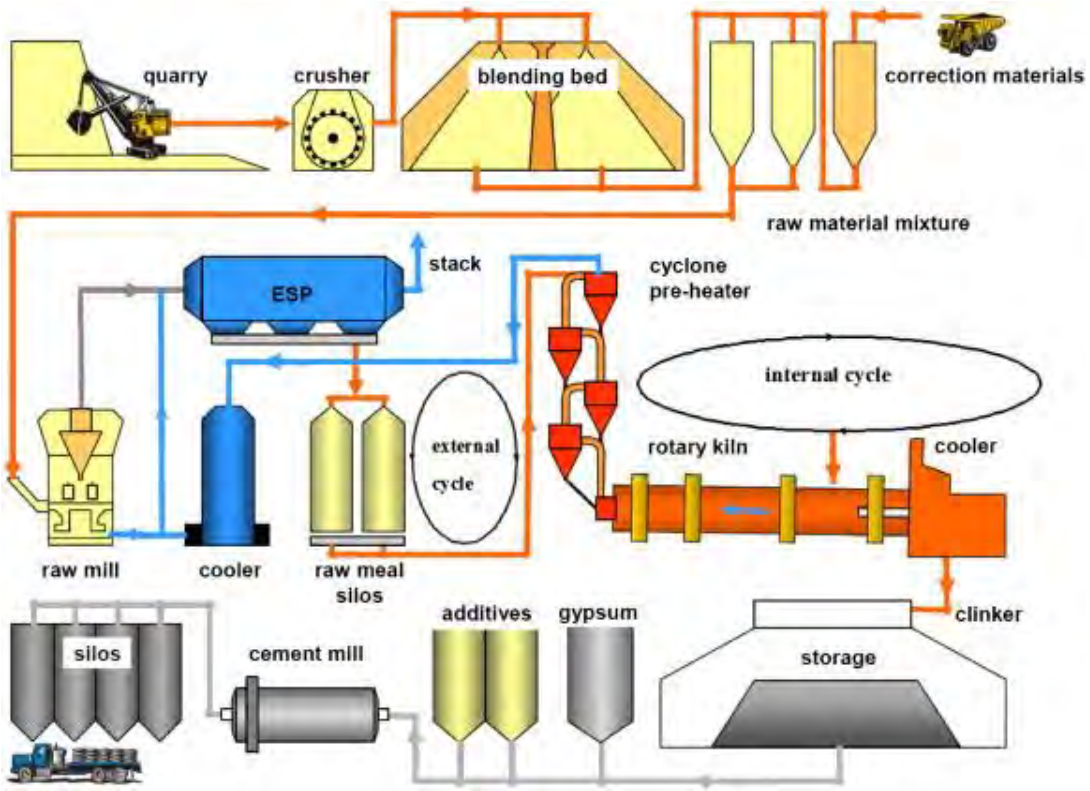


Figure 2.8. Typical dry process cement manufacture schematic (Source Zheng *et al*., 2012).

Basic constituents such as limestone and alumino-silicates, and compositional corrective additives such as bauxite, iron ore and sand enter the initial kiln in a dry powdered form. The raw meal is then collected via FF or ESP, and transferred to cyclonic pre-heaters (Achternbosch *et al*., 2003). Gas temperatures in the pre-heaters range from 850 down to 350°C at the exit, with residence times up to 25 s. This is followed by a cooling zone that also contains air quality devices where temperatures range from 350 to 90°C

The calcination process is completed in the main rotating kiln to produce clinker, with the relevant required temperature around 1450°C to ensure complete sintering reactions. Combustion gases have a residence time of between 5-10 s, retain temperatures above 1200°C during residence, and require 2-4% volume of oxygen. This

is longer and a higher temperature than for coal fired power stations (Senior *et al*., 2000). The clinker is then cooled and ground with additives such as gypsum to produce Portland cement, and other blended cement formulations using fly ash, slag, limestone and other pozzolans.

Mercury relevant flue gas composition of cement kilns as compared to coal fired power stations can be summarized as higher H_2O, SO_x and NO_x, with lower HCl and O_2. Temperatures are also up to 100°C higher at entry to the flue gas stack (Zheng *et al*., 2012). HCl content is much reduced through capture by high concentrations of alkaline solids in a turbulent hot environment, ideal conditions for absorbing acid gases. Combustion gases and solids move in a countercurrent flow, with volatiles such as Hg transported from the hot end of the kiln to the cold end. During this cooling phase, adsorbance or condensation of volatiles on particulates can occur, and while some passes through the system entirely as gas, any bound Hg fractions are eventually reintroduced to the meal as cement kiln dust (CKD), establishing an internal cycle. CKD can typically account for 7% of solids in the system (Senior *et al*., 2009).

There are also two basic operating modes of cement mills, compound and direct operation. Compound operation uses off-gas containing dust to transport meal with no cooling water, while direct operation cools gas down in a bypass fashion where water is used. The water importantly also de-dusts the gas. This greatly affects elemental mass flows, with direct operation creating higher mercury emissions than compound mode (Achternbosch *et al*., 2003). Switching modes also establishes further mercury internal cycling (Zheng *et al*., 2012).

Average cement raw material mercury content is 80ppb, based on a study of hundreds of materials, excluding fly ash and recycled CKD from 57 plants in Canada and the US (Hills and Stevenson, 2006). Mercury content can be greatly concentrated in fly

ash and CKD unless pretreated. Fuel sources also contribute mercury, such as coal, tyre-derived fuel and petroleum coke that contain substantial concentrations (Zheng *et al*., 2012). A snapshot of mercury content of most common primary and secondary raw materials and fuels as described by Zheng are detailed in Table 2.3, although no information is provided for relative quantities or their contribution to fugitive emissions.

Table 2.3 Mercury content of typical cement raw materials. All values dry weight ppm.

Material	Category	Av. Hg ppm	Max. Hg ppm
Limestone	Primary raw material	0.017	0.391
Sand	Primary raw material	0.029	0.556
Clay	Primary raw material	0.052	0.270
Shale	Primary raw material	0.057	0.436
Slag	Secondary raw material	0.012	0.054
Bottom ash	Secondary raw material	0.048	0.382
Iron ore	Secondary raw material	0.078	0.672
Fly ash	Secondary raw material	0.205	0.685
Recycled CKD	Secondary raw material	1.530	24.560
Petroleum coke	Fuel	0.050	0.200
Sub-bituminous coal	Fuel	0.070	0.900
Bituminous coals	Fuel	0.120	1.120
Tyre-derived fuel	Fuel	0.097	0.400

CaO is created by the heating process, and modelling by Larsen *et al*. (2007) of mercury in cement preheater kilns shows while CaO is in excess, HCl and SO_2 are sequestered from Hg related reactions. The dominant species below 180°C are oxidized

to HgO and $HgCl_2$, while above 200°C, they are predominantly gaseous. Above 300°C, Hg exists predominantly as the evasive GEM. This is problematic in that this represents the species at the typical bypass extraction point. Also, mass balance studies indicate raw material might react with mercury at high temperature to form new solid compounds, such as with silicates (Angel *et al*., 1990). During the cooling phase, particularly below 100°C, elemental mercury can adsorb or condense on the dust, as can oxidized species such as $HgCl_2$, HgO and $HgSO_4$. Mercury sorbtion rates on dust range from 35-85% of total mercury (Zheng *et al*., 2012). The dust is recycled, and mercury is only intermittently scrubbed when the CKD is removed and treated before reintroduction. It is estimated that half the total mercury content is either emitted or remains in the meal and ends up in the final product, with the other half trapped in dust within internal cycling (Renzoni *et al*., 2010).

Abatement of mercury can initially be achieved by cleaning coal used as fuel. Density gravity is used in bituminous coals to separate pyritic sulphurs, and some fraction of mercury will be bound and can be partitioned in this way. However, waste water treatment costs are significant (USEPA, 1997), and as higher mercury contributions are from the raw materials, fuel cleaning is not often done for cement manufacture. Alternatively, use of fuels with less mercury content, such as tyre-derived fuels (TDF) and gas, could moderately impact net emissions. Additionally, TDF can result in additional Hg oxidation rates, as shown by Zhuang and Miller (2006) where oxidation rates of 85% were seen with 10% blend of TDF and coal, likely due to high HCl content. This allows for enhanced mercury scrubbing with traditional technologies as discussed for coal fired power stations. However, benefits can be overshadowed by speciation from the variable intrinsic Hg content and chemistry of the TDF (Richards *et al*., 2008).

As carbon is deleterious to cement, PAC based solutions for mercury abatement are not ideal for cement manufacture due to cross contamination issues. Therefore other

substrates have been used as sorbents, particularly those that are attractive as cement additives. Modified non-carbonaceous materials and mineral oxides, including manganese oxide powder, aluminium oxide, silica gel, and molecular sieves such as zeolites, have been functionalized in efforts to remove mercury vapour (Lee *et al*, 2006). Silicate sorbents are prepared through ion exchange in mediums containing polyvalent metals, accompanied by the controlled addition of sulfide ions (Holmes and Pavlish, 2004).

Additionally, for gaseous $HgCl_2$ capture, those sorbents containing high Ca have been shown to be up to half as effective as carbon sorbents, but still 20 times less efficient at Hg^0 capture (Zheng, 2012). Injected sodium tetra-sulfide has been used as a sorbent in flue gasses, and captures both elemental and divalent mercury, forming solid and insoluble mercuric sulfide that is removed by ESP or FF (Licata *et al*., 2000). However this is unsuitable for cement production as sodium is deleterious to cement quality. More traditional wet scrubbing by FGD is used, however this is entirely useless for Hg^0 capture, and similar to coal fired power stations, requires mercury oxidation. Further mercury reductions can be made by partitioning CKD and thermally treating before re-introduction to the rotary kiln.

Other industrial emissions

Other major single contributors to mercury emissions are less well documented. Artisanal and small-scale gold mining (ASGM) operations account for one third of estimated emissions, however abatement is not well canvassed in the literature. The chemistry is simple in that apart from ore dependent emissions during ore crushing, the basic species emitted is GEM after heating mercury that has been used as an amalgam. Large-scale mining operations often use gas impervious capping to ameliorate fugitive emissions, such as from heap leaching pads.

The other major Hg emitter not well represented in the literature is from non-ferrous metal production. Fukuda *et al*. (2011) reviewed practices in Japan, and found raw materials averaged 30 μg kg^{-1}, while fuel averaged 58 μg kg^{-1}. It is estimated that half the mercury input in the process is emitted as GEM. The sintering process accounts for 90% of mercury emissions, which are partially captured by a combination of activated coke towers, and during desulphurizing by FGD with ESP.

Given the evasive nature of Hg^0 and mercury's low solubility, together with difficulties in efficient catalytic oxidation in a complex and evolving flue gas environment, it is unusual that previous research has not been more vigorously applied to GEM capture without the need for catalysis. While there has been mercury speciation and partitioning analysis done for activated carbons and fly ash, underlying sorption chemistry is not well understood. Similar to carbon, research is emerging for other functional solid substrates that act both as catalytic and capture devices, such as bulk minerals and synthetics like zeolites. The research contained herein is partially aimed at addressing our gap in knowledge about devices for direct Hg^0 capture without the need for catalytic oxidation.

2.5 Literature Summary

Although various physico-chemical methods have been devised to remove mercury from soils, many are destructive to the matrix itself. *Ex-situ* remediation is expensive and disruptive, while *in-situ* remediation requires solvents or binding chemistry that is often detrimental to soils. Less intrusive phytoremediation techniques are limited geographically with low efficiency, and biomass waste can be voluminous. Alternatively, microbial remediation is comparatively non-intrusive and low cost. The implementation of microbial remediation has challenges, such as viability and efficiency, and delivery to remote sites can be difficult. However, it seems possible these challenges may be overcome.

Microbial resistance mechanisms to mercury are well established, and this biochemistry can be utilized to design biomimetic tools that may assist remediation practitioners. It may be possible to harness methylmercury transformation biochemistry to design solid state products that potentially can be employed in environmental areas, such as wetlands. Immobilising cells and enzymes on solid substrates is well established technology, but has been under-utilised for remediation purposes, particularly for mercury related issues.

Finally, capture of gaseous elemental mercury has proved to be a very difficult exercise. Retort cooling is not always practicable, and solid sorbents such as activated carbons are expensive, and can result in voluminous waste, and this area suffers from a lack of detailed knowledge about sorption and desorption mechanisms. Catalytic oxidation of mercury can be effective, but is not highly efficient, and generally arises only incidentally in industrial settings as operators deal with other pollutants. A non-catalytic capture medium might prove beneficial in this area.

Chapter 3 - Research Aims

3.0 Research rationale

Biogeochemical cycling of mercury means it is naturally present in aquatic settings, in soils, and in the atmosphere. Anthropogenic activity has increased Hg mobility such that these areas are now heavily impacted. This thesis aims to contribute knowledge relevant to remediation of all three environmental compartments.

Specifically, logistical problems in inoculating terrestrial Hg contaminated sites with microbial loads is addressed with research using immobilised zeolite-bound cells, encapsulated in a biodegradable biopolymer. There is a wealth of research around distribution, cycling, health implications, and the difficulty with oxidizing gaseous elemental mercury to facilitate electrostatic capture, but virtually no research into non-catalytic capture, except for incidental work on activated carbons. Alternative capture methods are canvassed in the literature, however these invariably require oxidation and catalysis and are outside the scope of this work (Xu, *et al*., 2018; Li, *et al*., 2019; Liu, *et al*, 2019), and none of these are adaptable to diffuse emissions sources. The enclosed research on modified coir fibre material is aimed at addressing that particular problem, and potentially provides a cheap and facile alternative to carbon and oxidative based solutions. Research for aquatic settings is overwhelmingly aimed at divalent mercury capture, but research into direct remediation of methylmercury is virtually non-existent. The biomimetic device proposed is aimed at partially addressing that gap in knowledge.

3.1 Stated Aims

I. To show proof of concept for binding *Psuedomonas veronii* cells to zeolite by encapsulation in a biodegradable biopolymer

II. To examine the viability of those immobilised cells during storage

III. To evaluate the effectiveness of zeolite-bound cells as an inoculant in removing mercury from contaminated mine tailings

IV. To design a synthetic gene that could be cloned into *E. coli* cells for production of an enzyme with methylmercury transformation capabilities

V. To evaluate that organo-mercurial lyases ability to bind to zeolite particles

VI. To examine the biomimetic particles for methylmercury transformation functionality

VII. To develop an alternative to activated charcoals to capture gaseous mercury emissions without prior catalytic oxidation or other physico-chemical treatment

Chapter 4 – Research paper 1

4.0 Statement of contribution

Damien McCarthy - Concept and design of experiments, background research, experimental work, data analysis, instrument set up and calibration, manuscript production

Grant C Edwards - Concept and design of experiments, data analysis, instrument assistance, manuscript editing, general oversight

Mae S Gustin - Logistical support

Andrew Care - Laboratory guidance

Matthieu B Miller - GOM filter analysis

Anwar Sunna – Concept and design, biopolymer formula, manuscript editing.

4.1 An innovative approach to bioremediation of mercury contaminated soils from industrial mining operations

Chemosphere 184 (2017) 694–699

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

An innovative approach to bioremediation of mercury contaminated soils from industrial mining operations

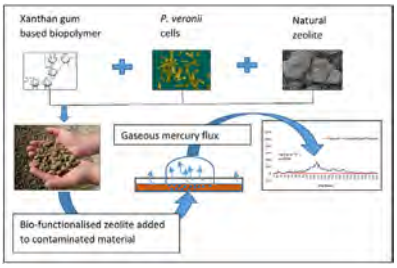
Damien McCarthy [a, *], Grant C. Edwards [b], Mae S. Gustin [c], Andrew Care [a], Matthieu B. Miller [b], Anwar Sunna [a, d]

[a] Department of Chemistry and Biomolecular Sciences, Faculty of Science and Engineering, Macquarie University, Sydney, NSW 2109, Australia
[b] Department of Environmental Sciences, Faculty of Science and Engineering, Macquarie University, Sydney, NSW 2109, Australia
[c] University of Nevada, Department of Natural Resources and Environmental Science, Reno, NV, USA
[d] Biomolecular Discovery and Design Research Centre, Macquarie University, Sydney, NSW 2109, Australia

HIGHLIGHTS

- A bulk natural substrate, zeolite, was bio-functionalised with encapsulated *P. veronii*.
- This remained viable and functional after long term storage.
- Bio-functionalised zeolite increased GEM flux by 10^4.
- A concomitant reduction in GOM emissions was also observed.
- Bio-functionalised zeolite shows potential for remediation of mercury-contaminated sites.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 29 December 2016
Received in revised form
11 June 2017
Accepted 13 June 2017
Available online 14 June 2017

Handling Editor: J. de Boer

Keywords:
Volatilisation
Mercury bioremediation
Xanthan gum
Zeolite
Biopolymer

ABSTRACT

Soils contaminated with mercury (Hg) have proved expensive and logistically difficult to remediate. Research continues into finding suitable environmentally-friendly and efficient ways of achieving this end. Bioremediation is an option, which employs the strategies microorganisms have evolved to deal with Hg. One microbial strategy involves uptake and intracellular volatilisation of mercuric ions, which passively diffuse from the cell and back into the atmosphere. In this work, *Pseudomonas veronii* cells grown to stationary phase were immobilised in a xanthan gum-based biopolymer via encapsulation. The *P. veronii*-biopolymer mix was then coated onto natural zeolite granules. Zeolite immobilised cells remained viable for at least 16 weeks stored under ambient room temperature. Furthermore, the immobilised cells were shown to retain both viability and Hg volatilisation functionality after transportation from Australia to the USA, where they were applied to Hg contaminated soil. Maximum flux rates exceeded 10 μg Hg m^2 h^{-1} from mine tailings ($\approx$7 mg kg^{-1} Hg with 50% v/v water). This was 4 orders of magnitude above background flux levels. It is envisioned that emitted gaseous elemental mercury (GEM) can be readily captured, and transformed back into metallic Hg, which can then be stored appropriately or recycled. This breaks the Hg cycle, as GEM is no longer translocated back to the atmospheric compartment. The immobilising excipients used in this research overcome many logistical issues with delivery of suitable microbial loads to locations of mercury contamination and presents a facile and inexpensive method of augmenting contaminated sites with selected microbial consortia for bioremediation.

* Corresponding author.
E-mail address: damien.mccarthy@students.mq.edu.au (D. McCarthy).

http://dx.doi.org/10.1016/j.chemosphere.2017.06.051
0045-6535/

1. Introduction

Mercury (Hg) pollution constitutes a significant global problem, presenting serious risks to the health of both the environment and humans. Management of this risk is particularly problematic as the environmental fate of released Hg is not yet fully understood (Qianrui et al., 2004; Rutkowska et al., 2014).

Mercury in contaminated soils is subject to complex geochemical cycling, as well as biotic cycling, the pathways of which are heavily dependent on local environmental factors. This biogeochemical cycle can lead to the formation of highly toxic methylated mercuric compounds, which may enter the food-chain, particularly in aquatic environments (Selin, 2009; Zhang et al., 2010). Methylating microbes involved include ubiquitous sulphur and iron reducing bacteria (SRB and FeRB respectively), as well as methanogens and firmicutes (Trevors, 1986). Although methyl mercury represents a small fraction of mercury compounds, its toxicity demands attention to remediating legacy Hg polluted waste streams.

In recognition of the global issue of mercury pollution, the United Nations Environmental Programme (UNEP) 2013 Minamata Convention on Mercury (www.mercuryconvention.org) was designed to exert pressure on nation states to address contamination. The treaty calls for emission reduction, waste stream remediation, and enhanced mercury monitoring in human populations, wildlife, and the environment.

Mercury contaminated soils from industrial and mining activities present an opportunity for legacy pollution amelioration as the disbursal is somewhat attenuated by soils, at least temporarily, allowing for direct intervention. Much research has been directed at this problem, with some success (Wang et al., 2012). However, many physicochemical strategies are often destructive to soils, require engineering and major ground works, and are constrained by cost and site specific logistical issues, such as geographical location and the composition and character of *in situ* soils and surroundings. No universally effective physicochemical method that is both inexpensive and non-detrimental to soils has been developed to date.

Although Hg is a naturally occurring element, with no known biological function, many organisms have evolved strategies to modulate the metal toxicity of their surroundings (Fox and Walsh, 1982; Saouter et al., 1995; Barkay and Wagner-Dobler, 2005). Bio-inspired strategies for mercury stress has seen research efforts shift from physicochemical treatments to bioremediation, although relatively few field trial successes have been noted to date. One bio-inspired approach, and the focus of this research, is Hg volatilisation mediated by microorganisms. Many microorganisms can detoxify their surroundings by translocation of contaminants from the soil, including metals, in a process whereby toxicants are volatilised by intracellular enzymatic processes and released via passive diffusion (Meagher, 2000). Selenium, arsenic and Hg are examples of metals that are transported from the soil back to the atmosphere in this way. In relation to Hg, many microorganisms can achieve such volatilisation, a process genetically mediated by the well characterised *mer* operon containing a cluster of genes responsible for cellular transport and reduction of oxidised Hg^{2+} to Hg^0, whereupon it naturally volatilises and diffuses from the cell, then from the soil back into the atmosphere as GEM. The organism used in this work, *Pseudomonas veronii*, is a non-pathogen endemic to soil that carries the *mer* operon.

One problem with previous approaches using microbial bioremediation relates to the logistics of the approach. Mercury contamination is scattered over thousands of sites worldwide and bioaugmenting soils with microbial consortia to deal with this pollution is logistically very difficult. Immobilising cells on a suitable transportable substrate might help overcome this issue and provide a much simpler bioaugmenting delivery strategy. Natural zeolites are a relatively inexpensive aluminosilicate mineral found in Australia and elsewhere, which have the extraordinary ability to absorb, hold, release, and exchange different chemicals, nutrients, toxins and ions. The surface topology of zeolites results in high surface area-to-volume ratios, allowing for very high cell density immobilisation in a small volume. Biopolymers of varying excipient makeup are well characterised in the literature as being suitable immobilising matrices for long term survival of cells (Cassidy et al., 1996). The aim of this work was to assess the efficacy of immobilising a *mer* operon carrying microorganism on a natural bulk substrate like zeolite using a xanthan gum-based biopolymer, the product of which was then applied to mercury contaminated soil. The combination of a zeolite solid immobilisation matrix and a xantham gum-based biopolymer resulted in a product can then be stored and transported readily, providing an ideal delivery mechanism to contaminated sites.

2. Materials and methods

Miller's Luria-Bertani (LB) base medium was purchased from Invitrogen and bacteriological agar from Sigma-Aldrich. Xanthan gum was purchased from Danisco (Nanyang, China) and Lupi Extra Virgin Olive Oil (Italy) from a food supply retailer. A granular (2–4 mm) natural zeolite (Zeolite Australia, Werris Creek, Australia) containing clinoptilolite and minor amounts of mordenite was used throughout the study. The cation-exchange capacity for the zeolite was 120 cmolc kg^{-1} with a hardness of 7 Mohs as reported by the supplier. Flux was measured in material obtained from central Nevada, Twin Creeks mine (41°15′ N, 117°9′ W, 1560 m), operated by Newmont Mining Corporation, with no pretreatment. Cell viability was measured in naturally mercury-enriched soil from Pulganbar, NSW, Australia, that was sterilised by gamma irradiation at a dose of 50 kGy (Steritech Pty Ltd, Wetherill Park, NSW, Australia). This material was homogenised into a whole bulk sample using Rotator/Endecott Test Sieve Shaker, with two sieves (2 mm and 19 mm) to obtain three size fractions, which were pulverised using a bar mill, and reintroduced according to dry weight ratio using a sample splitter. Sterility of the irradiated soil was confirmed by plating soil dilutions onto LB-Miller agar. Soil was considered sterile if no colonies were observed after 48 h of incubation at 28 °C.

2.1. Zeolite preparation

In order to remove fine particulate matter and sterilise the zeolite, 2–4 mm granule size zeolite was placed in a sieve (mesh size 20 μm), and washed under running hot water for 10 min. The zeolite was allowed to drain for 10 min and the procedure was repeated once, followed by autoclaving at 121 °C for 60 min. The zeolite was dried at 120 °C for five days and autoclaved once more. The autoclaved zeolite was then dried at 180 °C for 48 h and allowed to cool at room temperature before storage.

2.2. Zeolite bio-functionalisation

Pseudomonas veronii (strain CIP 104663) was cultured in liquid LB media at 28 °C with shaking at 85 rpm, and the optical density of the bacterial culture at 600 nm (OD_{600}) was monitored until the beginning of stationary phase began (18–24 h), and then incubation was stopped.

Biopolymer-mediated coating: P. veronii was coated to onto the zeolite as described by Swaminathan and Jackson (2008), and modified by Stelting et al. (2012 and 2014). Briefly, the zeolite granules (2–4 mm) were rehydrated with 4% (w/w) sterile water

and then a sample of *P. veronii* culture containing 4% (w/w) each of xanthan gum and olive oil was applied at a ratio of 1:25 to zeolite. The biopolymer was evenly distributed onto the zeolite surface after gentle tumbling the material for 10 min.

Biopolymer-free coating: P. veronii was coated onto the zeolite directly (without biopolymer) from an early stationary phase culture prepared as described above.

The biofunctionalised zeolite used in the flux experiments was prepared in Australia and stored in ambient conditions in sealed plastic containers for a period of six weeks before transport to the USA. No special treatment was required for transportation or storage, other than using sealed containers to avoid potential contamination.

2.3. Viability of *P. veronii* on low Hg contaminated soil

The viability of *P. veronii* on Hg contaminated soil was assessed using a previously sterilised soil sample containing 55 μg kg^{-1} Hg. Soil samples were mixed at a ratio of 1:1 (v/v) with either *P. veronii* immobilised onto zeolite with or without the biopolymer, and *P. veronii* cells without zeolite. Soil formulations were stored in airtight plastic containers at room temperature and viable counts were determined at the time of sample preparation and weekly thereafter for over nine weeks.

2.4. Viable counts

Viable cell counts were assessed by mixing 1 g (dry weight) of soil sample with 9 mL of 0.1 M phosphate buffer (pH 7.2). The cells were extracted by mechanical shaking using a multi-wrist shaker (Burrell Corp., Pittsburgh, PA) at maximum speed for 10 min. The sample was allowed to stand for 10 min to allow particulates to settle out followed by serial dilution (10^{-3}–10^{-6}) using the same buffer to facilitate obtaining a plate that could be manually enumerated. Dilutions (volume 10 μL, in triplicate) were plated out on LB agar using the tilt plate technique as described by Carvalhal et al. (1991). Plates were incubated at 28 °C and inspected at 24 h. Averages of the 3 readings per plate were used to calculate viable cell counts (cfu g^{-1}). The same method was used for viable counts on stored bio-functionalised zeolite.

2.5. GEM/GOM flux measurements

Mine tailings containing 7 mg kg^{-1} total mercury was mixed 1:1 (v/v) with biopolymer mediated bio-functionalised zeolite granules and watered with sufficient quantity to 15% or 50% volumetric soil moisture (VSM). As control, a similarly treated sample of sterile zeolite granules was used. The experimental set up for flux measurements was similar to that described by Miller and Gustin (2013) and shown in Fig. 1. Both GEM and gaseous oxidised mercury (GOM) air-surface exchange were measured. GEM concentrations were measured using a Tekran 2537 A analyser, with chamber inlet and outlet samples taken in sequential 10 min intervals. Hg flux was calculated for 20 min periods using:

$$F = Q \times \Delta C/A$$

where F = total flux (ng m^{-2} h^{-1}), Q = sample flow rate (m^3 h^{-1}), A is the chamber footprint (m^2), and ΔC is the difference between outlet and inlet samples (ng m^{-3}) as per Eckley et al. (2010). GOM fluxes were measured using polyethersulfone cation exchange membranes (CEM; Mustang S, Pall Corporation) over 281 h. Cation exchange membranes were subsequently analysed using EPA Method 1631 (US EPA, 2002) to quantify GOM as per Gustin et al. (2013). Total Hg concentrations were measured by Atomic Absorbance Spectroscopy (AAS) before and after the trial using a Milestone DMA-80 Direct Mercury Analyser with San Joaquin Soil SRM 2709a used as reference material.

3. Results and discussion

3.1. *P. veronni* viability

P. veronii encapsulated in biopolymer and immobilised onto zeolite was mixed with sterile Hg-contaminated soil (55 ppb Hg). Under these conditions, the viability of *P. veronii* showed a gradual decline in cell numbers from 10^9 to 10^6 cfu mL^{-1} followed by stabilisation around this population size (Fig. 2). Furthermore, after four months, the number remained stable at 10^6 cfu mL^{-1} (data not shown). In contrast, *P. veronii* cells directly applied to the same soil showed a rapid viability decline, followed by slight gradual increase in numbers of about 2 log units followed again by rapid decline. *Pseudomonas veronii* cells that absorbed straight onto zeolite without biopolymer and applied to the Hg-contaminated soil showed an immediate and steady decrease, which led to total population collapse.

Stelting et al. (2014) showed that a biopolymer immobilised *Pseudomonas* strain ADP remained viable for 10 weeks at 25 °C. Generally, a 1–2 log cfu decline was noted followed by maintenance of viability, even when applied to soils, although in previous studies the population stabilised earlier (Stelting et al., 2012, 2014).

P. veronii cells applied directly to the Hg-contaminated soil without zeolite displayed an initial rapid decline in the first 3 weeks, but then a partial increase was observed. This might be due to the lack of competition in the sterile soil that may have influenced the population's ability to rapidly establish. The decline in viability toward the last 2 weeks may be explained by an increasing fungal competition from airborne contamination that was noted towards the end of the experimental period.

Biopolymer-mediated immobilisation of *P. veronii* onto bulk natural zeolite offers several advantages over other substrates, notwithstanding the fact those substrates have been shown to be effective. Besides zeolites, the two most promising cell immobilising techniques for bioremediation is the use of live cells in alginate beads (Sinhaa et al., 2012; Giovanella et al., 2015), or co-cultured organisms bound to an organic waste substrate, such as cocopeat (Nunal et al., 2014). These approaches offer several advantages from a logistical and environmental perspective in that alginate itself acts as the delivery substrate, is fully biodegradable, and has been shown to be effective in immobilising organisms with retained viability and functionality, while is storable and transportable. However, they are more expensive and difficult to prepare. Co-cultured products offer environmental advantages and additionally, theoretical ease of preparation as it is a one-batch process. However, the quantity required is the key problem, as it is the microbial load that is ultimately going to determine efficacy of this remedial approach.

Immobilising cells onto zeolites using a biopolymer seems to offer advantages over these other approaches in that it is a facile technique proven to sustain cell viability for sufficient time allowing delivery to sites and for high volumes of cells to be delivered using a small volume of end product.

The seemingly rapid decline of cell viability over seven days is likely simply due to the very high Hg concentration of the tested mine tailings, which was approximately 7 mg kg^{-1} Hg compared to global averages for soil of approximately 8 μg kg^{-1} Hg. It should be noted that in this work, no attempt was made to find a bacterial strain with very high tolerance to Hg, and further research is required to find an appropriately tolerant strain carrying the *mer* operon that may also be amenable to immobilisation. Due to

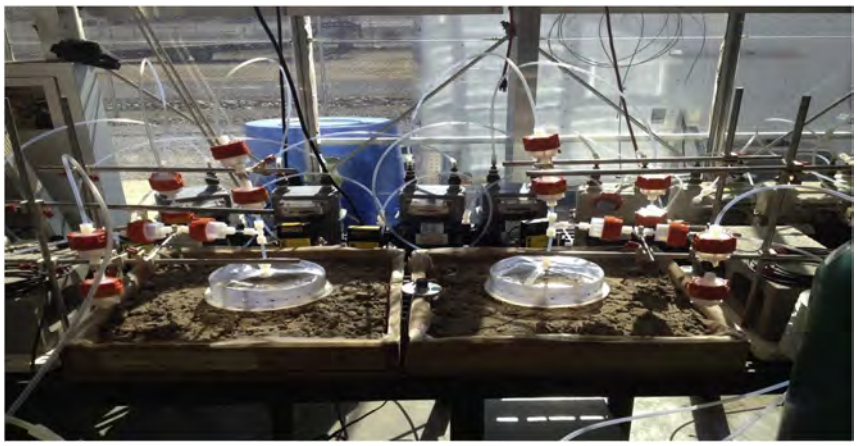


Fig. 1. Mercury dynamic flux chamber set up. Flux was measured using a Tekran 2537 A Hg analyser (0.1 ng m^{-3} LDL) with a Tekran Automated Dual Sampling Unit (TADS) attached to cylindrical Teflon dynamic flux chambers via a 2 m Teflon tubing (a full description can be found in Eckley et al., 2010). Trays were separated by several metres during experiment to avoid cross contamination.

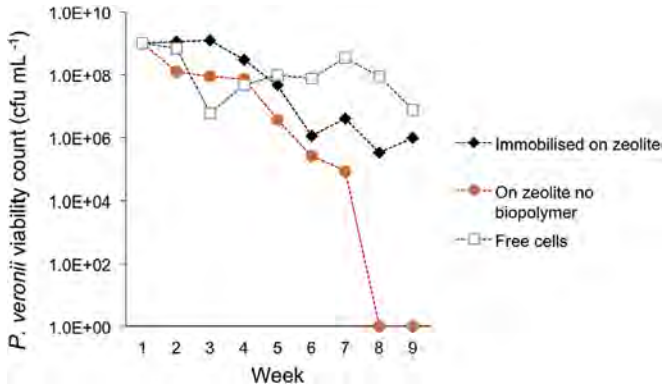


Fig. 2. Viable count of *P. veronii* over a 9-week treatment of dry sterile soil containing relatively low Hg concentration (55 ppb).

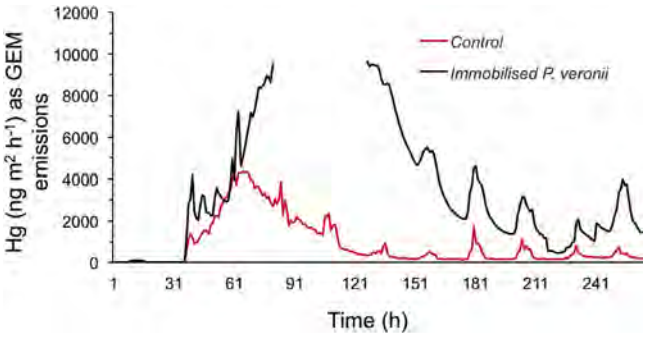


Fig. 3. GEM emissions after application of zeolite-immobilised *P. veronii*. 15% (v/v) water was added to samples once at beginning of the experiment. Background emissions were positive flux of about 2 ng Hg m^2 h^{-1}. The application of zeolite-immobilised cells increased this rate by several orders of magnitude to beyond 3500 ng m^2 h^{-1}. A diel cycle is noted with daylight corresponding to higher emission rates.

bacterial community dynamics, it is preferable that suitable niche communities are also identified that may be applied as consortia rather than applying single microbial strategies which tend to fail unless in bioreactors and similar. However, this aspect requires extensive bioinformatics and soil characterisation across a wide geographical subset to obtain the true community dynamics of Hg contaminated soils such as may be required for successful *in situ* remediation across the spectrum of differing contaminated soil scenarios. It would be prudent to research optimised conditions for *P. veronii* in any event.

3.2. Functionality

In terms of functionality, the application of zeolite with biopolymer immobilised *P. veronii* resulted in a sharp increase in GEM emissions, with peak emissions exceeding 3 μg Hg m^2 h^{-1} for 15% water treatment (Fig. 3) and for 50% water (Fig. 4), emissions exceeded at least 10 μg Hg m^2 h^{-1}, although probably much higher, but unfortunately readings were above the upper detection limit of the instruments. The baseline control consisted of a duplicate contaminated material with sterile zeolite added but without cells. Considering background emissions were roughly 1 ng Hg m^2 h^{-1}, this represents a dramatic GEM increase using bio-functionalised zeolite. Increased GEM positive flux was detectable over the 12-day period of the experiment. There was also a large concomitant decrease (>2 orders of magnitude) in GOM flux. This was not anticipated and the authors are unaware that this observation has been made before in relation to reactive mercury flux, nor are the authors aware of direct Hg flux measurements being taken from bio-remediation trials of this nature. These data accord well with the basis of the remediation mechanism proposed in that Hg^{2+} is

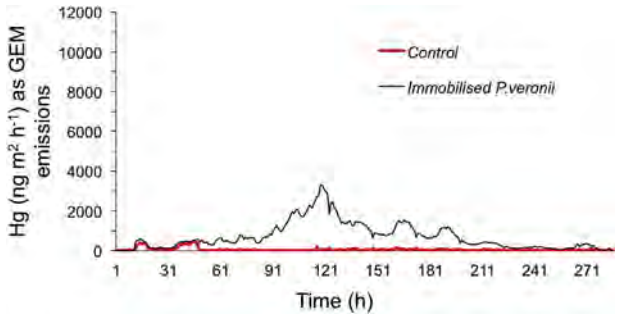


Fig. 4. GEM emissions after application of zeolite-immobilised *P. veronii*. 50% (v/v) water was added to samples once at the beginning of experiment. Background emissions were positive flux of about 2 ng Hg m^2 h^{-1}. A smaller flux from the addition of water (red) needs to be recognised as contributing to overall flux. A diel cycle is again noted. There is a gap in the data due to the higher than anticipated flux exceeding the upper detection level of the instruments.

taken up by *P. veronii*, thereby reducing the overall amount, which may passively escape as GOM. This tends to imply the take up of oxidised mercury by the organism is active rather than passive, as a greater proportion of GOM than was observed would be expected to be emitted if the Hg-microbial interaction were purely random.

The application of zeolite-immobilised cells increased this rate by several orders of magnitude to beyond 10^4 ng m^2 h^{-1}. The emitted GEM obviates the need for a capture mechanism for the liberated gaseous Hg, and several existing technologies could theoretically be employed for this purpose. These include exhaust extraction whereby emitted GEM is extracted and cooled such that metallic Hg is formed for subsequent removal, or by exhaust extraction followed by mechanical processing such as developed by the US Environmental Protection Agency (US EPA) for smaller scale operations with the use of their Gold Shop Mercury Capture System (MCS, US EPA, 2017). Alternatively, a bulk capping material such as activated carbon could be employed, as this is known to have GEM capture characteristics, however this approach would require subsequent removal and treatment of the carbon substrate. One promising technique currently under investigation is the use of a fibrous geotextile, such as a mat, with GEM capture capability that could readily be deployed over the area being remediated and simply rolled up and removed after Hg concentrations in the soil were reduced to acceptable levels. The geotextile could then be easily removed and treated off-site to allow for removal and storage of captured Hg, and potentially re-use of the geotextile.

Quite apart from the GEM increased flux, results for gaseous oxidised mercury (GOM) flux tests (Fig. 5.) using the CEM filters showed a concomitant large fall in GOM after treatment with zeolite-immobilised cells. This strongly indicates the successful capture of much of the oxidised mercury by *P. veronii* cells and subsequent intracellular reduction to GEM. GOM flux from Hg contaminated soil (7 mg kg^{-1} Hg) treated with sterile zeolite was 1863 pg Hg m^2 h^{-1} compared to 186 pg Hg m^2 h^{-1} for soil treated with bio-functionalised zeolite in the 15% water treatments. GOM for untreated material was not measured in this experiment, however previous work by Miller and Gustin (2013) alludes to untreated materials. The same material was used in this experiment, however, the time between this and the 2013 experiment rendered any meaningful comparison of GOM too difficult.

For gaseous elemental mercury, the result seems to confirm that volatilisation is the mechanism for soil Hg reduction by the immobilised cells used in this research. It is hard to explain any other reason for the dramatic flux increase of GEM. Emissions were not uniform and fluctuated in a seemingly regular pattern of increased then reduced GEM emission rates, the intensity of which gradually reduced over time. This could be due to diel cycles where bacterial activity is influenced by temperature – increased daytime temperature causes increased biological activity and hence increased Hg reduction. Although no cell viability measurements were taken in this contaminated soil experiment, it is assumed increased temperature alone does not account for increased GEM emissions, as this would have been evident in the control soil that showed no such fluctuation pattern, and so increased bacterial activity must be taking place.

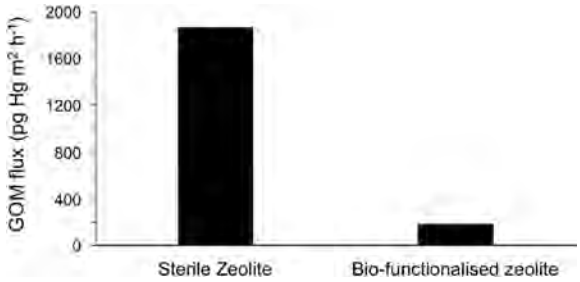


Fig. 5. Gaseous oxidised mercury (GOM) emissions. GOM emissions from Hg contaminated soil showed a considerable decrease when treated with zeolite-immobilised cells when compared to sterile zeolite only (no cells) treatment.

3.3. General considerations

The bench scale experimental set up did not take account of flooding and seasonal dynamics, such as might be encountered in natural settings. Rinklebe et al. (2009) showed the significance of such factors in Hg flux rates, as well as soil composition and vegetation cover, which would need consideration in any kinetic studies using this bioremediation technique. Further, the material used in this study was mixed (but not sieved) to produce replicate samples for the experiments, however in natural settings this would not be the case. During et al. (2009) showed that Hg flux was spatially variable even at small distances due to soil heterogeneity, for example in terms of pore size distributions, substrate characteristics including particle size and type, and organic matter constituents.

An important aspect for bioremediation practitioners is the continuous management of remediated sites, and these could readily be monitored by the simplified method developed by Böhme et al. (2005) and optimised by Rinklebe et al. (2008) using flux chambers that can be deployed on site without the need for stationary AAS equipment such as used in this method. Further, their work showed that background emissions can be measured without the need for a switching unit between inflow and outflow for flux chambers, greatly simplifying the measuring techniques. Their method provides a very practical tool for remediation practitioners for continuous Hg flux measurement of sites under remediation.

4. Conclusions

This work focused on establishing the effect on mine tailings Hg concentrations by augmenting soil with organisms having Hg^{2+} reduction and volatilisation capacity after biopolymer encapsulation and immobilisation onto a natural zeolite substrate. This method shows great promise; it was demonstrated that cells could be stored for at least four months and transported intercontinentally without loss of functionality of the immobilised cells. Increased GEM emissions using immobilised *P. veronii* were 10^4 above background levels. The biogeochemical cycling of Hg that potentially leads to highly toxic derivatives must be interrupted by not only isolation but also removal of Hg. In this context, immobilised cells that volatilise Hg promise an alternative to overcome at least some of these issues, as the technique employed in this research liberates Hg from soils and can easily be scaled up at low cost and with low technical expertise required on the ground for delivery. The overall objective was to increase GEM emissions from contaminated soils to facilitate remediation of contaminated soils and mine waste streams. Further research is required to elucidate a suitable emitted GEM capture mechanism that breaks the biogeochemical cycle of Hg.

Acknowledgements

The authors would like to thank Newmont mining for their kind provision of mine tailings material; the Gustin Mercury Research Laboratory at the University of Nevada, Reno; Zeolite Australia Pty Ltd for provision of zeolite granules. Support for this work came from Macquarie University and the United States National Science Foundation grant number 629679.

References

Barkay, T., Wagner-Dobler, I., 2005. Microbial transformations of mercury: potentials, challenges, and achievements in controlling mercury toxicity in the environment. Adv. Appl. Microbiol. 57, 1–52.

Böhme, F., Rinklebe, J., Stärk, H.J., Wennrich, R., Mothes, S., Neue, H.U., 2005. A simple field method to determine mercury volatilization from soils. Environ. Sci. Pollut. Res. Int. 12, 133–135.

Carvalhal, M.L.C., Oliveira, M.S., Alterthum, F., 1991. An economical and time saving alternative to the most-probable-number method for the enumeration of microorganisms. J. Microbiol. Methods 14, 165–170.

Cassidy, M., Lee, H., Trevors, J., 1996. Environmental applications of immobilized microbial cells: a review. J. Microbiol. 16, 79–101.

During, A., Rinklebe, J., Böhme, F., Wennrich, R., Stärk, H.J., Mothes, S., Laing, G.D., Schulz, E., Neue, H.U., 2009. Mercury volatilization from three floodplain soils at the Central Elbe River, Germany. Soil Sediment. Contam. 18, 429–444.

Eckley, C.S., Gustin, M., Lin, C.-J., Li, X., Miller, M.B., 2010. The influence of dynamic chamber design and operating parameters on calculated surface to air mercury fluxes. Atmos. Environ. 44, 194–203.

Fox, B., Walsh, C., 1982. Mercuric reductase. Purification and characterization of a transposon – encoded flavoprotein containing an oxidation-reduction-active disulphide. J. Biol. Chem. 257, 2498–2503.

Giovanella, P., Costa, A.P., Schäffer, N., Peralba, M.C.R., Camargo, F.A.O., Bento, F.M., 2015. Detoxification of mercury by bacteria using crude glycerol from biodiesel as a carbon source. Water Air Soil Pollut. 226, 224.

Gustin, M.S., Huang, J., Miller, M.B., Peterson, C., Jaffe, D.A., Ambrose, J., Finley, B.D., Lyman, S.N., Call, K., Talbot, R., Feddersen, D., Mao, H., Lindberg, S.E., 2013. Do we understand what the mercury speciation instruments are actually measuring? Results of RAMIX. Environ. Sci. Technol. 47, 7295–7306.

Meagher, R., 2000. Phytoremediation of toxic elemental and organic pollutants. Curr. Opin. Plant Biol. 3, 153–162.

Miller, M.B., Gustin, M.S., 2013. Testing and modelling the influence of reclamation and control methods for reducing nonpoint mercury emissions associated with industrial open pit gold mines. J. Air Waste Manag. Assoc. 63, 681–693.

Nunal, S., Mae, S., Santander-de-leon, S., Bacolod, E., Koyama, J., Uno, S., Hidaka, M., Yoshikawa, T., Maeda, H., 2014. Bioremediation of heavily oil-polluted seawater by a bacterial consortium immobilized in cocopeat and rice hull powder. Biocontrol Sci. 19, 11–22.

Qianrui, W., Daekeun, K., Dionysios, D., Dionysiou, G., Sorial, A., Timberlake, D., 2004. Sources and remediation for mercury contamination in aquatic systems: a literature review. Environ. Pollut. 131, 323–366.

Rinklebe, J., During, A., Overesch, M., Wennrich, R., Stärk, H.J., Mothes, S., Neue, H.U., 2008. Optimization of a simple field method to determine mercury volatilization from soils – examples of 13 sites in floodplain ecosystems at the Elbe River (Germany). Ecol. Eng. 35, 319–328.

Rinklebe, J., During, A., Overesch, M., Laing, G.D., Wennrich, R., Stärk, H.J., Mothes, S., 2009. Dynamics of mercury fluxes and their controlling factors in large Hg-polluted floodplain areas. Environ. Pollut. 158, 308–318.

Rutkowska, M., Dubalska, K., Bajger-Nowak, G., Konieczka, P., Namieśnik, J., 2014. Organomercury compounds in environmental samples: emission sources, toxicity, environmental fate, and determination. Crit. Rev. Env. Sci. Technol. 44, 638–704.

Saouter, E., Gillman, M., Turner, R., Barkay, T., 1995. Development and field validation of a microcosm to simulate the mercury cycle in a contaminated pond. Environ. Toxicol. Chem. 14, 69–77.

Selin, N., 2009. Global biogeochemical cycling of mercury: a review. Annu. Rev. Environ. Resour. 34, 43–63.

Sinhaa, A., Pantb, K., Kharea, S., 2012. Studies on mercury bioremediation by alginate immobilized mercury tolerant *Bacillus cereus* cells. Int. Biodeterior. Biodegrad. 71, 1–8.

Stelting, S., Burns, R., Sunna, A., Visnovsky, G., Bunt, C., 2012. Immobilization of *Pseudomonas* sp. strain ADP: a stable inoculant for the bioremediation of atrazine. App. Clay Sci. 64, 90–93.

Stelting, S., Burns, R., Sunna, A., Bunt, C., 2014. Survival in sterile soil and atrazine degradation of *Pseudomonas* sp. Strain ADP immobilized on zeolite. Bioremediat. J. 18, 309–316.

Swaminathan, J., Jackson, T.A. A composition to improve delivery of an active agent. International Patent WO2008023999; 2008.

Trevors, J.T., 1986. Mercury methylation by bacteria. J. Basic Microbiol. 26, 499–504.

United States Environmental Protection Agency, 2002. US EPA: Method 1631: Revision E, Mercury in Water by Oxidation, Purge and Trap, and Cold Vapor Atomic Fluorescence Spectrometry, pp. 1–33.

United States Environmental Protection Agency, 2017. Reducing Mercury Pollution from Artisanal and Small-scale Gold Mining. https://www.epa.gov/international-cooperation/reducing-mercury-pollution-artisanal-and-small-scale-gold-mining (Retrieved May 2017).

Wang, J., Feng, X., Anderson, C.W., Xing, Y., Shang, L., 2012. Remediation of mercury contaminated sites – a review. J. Hazard. Mater. 221, 1–18.

Zhang, H., Feng, X., Larssen, T., Shang, L., Vogt, R., Lin, Y., Li, P., Zhang, H.G., 2010. Fractionation, distribution and transport of mercury in rivers and tributaries around Wanshan Hg mining district, Guizhou Province, Southwestern China: Part 2-Methylmercury. Appl. Geochem. 25, 642–649.

Chapter 5 – Research paper 2

5.0 Statement of contribution

Damien McCarthy - Concept and design of product, background research, conceptual design of experiments, experimental work, data analysis, instrument set up and calibration, manuscript production

Grant C Edwards – Concept and design of experiments, manuscript editing, oversight.

5.1 Gaseous mercury capture by coir fibre coated with a metal-halide.

Damien N. McCarthy [a,*], Grant C. Edwards [a]

[a] Department of Environmental Sciences, Faculty of Science and Engineering, Macquarie University, Sydney, NSW 2109, Australia.

* Corresponding author:

Damien McCarthy

Department of Environmental Sciences

Faculty of Science and Engineering

Macquarie University, Sydney, NSW, 2109

Australia

Phone: +61 0415 126949

Fax: +61 2 9850 8420

Email: damien.mccarthy@hdr.mq.edu.au

Abstract

Toxic gaseous elemental mercury (GEM) is emitted to the atmosphere through a variety of anthropogenic and natural routes at rates estimated at over 5000 tonnes per annum, a large fraction of which is Anthropogenic. It is then widely disbursed atmospherically and eventually deposited, where it is subject to further biogeochemical cycling, including re-emission. Research into capture of point source mercury emissions revolves almost exclusively around the use of activated carbons, various catalytic oxidation substrates, or as a by-product of acidic treatments of flue gas during SOx and NOx reduction methods. GEM is very non-reactive in its native state, but capture rates are greatly enhanced if GEM is first oxidised, or at least where oxidation states play a role at the substrate GEM interface. Little research has been devoted to capture of GEM directly, however, presented here is a novel adaption of coir fibres for use as a substrate in capturing GEM emissions directly. Various coir modifications were investigated, with the most effective being fibres coated with CuI crystals dispersed in a non-crosslinked poly-siloxane matrix. Scanning electron microscopy was used to view surface morphologies, and sorbtion characteristics were measured using atomic absorption spectroscopy (AAS). These results indicate that coir fibres modified by CuI-$[SiO_2]_n$ show great promise in their ability to efficiently sorb GEM, and could potentially be utilised in a variety of configurations and settings where GEM emissions need to be captured.

Keywords

Coconut fibre, volatilisation, bioremediation, environmental toxicology, atmospheric pollution, adsorbtion.

1. Introduction

Gaseous elemental mercury (GEM) capture is difficult due to the physical properties of this particular heavy metal. For example, mercury (Hg) has an extremely high surface tension of 485.5 mN m^{-1} at standard temperature and pressure, which greatly reduces solubility. The very high volatility of elemental mercury ($Hg^0_{(g)}$) is due to quantum effects caused by its unique e^- subshell configuration (Norrby, 1991). As Hg resides atmospherically mainly in gaseous form rather than as a particulate (Hg_p), long-range transport is made possible by extended atmospheric times of > 1 yr (Slemr *et al*., 1985), and in particular due to its interaction with tropospheric bromine (Amos *et al.*, 2013). This toxic element is subsequently transformed into more reactive species upon photo-oxidation, and reactions with atmospheric halogens. This compares to other metals that predominate as particulates and have shortened atmospheric residence times in the range of hours to months (Galloway *et al*., 1982), although the picture is somewhat complicated by particulate matter size, season, and re-emission factors such as wind (Prabhakar *et al.*, 2014). The long-range transport of Hg causes widespread environmental toxicology impacts, enhanced by biogeochemical cycling.

The disbursal of GEM can be somewhat attenuated by point source emission reduction and capture such as is used for coal-fired power station flue gasses (activated carbons and other scrubber technologies). However, legacy pollution stemming from industrial uses of Hg dating back millennia remains an ongoing problem for environmental remediation specialists. Direct treatment of soils can be difficult and destructive. Treatments such as volatilising Hg by thermal stimulation can be effective and relatively

harmless to soils but the resultant GEM has to be captured. This can then be cooled to facilitate a return to metallic Hg but cannot be readily achieved for *in situ* treatments, and may require complete excavation of sites and transport to treatment facilities. However, GEM emissions can be increased via biological methods, such as the addition of $Hg^{2+} \rightarrow Hg^0$ reducing bacteria, making this a practical route for remediation (Dash and Das, 2012), but is again dependent on suitable capture methods.

Most current research into GEM capture centres around technologies developed for flue gas emissions from coal combustion. Although the Hg content of coal on average is low at about 0.5mg kg^{-1} (Finkelman, 1981), the sheer volume of coal combusted means large volumes of Hg are released atmospherically by this process. The main areas of active research are in catalytic oxidation of Hg^0 using fly ashes and solid state metal catalysts, combined with particle scrubbers and membrane type technologies. Such technologies include acid and solid infusion-based technologies to either solubilise Hg, or to provide a binding surface on particulates, respectively, for subsequent capture in membranes and filters (Gao *et al*., 2013). Little research is aimed at direct capture of gaseous Hg^0 in ambient conditions using sustainable technology, as is presented in the current study.

The use of renewable resources for environmental remediation of heavy metals makes sense from an economic and environmental perspective. Coir is derived from the mesocarp of germinated fruit of the coconut palm (*Cocos nucifera*), and is a readily available renewable resource available in bulk at low cost, and has useful properties such as high tensile and flexural strength. It can readily be made into rope and matting by facile mechanical processes and is widely used as a geotextile. However, when employed as a geotextile, being cellulosic, coir is prone to chemical and biological degradation over time mediated by fungi, bacteria, and substrate characteristics (Balan,

1995; Lekha, 2004). This degradation can be reduced by various fibre surface modifications and other treatments (Nicholas, 1982; Sumi, *et al*., 2016).

Surface modifications can also be beneficial for matrix adhesiveness if one desires fibre coatings, or for fibre use in polymer composites. For example, cellulosic fibres have surface waxes and oils, interfering with fibre matrix bonding, but these can be removed or reduced with various treatments such as alkali baths (Chandrasekar *et al*., 2017). Alkali treatment also increases fibrillation, which can increase surface area interlocking of matrix and fibre surfaces, and interfacial bonding is also improved by increased surface roughness (Liu *et al*., 2004; Punyamurthy *et al*., 2012). The challenge is to modify coir in such a way that it has GEM capture characteristics while reducing its tendency to degrade when applied in environmental or industrial settings.

The current work presents a facile and robust method of GEM capture using coir fibre coated with polymeric siloxane harbouring CuI crystals that forms solid and insoluble copper(I) tetra-iodide mercurate when exposed to GEM in ambient conditions.

2. Materials and Methods

2.0 Fibre pre-treatment

Coir fibre was obtained in pre-made mat form of 5mm thickness from a local supermarket, and subsequently cut into appropriate size fractions or prized apart. Fibres were soaked in dH_2O for 2 h, then rinsed under copious amounts of hot water to remove any grit and other particulates, followed by autoclaving at 121°C for 1 h, and thoroughly dried in a fume-hood for 48 h. All fibres not being immediately used were stored in sealed polypropylene bags.

2.1 Soxhlet extraction and alkaline treatment

Naturally occurring waxes and oils were removed by Soxhlet extraction prior to any other modifications. Fibres were added to a Soxhlet thimble and 100% C_3H_6O solvent was gently boiled for 24 h. Fibres were then washed under copious amounts of dH_2O and allowed to dry in a fume-hood for 24 h. A second solvent of 1:2 solution of 70% ethyl acetate (EtOH)(Fisher Scientific [E12420]) and 100% C_6H_6, was used for 48 h, after which time the fibre was washed and dried as before. Dried fibres were totally submerged in each of 5% and 10% $^{w/v}$ solutions respectively of dissolved NaOH (Fisher Scientific as pellets [Lot 124515]) at 25°C, and gently agitated on a VWR™ rocking platform for 1 h in sealed glass containers. Fibres were then washed under copious amounts of dH_2O and allowed to dry for 24 hours in a fume-hood.

2.2 Coir fibre modifications

2.2.1 Poly-isoprene

Pure latex liquid (cis-1,4 poly-isoprene) (Shintani Laboratory, University of Nevada, Reno, Nevada) was added to 200mL 100% tetrahydrofuran (THF) (Mallinckrodt Inc. (2858-06)) such that 1%, 5%, 10% and 50% $^{w/v}$ solutions were made. These were placed in sealed glass jars and gently agitated on a VWR™ rocking platform overnight to fully dissolve poly-isoprene. Untreated coir mat was cut into appropriate radius discs. The poly-isoprene plus THF solutions were then poured over the coir discs seated on glass trays, and placed in a fume-hood overnight to allow the THF to evaporate. A glass spreader was used to assist coating for the 50% solution due to high viscosity. The procedure was repeated with the addition of a mass equivalent to latex of either activated charcoal (Sigma-Aldrich [C6289]), or activated coconut pith (Fisher Scientific [5-690-A]) or CuI crystals (Strem Chemicals [93-2936]), with magnetic stirring during dissolution phase to keep particulates suspended.

2.2.2 Carboxymethylcellulose

Carboxymethylcellulose (CMC) sodium salt (Sigma-Aldrich [C-5103]) was slowly added to 200mL dH_2O in sufficient quantity to make 1%, 2%, 3% and 4% $^{w/v}$ solutions, that

were gently heated and stirred until CMC was fully dissolved. CMC was poured over the fibres seated in glass trays and placed in the fume-hood for 48 h to allow the water to evaporate. A glass spreader was needed for the 3% and 4% CMC solutions due to high viscosity. The procedure was repeated for the 4% CMC solution, with the addition of CuI crystals or biochars as previously described.

2.2.3 Siloxane

Siloxane (as Ge® Silicone II 100% silicone caulking gel) was added to 200mL ethyl acetate in sufficient quantity to make 1%, 5%, 10% and 50% $^{(w/v)}$ solutions, and stirred with a glass rod until silicone was dissolved and a homogenous mixture was obtained. The solutions were poured over the discs with the use of a glass spreader to ensure even coverage. The discs were placed in a fume-hood overnight to allow the ethyl acetate to evaporate. The procedure was repeated using the addition of CuI crystals and biochar as previously described. The ratio of ingredients can be summarised as 1:1:1 (SiO_2:CuI*:Coir $^{w/w}$). [i]

2.3 *Hg exposure and quantification*

2.3.1 GEM capture per formulation

Approximately 50g liquid Hg was placed in a small beaker seated in a water bath and heated to 50°C to facilitate off-gassing of GEM. Raw and NaOH treated fibres, as well as all other modified coir fibre samples were sequentially placed over the mouth of the beaker to allow for direct exposure to the GEM plume emanating from the beaker. Exposure time was 1 h, except for 50% siloxane-coated fibre with CuI, which was also exposed for 24 h. Post exposure, samples were placed in sterile 15mL tubes and kept away from sunlight and excessive heat until analysed using a pre-calibrated Milestone DMA-80 direct mercury analyser (EPA Method 7473) with reference material being NIST San Joaquin 1400 ± 80 ng g^{-1} Hg.

2.3.2 GEM capture per Siloxane plus CuI formulation

Liquid mercury in a Teflon flask was secured in a water bath heated to 50°C. Fibres were tightly packed in short lengths of polytetrafluoroethylene (PFTE) tubing that were snugly secured over the flask opening to facilitate exposure to GEM vapour in the headspace. Mercury content of exposed fibres was measured using the DMA-80. Surface area of top of liquid Hg was approximately 28 cm^2, with exposed front of samples about 5cm from the surface. A range of exposure times per sample were conducted. Samples were tested for mercury content using a DMA-80 direct mercury analyser. A second experiment measured real time Hg concentrations of vapour before and after passing through the treated fibres using an automatically calibrated Tekran® 2735X mercury vapour analyser, with exposure per sample 45 h. (Tekran®, Toronto, Canada) as depicted in Figure 1. Tubing and sample holders were made of 100% Teflon®. Fresh clean lines were installed at each iteration of the experiment to avoid contamination issues, with a period of 20 minutes allowed after each filter was put in place to ensure residual mercury vapour was purged from the line directly adjacent to the detector.

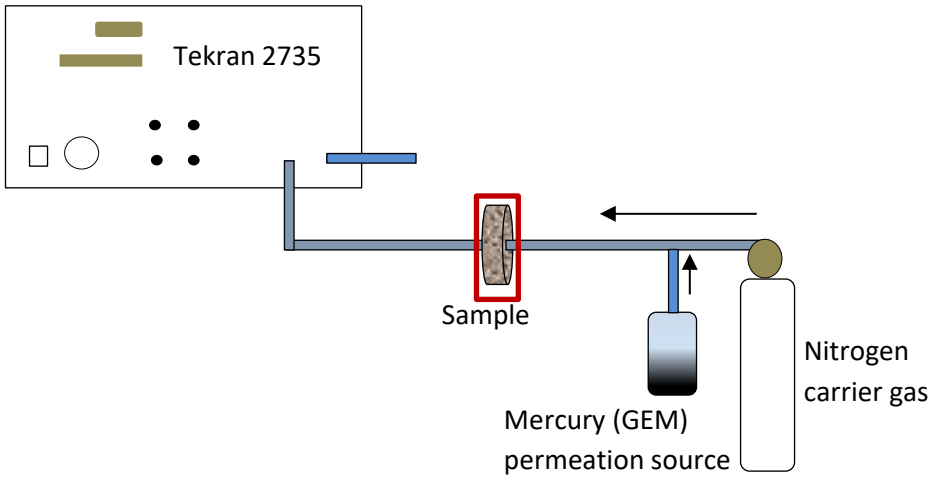


Figure 1. Abridged experimental set up using Tekran 2735X mercury vapour analyser. Permeation source was liquid mercury secured in a thermally controlled water bath at 50°C. Sample carrier gas was pure nitrogen. Tekran internal carrier gas was pure argon, with sample flow rate 0.5L min^{-1}, Samples dimensions were 28 cm^2 of lineally compressed 5mm thick discs of coir treated with non-cross linked polymeric siloxane and CuI.

2.4 Imagery

Scanning electron microscopy (SEM) was undertaken of platinum sputter coated samples using an S-4700 II Scanning Electron Microscope (Hitachi) at the Electron Microscopy & Microanalysis Facility, University of Nevada, Reno, Nevada.

3 Results and Discussion

3.1 *Coating and adhesion*

Poly-isoprene coated coir fibre was manually inspected to view coating homogeneity characteristics and to compare flexural changes between coated vs uncoated fibres. It was found that the most appropriate coating was a 10% solution of poly-isoprene dissolved in THF. The 1% solution did not tend to cover the fibres well enough. The 5% solution left some small portion of fibres uncoated (agitating the media while drying may resolve this, although this was not attempted here). The 10% solution easily coated all fibres, and did not leave pores in the matting poly-isoprene matrix through which GEM might escape without interacting with the fibres. All subsequent work used the 5 and 10% solutions.

The dissolved CMC proved difficult to work with in terms of suitable coating characteristics. It was difficult to obtain an even coating with all solutions below 4%, while the 4% solution, although producing an improved coating outcome, was very difficult to make and administer due to low solubility and high viscosity, respectively. All coatings were uneven and tended to coagulate upon drying rather than producing a homogenous coating. It was also noted that these CMC coatings became contaminated over time with bacterial or fungal micro-organisms. This is problematic in terms of end use of the product, because the concept of coating is not only to provide a matrix for GEM binding additives but also to protect fibres from degradation while employed as a geotextile. Although no attempt was made to identify contaminating species, the fact the CMC was so readily colonised suggests this is a poor surface coating for the intended purpose without further efforts to decrease susceptibility to contamination by micro-organisms.

Siloxane coatings were found to be the most suitable in any dilution in terms of homogeneity of the coating. However, the 1% and 5% solutions left pore spaces in the matrix through which GEM could pass without interacting with the fibres. The 10% siloxane solution proved the most effective in terms of overall coverage, and the flexural characteristics were little changed compared to the uncoated fibres in that the matting was not noticeably stiffer. However, it proved very difficult to retain CuI crystals in suspension using any concentrations during the solvent evaporation step as they tended to drop out of the solution and remained as detritus. The 50% solution proved better in retaining CuI crystals in solution, but was difficult to coat using pre-formed matting due to higher viscosity and so matting was teased apart prior to coating. This also had undesirable flexural and structural characteristics upon drying. Table 1 summarises the results. GEM capture is discussed in more detail in 3.2

Coating	Cover	Additive adhesiveness	Substrate adhesiveness	Fungal contamination	Surface morphology	GEM capture
(1)						
1%	Poor	Moderate	Poor	No	Smooth	-
5%	Moderate	Moderate	Moderate	No	Smooth	-
10%	Good	Moderate	Good	No	Smooth	-
(2)						
4%	Poor	Poor	Poor	Yes	Rough	-
(3)						
1%	Poor	Good	Good	No	Rough	-
5%	Moderate	Good	Good	No	Rough	-
10%	Good	Good	Good	No	Rough	Moderate
(4)	-	-	-	No	Smooth	Moderate
(5)	-	-	-	No	Rough	Low
(6)	Poor	Poor	Poor	No	Smooth	-
(7)	Poor	Poor	Poor	No	Rough	-
(8)a	Good	Good	Good	No	Rough	Good
(8)b	Good	Good	Good	No	Rough	Excellent
(8)c	Good	Good	Good	No	Rough	Excellent

Table 1. Summary results. Treatments as follows: (1) poly-isoprene, (2) CMC (3) poly-siloxane, (4) untreated fibres, (5) solvent and NaOH treated fibres, (6) 10% polyisoprene and biochar (7) 10% poly-siloxane and biochar, (8)(a) 10% poly-siloxane +0.5g CuI g^{-1} coir, (b) +0.1g CuI g^{-1} coir, (c) +2.0g CuI g^{-1} coir.

Irrespective of coverage, raw fibres tended to produce poor adhesion outcomes and some portion of the coatings could readily be removed by rubbing through fingers. In contrast, dewaxed and NaOH treated fibres produced much better adhesion results,

and coatings were not easily removed through rubbing between fingers or other manual stresses such as folding, rubbing and chafing. However, it was noted that a 10% NaOH tended to reduce the structural integrity of fibres, whereas the 5% solution did not alter these characteristics.

Figure 2 clearly shows the difference in surface morphology between untreated coir fibre and dewaxed and 5% NaOH treated coir fibre. These results are similar to those obtained by Rout *et al*. (2001), with treated fibres showing greatly reduced surface waxes and oils, greater pore morphology, and increased fibrillation together with removal of tyloses. These characteristics should all improve coating adhesion, and this does seem to be the case in this set of trials. Coating dewaxed and 5% NaOH treated coir fibres with either poly-isoprene, CMC or silicone resulted in much better adhesion characteristics, although CMC-coated fibres proved less favourable in that some portion of the coating could still be readily removed through friction.

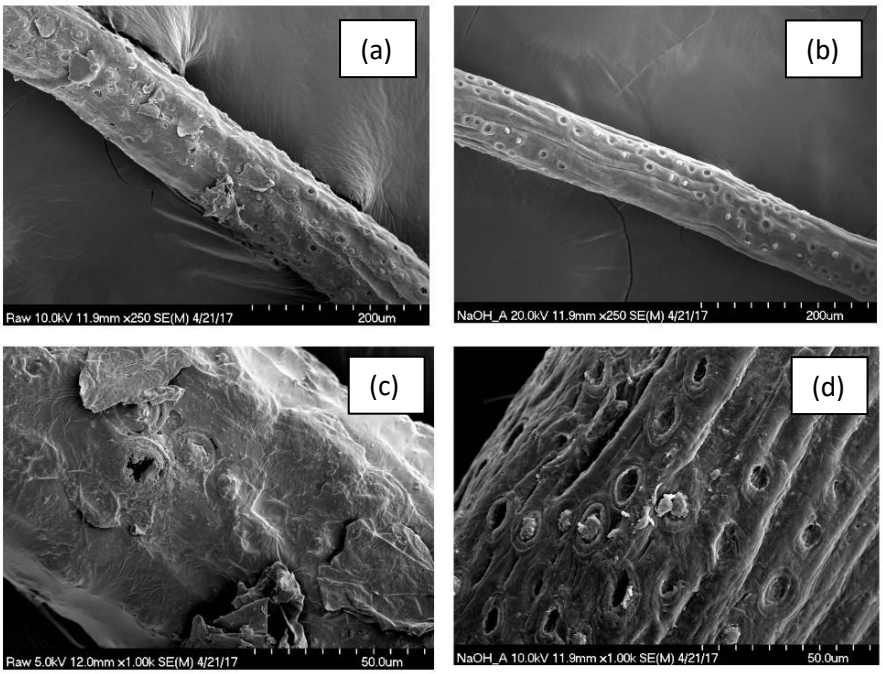


Figure 2. SEM images of untreated coir fibre at magnification x250 (a) and x1000 (c), and Soxhlet dewaxed and 5% NaOH treated coir fibre at magnification x250 (b) and x 1000 (d).

The addition of CuI crystals resulted in a much greater surface area that might be exposed to GEM as compared to biochar or activated charcoal additive, as seen in Figure 3. This was in part due to higher size fraction of the biochar particles, estimated to be >150μm on average as compared to <10μm for CuI crystals, also aided by greater surface area from siloxane morphology. Coverage might be helped through crushing biochar into a smaller particle size prior to use, although no attempt was made in the current work to obtain smaller uniform particle sizes. Further, overall coverage appeared to be better for CuI crystals, as Figure 3 also shows crystals (Figure 3(b,c)) are more homogenously coated on the fibre as compared to biochars (Figure 3(a)). Also noted was a loss of biochar when exposed to rubbing and chafing, as compared to no loss for CuI crystals when manually rubbed between fingers. Greatly increased loss of both biochar and CuI was noted for CMC-coated fibres, strongly indicating this is not an ideal matrix for coating or holding GEM capture additives such as CuI crystals or biochar particles.

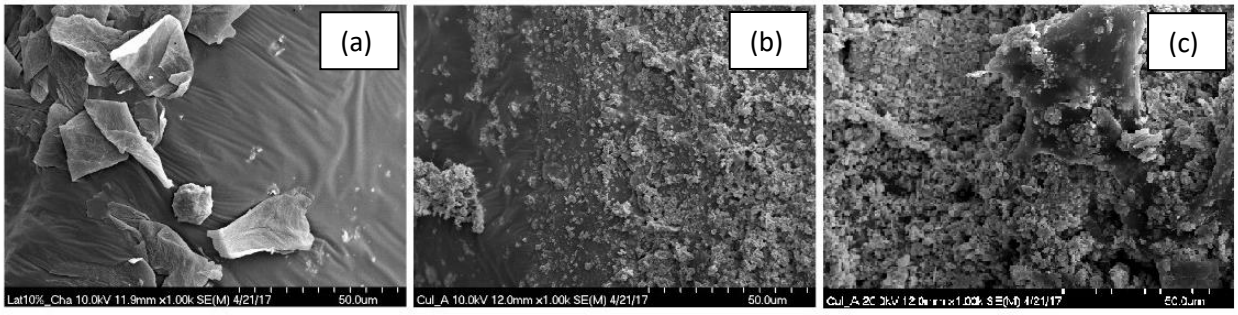


Figure 3. SEM images of (a) 10% poly-isoprene with biochar addition and (b) 10% poly-isoprene with CuI crystal addition and (c) 10% poly-siloxane with CuI (magnification x1000).

When coated with 10% siloxane (Figure 3(c)), a denser CuI distribution seems to have been achieved as compared to using poly-isoprene (Figure 3(b)) or lower siloxane concentrations. This is in part due to the nature of the dissolved product applied to the fibres, whereby the lower viscosity solutions resulted in a good proportion of the CuI

crystals in the suspension settling out as particulates, and were left as detritus in the glass trays after evaporation of the solvent. This was a similar problem for biochar suspensions. Even so, 10% solution of siloxane proved difficult to directly coat, as much of the CuI crystals also settled out as detritus during the solvent evaporation stage. The solution found to rectify this problem was to add CuI crystals during the drying process. Ideal coating material was found to be a 2:1 solution of ethyl acetate and siloxane (33% solution), followed by addition of a mass of CuI equal to the mass of siloxane used, as this appeared to give the most even and dense coating while reducing any unbound fraction to a minimum. This was applied to a mass of coir fibre equal to the mass of siloxane used. Figure 4 is an image of a typical sample prepared in this manner.

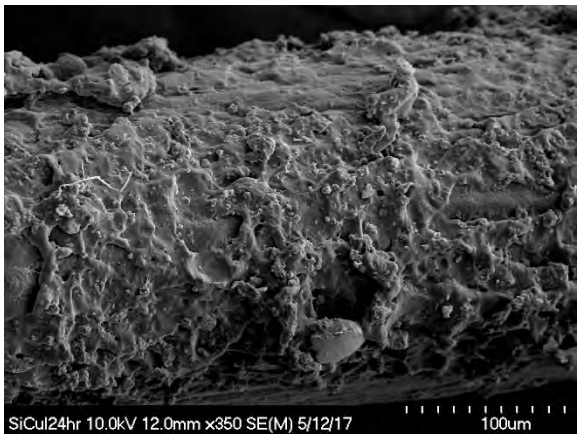


Figure 4. SEM images of typical 10% siloxane-coated fibre with CuI addition at magnification x350.

3.2 *GEM capture per treatment*

Upon visual inspection of the samples after initial exposure, it was clear that the siloxane plus CuI formula had caused a visual discolouration, as seen in Figure 5 which represents a sample exposed to mercury vapour for 24 h. This colour change was in accordance with expectations, as copper(I) tetra-iodide mercurate has a reddish brown colour as compared to white CuI crystals, however the vivid discolouration was not expected. No other colour variation was detected by eye on other samples only exposed for one hour to the GEM plume.

Figure 5. Unexposed coir containing siloxane plus CuI (left) compared to coir exposed to GEM for 24h (right). A clear colour change is noted following exposure, indicating the formation of copper(I) tetra-iodide mercurate.

Please note, although mercury speciation was not characterised, in the discussion following it is assumed emissions are GEM, although it is likely there is trace gaseous oxidised mercury and other minor mercury constituents, but these are ignored at this scale as they likely represent pg m^{-3} concentrations. Figure 6 indicates that fibres that had not undergone solvent extraction had high intrinsic mercury content, of about 400 to 500 ppb Hg, compared to those that had undergone extraction and NaOH treatment,

where Hg levels were reduced to 40 to 50 ppb. This indicates prior Hg contamination, either during growing or manufacturing processes to produce raw coir matting. The solvent extraction process removes existing mercury to roughly 90% efficiency. It was also noted that all samples retained some capacity to adsorb more mercury, irrespective of treatment. This might be related to existing surface oils and waxes having some capacity to adsorb mercury, as when these natural waxes and oils had largely been removed, GEM adsorbance is lower.

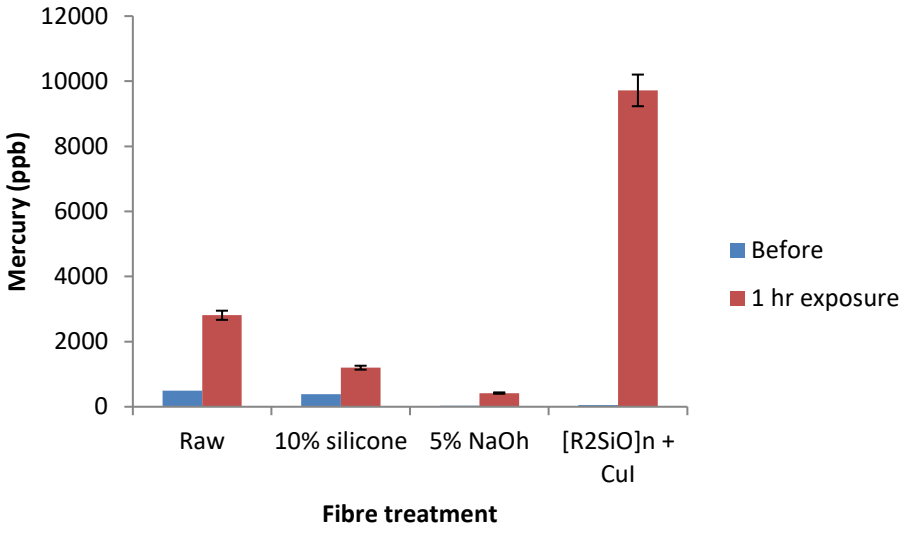


Figure 6. Mercury capture per treatment. Raw and 10% silicone used non pre-treated coir, while 5% NaOH and $[R2SiO]_n$ + CuI were solvent and NaOH treated prior to coating application. Samples exposed to a highly concentrated plume of GEM vapours for 60 minutes.

It also appears that while silicone can adsorb mercury, it is not as efficient as natural waxes and oils, but more efficient than solvent and NaOH treated samples. Figure 6 also indicates that by far the best performing in terms of GEM capture was the siloxane plus CuI samples, where post exposure mercury levels were much greater than for other samples. This data suggests coir fibre coated with a poly-siloxane coating containing CuI crystals may be a good way to capture GEM emissions.

The data in Figure 6 should be treated cautiously, as the sampling caused problems with the DMA-80, likely due to either high silicone or copper contamination, which got progressively worse until sampling could not continue, and explains the truncated results presented. The dataset shown is the average of triplicate successful measurements, over two replicate experiments, also averaged to give final figures as presented. An attempt was made to overcome issues with the DMA-80 contamination problem using a Tekran® Series 2600, through solubilising mercury, however continued contamination problems were encountered, again likely due to high copper content, so surmised due to the distinctive dark discolouration of reagents, and the continued failure of the instrument.

To better evaluate the adsorbance characteristics of the most promising material, an experiment was set up to measure the GEM concentration of a gaseous mercury flow in real time, and compare that after passing it through a volume packed with fibres coated with silicone + CuI of varying concentration. Figure 7 shows the results of those experiments. Note the $\log_{10}$ y-axis scale is used to present the data. As can be seen, the filters were very effective in capturing GEM, with the best performing being the filters coated with 2.0 g CuI g^{-1} coir, capturing close to 100% of extremely high emissions over a 45 h period. The data presented is from a single trial whereby some breakthrough could be detected for the 1.0 and 2.0g CuI versions. Other trials showed no such breakthrough. Breakthrough for the 0.5g CuI sample was similar for all trials.

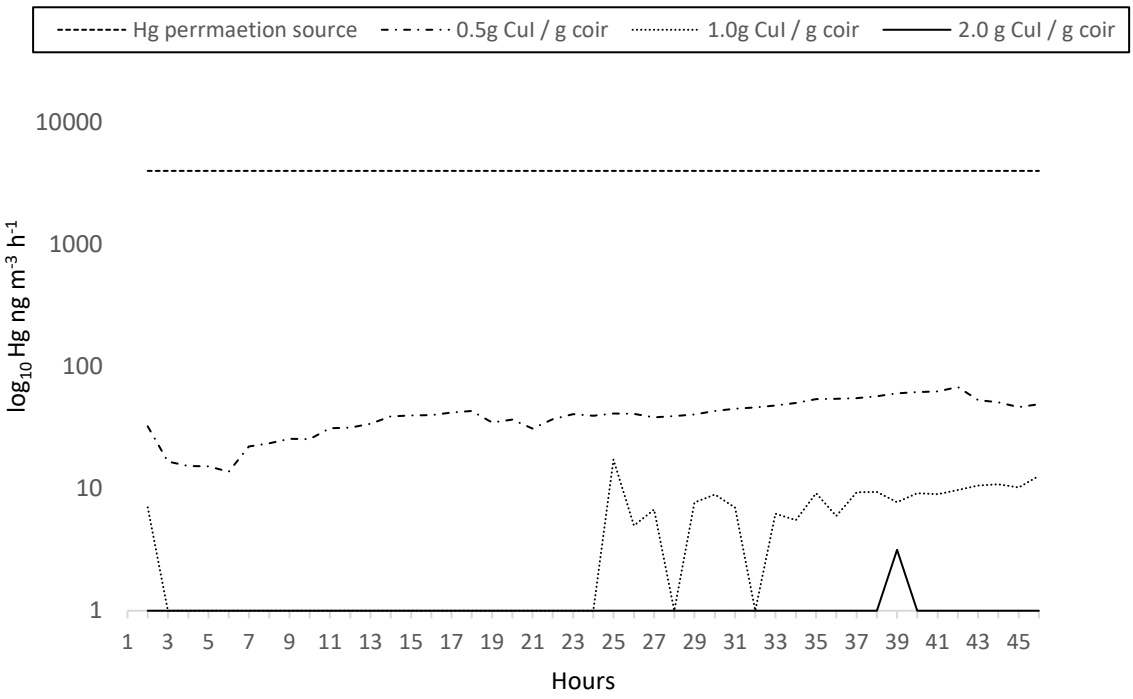


Figure 7. Concentration of GEM after passing through coir-based filters. The permeation source averaged 3973 ng m^{-3} Hg h^{-1}, and the flow rate was 0.5L min^{-1}. Filters were constructed from solvent and NaOH treated coir, with a coating of poly-siloxane with 0.5, 1.0 and 2.0g CuI g^{-1} coir respectively. Carrier gas was nitrogen (Hg), set to an ultra-low flow rate. Tubing, fixtures and sample housing was made from 100% Teflon®. Exposure time per sample was 45 h. Concentrations measured using a Tekran® 2735X mercury vapour analyser.

We interpret this to represent under-coverage of the available surface area at 0.5g CuI g^{-1} coir. Further, samples were compressed tightly in the holding chambers, meaning some distortion of the substrate may have taken place to induce patches of less homogenous thickness and reactant coverage. When two similar filters were housed together linearly, no such breakthrough was detected after 45 h, even for the 0.5g CuI g-1 coir samples. Of course, it is envisaged actual filters could be made of any geometric shape, density, and volume to suit.

The next best performing were those filters coated with 1.0 g CuI g^{-1} coir formula, which had a small breakthrough after 24 h of about 10 ng m^{-3} h^{-1}, however considering the

starting concentration of the gas, this represents a negligible fraction. The filters using 0.5 g CuI g^{-1} coir consistently performed worst, although still captured approximately 99% of the mercury. Somewhere between 1 and 2 g CuI g^{-1} coir appears to be the optimal level of CuI, but somewhat below 2 g CuI g^{-1} coir, as it was noted that there was residual CuI that could be readily shaken off prepared samples at this level, while no such problem was encountered with those prepared at 1 g CuI g^{-1} coir. It should be noted that coverage will also depend heavily on application method. The stoichiometry suggests even at low efficiency, each gram of CuI could potentially react with and capture at least tens of mg Hg. No attempt was made to ascertain saturation point of the filters due to time constraints. Estimated total exposure (Q) was 5.4 µg Hg, given Q= FCT, where F is sample flow rate m^{-3} h, C is concentration GEM ng m^{-3}, T is exposure time (hours).

Once the reaction takes place to form copper(I) tetra-iodide mercurate, the Hg is bound in a virtually insoluble and stable solid form. This is particularly important for large-scale outdoor applications such as matting, as they are exposed to weather. Hg in this form will not re-volatilise readily, and is strongly bound to the silicone coating. This is critical, as CuI has serious deleterious effects to aquatic life, as does Hg Manufacturing processes could mitigate against this problem, but in environmental applications of scale, management of any risk by loss of CuI from the substrate would be important.

While it was difficult to explore the full characteristics and operating parameters here, future exploration and detailed comparison under thermal and chemical environments likely to be encountered in industrial settings is the obvious extension to this proof of concept work. These more complex environments, such as hot, evolving, and chemically complex flue gasses would require some detailed analysis regarding the GEM capture performance of the proposed technology, however this might be well

worth exploring given the expense and limited efficiency of the current technology, where employed.

Much research has been conducted into capture of industrial GEM emissions, largely focusing on catalytic oxidation followed by appropriate scrubbing. Similarly, activated carbons have been the subject of detailed examination for their intrinsic and induced mercury capture properties, although the detailed mechanisms are still the subject of debate. However, little research has been applied to the capture of GEM without the need for prior oxidation. If the technology described in this work is used as a geotextile matting, for example, the product could be used to capture GEM at large scale, such as in gold mining operations, or in the remediation of soils. Alternatively, one could envisage artisanal gold mining applications, where GEM emissions are known to have serious consequences for those participants. Such devices might include packed bed columns or face masks, among other configurations. Similarly, for point source industrial emissions including from energy production, a wide variety of applications could potentially be explored.

4 Conclusion

Coir fibre was investigated as a substrate to construct a GEM capture device capable of working in ambient conditions without prior catalytic oxidation. Over 99% of Hg was effectively scrubbed from a plume containing 4000 ng Hg m^{-3}. The product is made from a renewable resource available in bulk at low cost, with readily available and inexpensive additives. The results suggest this technology shows potential for application in a variety of settings, and can be manufactured in a facile manner with existing technology.

Acknowledgements

The authors wish to thank Prof. Glenn Miller, Applied Research Facility, Dept. Natural Resources and Environmental Science, University of Nevada, Reno, and Zachary Karmiol, Department of Chemical and Materials Engineering, University of Nevada, Reno, for SEM imagery. Support (non-grant) for this work came from the Australian Government Research Training Program (RTP) and Macquarie University, Sydney, Australia, as well as logistical support from University of Nevada, Reno. Funding provided via Macquarie University Research Training Program Scholarship (MQRTP).

Note. Patent Cooperation Treaty (PCT) International Application No.: PCT/AU2018/000169. Intellectual property owned by Macquarie University, Australia.

References

Amos, H.M., Jacob, D.J., Streets, D.G., Sunderland, E.M., (2013) Legacy impacts of all-time anthropogenic emissions on the global mercury cycle. *Global Biogeochemical Cycles,* 27, p 410.

Balan, K., (1995) *Studies on engineering behaviour and uses of geotextile with natural fibres*. Ph.D. Thesis, Indian Institute of Technology Delhi, India.

Chandrasekar, M., Ishak, M.R., Sapuan, S.M., Leman, Z. & Jawaid, M., (2017) A review on the characterisation of natural fibres and their composites after alkali treatment and water absorption. *Plastics, Rubber and Composites*, 46(3), pp. 119-136.

Dash, H.R. and Das, S., 2012. Bioremediation of mercury and the importance of bacterial *mer* genes. *International Biodeterioration & Biodegradation*, 75, pp.207-213.

Finkelman, R.B., (1981) *Modes of occurrence of trace elements in coal*. United States Geological Survey. Open File Report No. OFR-81-99. pp. 301

Galloway, J., Thornton, S., Volchok, H., McLean, R., (1982) Trace metals in atmospheric deposition: a review and assessment. *Atmospheric Environment*, 16 (7) pp 1677-1700

Gao, Y., Zhang, Z., Wu, J., Duan, L., Umar, A., Sun, L., Guo, Z., Wang, Z., (2013) A critical review on the heterogeneous catalytic oxidation of elemental mercury in flue gases. *Environmental Science and Technology*, 47, pp 10813−10823.

Lekha, K.R., (2004) Field instrumentation and monitoring of soil erosion in coir geotextile stabilized slopes: a case study. *Geotextiles and Geomembranes*, 22 (5), pp. 399–413.

Liu, W., Mohanty, A.K., Drzal, L.T. and Misra, M., (2004) Effects of alkali treatment on the structure, morphology and thermal properties of native grass fibers as reinforcements for polymer matrix composites. *Journal of Materials Science*, 39(3), pp. 1051-1054.

Nicholas, D.D., (1982). *Wood deterioration and its prevention by preservative treatments: Degradation and Protection of Wood* (Vol. 1). Syracuse University Press.

Norrby, Lars J., (1991) Why is mercury liquid? Or, why do relativistic effects not get into chemistry textbooks? *Journal of Chemical Education*, 68 (2), p 110.

Punyamurthy, R., Sampathkumar, D., Srinivasa, C.V. and Bennehalli, B., (2012) Effect of alkali treatment on water absorption of single cellulosic abaca fiber, *BioResources*, 7(3), pp. 3515-3524.

Prabhakar, G., Sorooshian, A., Toffol, Arellano, A.F., Betterton, E.A., (2014) Spatiotemporal distribution of airborne particulate metals and metalloids in a populated arid region. *Atmospheric Environment*, 92, (9) pp 339-347.

Rout, J., Tripathy, S.S., Nayak, S.K., Misra, M. and Mohanty, A.K., 2001. Scanning electron microscopy study of chemically modified coir fibers. *Journal of Applied Polymer Science*, *79*(7), pp.1169-1177.

Suni, S., Unnikrishnan, N., Mathew, L., (2016), Experimental investigations on biological resistance of surface modified coir geotextiles. *International Journal of Geosynthetics and Ground Engineering*, 2 (4) p.31.

Slemr, F., Schuster, G. and Seiler, W. (1985) Distribution, speciation, and budget of atmospheric mercury. *Journal of Atmospheric Chemistry*, 3, pp 401-434.

Chapter 6 – Research paper 3

6.0 Statement of contribution

Damien McCarthy - Design of product, background research, conceptual design of experiments, experimental work, data analysis, instrument set up and calibration, manuscript production,

Grant C Edwards – Manuscript editing, oversight.

NB – Dr Christie Howard, University of Nevada, Reno, USA, assisted with logistical support and troubleshooting experimental work, but is not named as co-author.

6.1 Immobilized organo-mercurial lyase on zeolite using a solid binding peptide

International Journal of Environmental Bioremediation & Biodegradation, 2018, Vol. 6, No. 1, 8-17
Available online at http://pubs.sciepub.com/ijebb/6/1/2

DOI:10.12691/ijebb-6-1-2

Immobilized Organo-Mercurial Lyase on Zeolite Using a Solid Binding Peptide

Damien N. McCarthy*, Grant C. Edwards

Department of Environmental Science, Faculty of Science and Engineering, Macquarie University, Sydney, Australia
*Corresponding author: mccarthyd001@gmail.com

Abstract Methylmercury (MeHg) compounds can form naturally, are highly toxic, and of concern because of their tendency to bio-accumulate. Certain bacteria have evolved mechanisms that can tolerate MeHg by first demethylating MeHg compounds, before further processing. Drawing inspiration from this demethylation mechanism controlled by a single organo-mercurial lyase in a protonolysis reaction, this research uses a recombinant gene that produces this lyase plus an additional polypeptide that selectively binds to zeolite particles, effectively tethering the enzyme to the solid substrate. This work is part of a broader attempt to create a fixed bed reactor for de-methylation of MeHg. Enzyme immobilization was achieved using a solid binding peptide (SBP) with high affinity for faujasite zeolite (FZ), the choice of binding substrate in the present work. The lyase is coded for by the *merB* gene, and a sequence with highly conserved active site homology was obtained from *E.coli* plasmid R8361b. The SBP plus *merB* sequence was designed such that the SBP was positioned either on the N or C-terminal of the construct. The DNA was synthesized commercially, and expressed in *E.coli* (BL21DE3 Star) using pET100® vector. Sanger sequencing was used to confirm construct in transformed cells using standard T7 oligos. Expression was lactose induced, and SDS-PAGE electrophoresis was used to confirm protein production and size. LC-MS/MS and sequence bio-analytics confirmed peptide sequence. Silica binding assays using SDS-PAGE confirmed binding of the enzyme to the silica substrate. Enzyme functionality results using a non-methylated mercuric compound were inconclusive, however the enzyme has not been assessed using MeHg compounds at this stage.

Keywords: *merB, methylmercury, enzyme immobilization, demthylation*

Cite This Article: Damien N. McCarthy, and Grant. C Edwards, "Immobilized Organo-Mercurial Lyase on Zeolite Using a Solid Binding Peptide." *International Journal of Environmental Bioremediation & Biodegradation*, vol. 6, no. 1 (2018): 8-17. doi: 10.12691/ijebb-6-1-2.

1. Introduction

Methylated mercury species (MeHg) are highly toxic compounds that form mostly via bio-genic mechanisms under certain conditions, particularly anoxic environments with high anionic ligand content [1]. Methylmercury formation is often mediated by microbial activity, in particular by sulfur and iron reducing bacteria (SRB and FeRB respectively), methanogens, and firmicutes [2,3]. The proclivity for such toxic compounds to bio-accumulate in the food web [4], and therefore impact human health, is one of the main factors MeHg compounds have received much research attention over the past decade. Apart from several transgenic plant studies aimed at bioremediation [5,6], few studies have focused on bio-remediation of MeHg in aqueous bodies.

One strategy that may prove beneficial in this regard is inspired directly from evolved microbial metabolic mechanisms that deal with MeHg via *de-methylation*. Such de-methylating bacteria are ubiquitous and represented by a wide range of taxa [7]. The overall microbial strategy ofen involves the intracellular reduction of Hg^{2+} -> Hg^0 by a mercuric reductase enzymatic reaction, whereby Hg^0 then naturally volatilizes and passively diffuses from the cell and out of the immediate surrounds into the atmosphere [8]. Where it is necessary for microbes to deal with MeHg, the reduction reaction is preceded by MeHg intracellular interaction with an organo-mercurial lyase, which cleaves the C-Hg bond through a protonolysis reaction [9], producing CH_4 and Hg^{2+}, where mercury is then passed to the mercuric reductase enzyme. It appears that the reductase enzyme activates release of the lyase bound Hg^{2+} [10]. The C-Hg enzymatic cleavage confers broad spectrum Hg resistance. HgR microbes with broad spectrum capabilities are characterized by the presence of the *merB* gene, which codes for the organo-mercurial lyase.

The mechanistic function of the *merB* product has not been fully elucidated, however it is highly likely to function as proposed by Parks *et.al.*, [9]. The enzyme itself is relatively small at around 24kDa, having been structurally characterized through crystallography, including various mutants, which have helped deduce the functional domain, key amino residues, and mechanistic architecture [11,12]. The may give this enzyme the ability to remain functional while immobilized on an extracellular solid substrate.

Immobilizing technologies greatly reduce the complexity of deploying this bio-catalytic strategy for remediation purposes, as living cells pose problems in terms of delivery to heterogeneous sites, geographical containment

within the contaminated plume (particularly so in a water column), nutrient supply, and competition from indigenous microbes etc. [13]. The concept explored in this work thus involves the extraction and immobilization of the *merB* gene product on a solid substrate, that being a faujasite type alumino-silicate zeolite (FZ) in particle form. This work represents the first stage of a broader project such that a fixed bed bio-catalytic reactor may be constructed to de-methylate aqueous MeHg compounds *in situ*.

Enzyme immobilization techniques often suffer from orientation and target accessibility issues [14]. An approach that may overcome these problems is the use of solid binding peptides (SBP's) that selectively recognize and bind to solid surfaces [15]. The SBP-enzyme conjugate can be either C or N terminal, depending on the active site location and orientation required, with the SBP used as a linker to tether the enzyme to the substrate. One such class of SBPs has a high affinity for certain zeolites and can thus be used to immobilize enzymes on silica-based substrates [16]. Zeolite is an ideal substrate for this work due to its inert and robust nature, and because it is readily synthesized and inexpensive. This particular SBP is highly amorphous, overcoming issues with accessibility and steric hindrance, and has been used to immobilize over 25 enzymes on silica based substrates which retained functionality [17]. The immobilization of the enzyme on a silica substrate can be achieved via the expression of a fusion protein that contains this SBP linker and the organo-mercurial lyase, which is then extracted and purified from *E.coli* cells and bound to solid particles. These particles may then be packed in a column, and it is envisioned that MeHg concentrations may be reduced or eliminated in aqueous media which interacts with these particles.

2. Materials and Methods

2.1. Recombinant Protein Construct

Two constructs were designed with the SBP flanking either N or C terminal of the *merB* sequence. The SBP used is a truncated version of one previously described [17,21]. *merB* sequence was derived from GenBank: U77087.1, *E.coli* plasmid R831b, and codon optimized for expression in *E.coli* BL21 DE3 Star. Constructs synthesized commercially by GeneArt®. Oligos designed as per requirements and synthesized commercially by Life Technologies Corporation. Constructs synthesized in a T7 mediated expression ready pET100® vector with existing N terminal polyhistidine-tag. These are designated N[FZ]SBP+*merB* and C[FZ]SBP+*merB* for N and C terminal zeolite binding SBP position respectively. *E.coli* DH5α (Life Technologies Corporation) cells as stock held in 20% glycerol at -80^{0}C. Expression was carried out using *E.coli* BL21 DE3 Star cells (Life Technologies Corporation). The SBP linker amino acid sequence is $(VKTQATSREEPPRLPSKHRPG)_3VKTQTAS$.

2.2. Transformation and DNA Confirmation

Chemically competent *E.coli* DH5α and BL21 DE3 Star cells transformed using heat shock method. S.O.C. media by Thermo Fischer (Cat No. 15544034). Incubation at 37°C (shaking at 120 rpm) with 100µg/mL Ampicillin (Amp) (Thermo Fischer Cat No. 11593027) doped media. Sanger sequencing was performed by University of Nevada, Reno, at the Nevada Genomics Centre (NGC), using approximately 500ng plasmid template to 10 Pmol primer. Plasmid extraction using Qiagen QIAprep® Spin Miniprep Kit (Cat No. 27104). Oligos supplied by NGC (Fwd): T7 primer 5' TAATACGACTCACTATAGGG. Pairwise sequence alignment was performed using Emboss Needle ©.

2.3. Expression and Purification

Lactose based auto-induction was performed using two-stage media. Cells were first grown to stationary phase using **RM** media plus glucose (per L: 20g casamino acids, 100mL 10x M9 salts, 1mL 1M $MgCl_2$, 10mL glucose, Amp to 1mM in dH_2O) at 37°C with shaking at 120rpm. Glucose ensures leaky T7 promoter induction can be tightly controlled, and was monitored regularly to ensure supply using glucose assays (Genzyme Diagnostics Glucose [Trinder] Assay Cat No. 22032). **ZYM-5052** Media ((per L: 2 mL 1M $MgSO_4$, 40mL 25x *M Sola* (per L: 88.75g $NaHPO_4$, 85g KH_2PO_4, 67g NH_4Cl, 17.75g Na_2SO_4), 2g lactose, 5mL 100% glycerol, 0.5g glucose)) was inoculated to $OD_{600} \approx 0.5$ with RM media stationary phase cells. Protein expression quantified using Thermo Fischer EZQ™ Protein Quantification Kit Cat No. R33200. Non induced cells, and cells containing neither N[FZ]SBP+*merB* or C[FZ]SBP+*merB* as controls. 500µL aliquots were removed at various time-points, pelleted ($7 \times 10^3 rpm^{-min} \times 2mins$), boiled in 80µL 2x SDS at 97°C for 7mins, centrifuged ($7 \times 10^3 rpm^{-min}$ for 3mins), and protein expression checked (for size) using 10µL soluble product on (NuPAGE ™ 12% Bis-Tris) protein gels (1X MOPS/SDS) at 120V, followed by Coomassie Blue™ staining. Pelleted samples were stored at -20°C where necessary.

Protein purified using Ni-NTA agarose (Invitrogen, Cat. No. R901-01) by gravity fed column (Bio-Rad Poly-Prep®chromatography column (Cat. 731-1550). Binding buffer (20mM Tris, 500mM NaCl, 10mM Imidizole, 10% glycerol, pH 8.0). Wash buffer (20mM Tris, 100mM NaCl, 30mM Imidizole, pH 8.0. Elution buffer (20mM Tris, 100mM NaCl, 300mM Imidizole, pH 8.0) Synthesis of the desired amino sequence(s) was 100% confirmed by tandem mass spectrometry after trypsin digestion of excised polyacrylamide gel bands. LC-MS/MS was performed by the Nevada Proteomics Centre at the University of Nevada, Reno, using an LC gradient of 60-90 min with data analysis performed using Scaffold Version 4.8.4 with peptide threshold of 80% and protein (min 2) threshold of 99%.

2.4. Zeolite Binding Assay

Post purification, the enzyme was mixed with silica particles (SiliaFlash® Irregular Silica Gels, 60Å (Cat No. R12030B); Zeolite Y CBV100, Zeolyst Int; Sigma-Alldrich Silica Gel, 60Å (Cat 9385); and Faujasite Type Zeolite, Sigma-Aldrich (BCR704-10G)). The binding assay is described by Sunna *et. al.* (2013). Briefly, approximately 100mg particles are first vortexed in 1ml

wash buffer (10 mM Tris–HCl, pH 7.5, 100mM NaCl and 1% Triton-X100), repeated twice, and dried under N_2. Soluble protein was mixed with particles and incubated by slow rotation at room temperature for 1h. Any unbound fraction was removed after centrifugation at 10 x 10^3 rpm for 30s. The residual silica pellet was washed by vortexing with 100µl of 100 mM Tris–HCl buffer, pH 8.0. Washing was repeated twice and wash fraction samples retained. Bound protein was eluted using 100µl of 2x SDS PAGE-loading buffer followed by incubation at 99°C for 10 m. Pellet was vortexed briefly every 2 m during this elution step. The various fractions were analyzed using SDS-PAGE with Coomassie Blue™ staining.

2.5. Functional Assay

Functionality was assessed using an indirect assay on 250mL culture. Cells were pelleted (5krpm 15mins at 4°C), resuspended and briefly washed in lysis buffer (50mM Tris-HCl (pH 7.5), 1mM β-mercaptoethanol, 1mM PMSF, 0.5mM EDTA, 5%$^{v/v}$ glycerol) before centrifugation (5krpm 15mins at 4°C). Cells were resuspended in 2mL lysis buffer and sonicated on ice for 15s. The mixture was centrifuged (15krpm 10mins at 4°C) and 20µL soluble fraction added to assay solution (50mM Tris-HCl (pH 7.5), 1mM L-Cys, 1µM 4-Chloromercuribenzoic acid (PCMB)) and absorbance at λ_{250} monitored every minute for 20 mins. Crude extract and FZ bound enzyme were tested.

3. Results and Discussion

3.1. Protein Expression

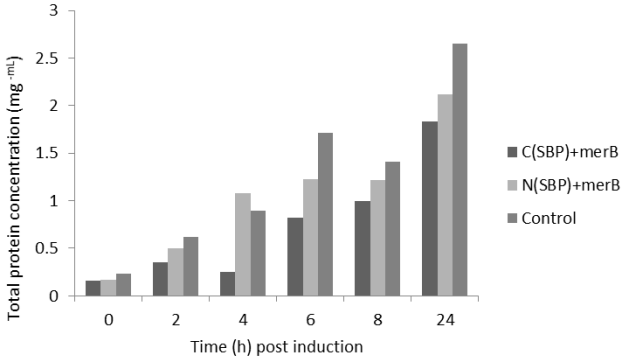


Figure 1. Average total protein expression (mg^{-mL}) over time where T_0 is OD_{600} = 0.5 for C[FZ]SBP+*merB* and N[FZ]SBP+*merB* transformants (control was IDOLDH+)

Protein expression was first unsuccessfully attempted using IPTG, where it was noted that growth rates and viability were so variable after induction at OD_{600} = 0.4 - 0.6 at varying concentrations between 0.1 and 2.0µM final concentration IPTG that data sets could not be replicated. There is evidence that elevated levels of merB product can be toxic, somewhat modulated by inclusion body formation [18]. Although the expected product is soluble, the insoluble fraction was also analyzed but no evidence was obtained for the expected product in this fraction. The T7 promoter used in this plasmid (pET100®) is known to be somewhat leaky [19], and to rule out toxicity problems that may be occurring, we used a glucose and lactose based induction method to tightly control expression, which proved much more successful.

Using total protein concentration as a proxy for growth rates, post induction batches were monitored every 2h for 8hrs, then sampled again at 24h. Figure 1., shows total soluble protein content after expression was induced using the glucose-lactose induction method. This result is much more in line with expectations, although of note is that the C[FZ]SBP+merB variant consistently does not seem to do as well, and in particular struggles consistently between 3 and 4h mark post induction. No attempt was made to solve that puzzle, although there is some evidence that C terminal additions to constructs are more difficult to express successfully [20]. Conversely, between 3 and 4h post induction appears to be the time with the highest growth rate for N[FZ]SBP+merB. Induction time was always at or adjusted to the same cell density so this cannot explain this variable. In any case, cells with N terminal construct performed consistently better using lactose based induction, and although C[FZ]SBP+merB cells did eventually grow at a comparable rate, they took additional time to recover post induction and appear to have suffered some considerable viability issues as well.

SDS-PAGE was used to help detect soluble protein fraction. Figure 2, is an image of the Coomassie Blue™ stained SDS-PAGE gel, indicating products of the expected MW of approximately 34.8kDa. Lane 14 is overspill and can be ignored. The induced bands are clearly not visible 2h post induction and we surmise this is due to residual glucose availability, thus repressing expression. Controls were both IPTG induced at OD_{600}=0.5 and these gel fractions represent 8h post induction.

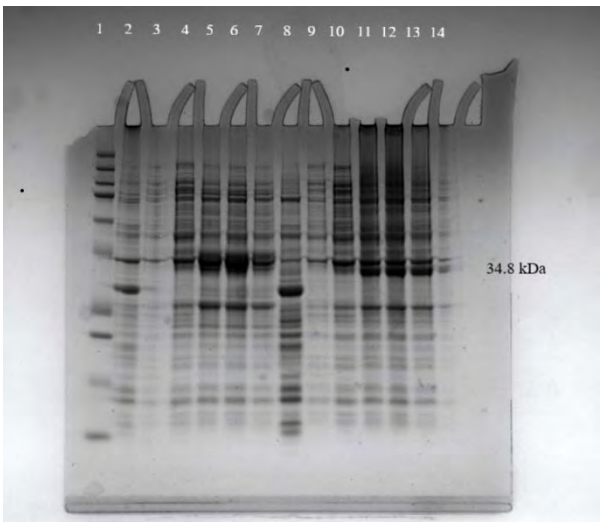


Figure 2. SDS-PAGE gel indicating product at the expected MW of 34.8 kDa for both C and N(SBP)+merB constructs. Lane 1 is Bio-Rad protein ladder, Lane 2 is pUC19 control, Lanes 3-7 are C(SBP)+merB 2,4,6,8 and 24h post induction (respectively), Lane 8 is IDOLDH(+) control, and Lanes 9-13 are N(SBP)+merB 2,4,6,8 and 24h post induction (respectively). Lane 14 is overspill and should be ignored

In order to confirm N[FZ]SBP+merB growth, cells were again grown until 8h post induction, this time using auto induction method only. As is clearly visible in Figure 3, the N terminal variant successfully grew, and the time series clearly indicates any toxicity issues seem to have been mitigated as progressively more protein is found in the soluble fraction as growing time is extended.

The wells were each loaded with 10µL sample ruling out inconsistent volumes, indicating the sample was progressively more concentrated with the desired product.

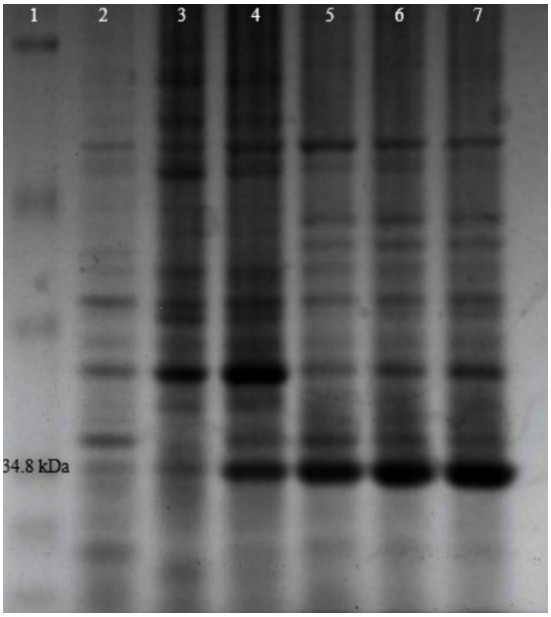


Figure 3. SDS-PAGE gel image of N[FZ]SBP+merB using auto induction media Lane 1. Bio-Rad Broad Spectrum protein ladder, Lane 2. Non-induced, Lane 3. 0.5 OD600, Lane 4. 2h post induction, Lane 5. 4h post induction, Lane 6. 6h post induction, Lane 7. 8h post induction

3.2. Sequencing

Cloning confirmation of the desired construct was obtained using Sanger Sequencing, and expression was confirmed using LC-MS/MS after trypsin digestion (data in supplementary). The results for C terminal variant were not in line with predicted results for either. In particular, C[FZ]SBP+merB variant mass spectrometry results were poor. There was no match for a peptide adjacent to the C term although one was predicted. MS results for N[FZ]SBP+merB were more conclusive, showing 8 peptide matches representing 80aa's with over 25% coverage, including coverage of the SBP conjugate. Due to the many problems associated with the C terminal variant it is likely the N terminal variant would be easier to scale up production.

The SBP has an amorphous structure which can assist with enzyme orientation and steric hindrance issues. This structure was confirmed theoretically using the Foldindex© [22] software to ascertain whether the N terminal SBP segment of the polypeptide conjugate is folded as opposed to the functional *mer*B derived segment. Figure 4 clearly indicates the amino sequence containing the SBP is predicted to be unfolded.

This amorphous structure is important because when tethering enzymes to solid substrates, maximum flexibility is required to ensure the target molecule, in this case methylmercury, is not hindered from interacting with the functional domain of the enzyme. The SBP structure allows for greater degrees of freedom to as opposed to direct binding on solid substrates.

As sequencing data indicated successful production of the enzyme with SBP for the N[FZ]SBP+*mer*B variant, a series of silica/zeolite binding assays were performed.

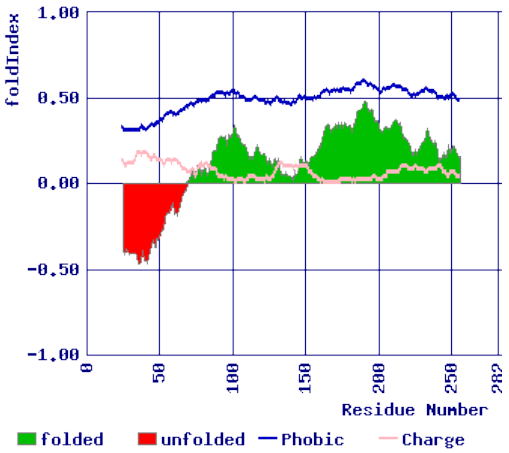


Figure 4. Foldindex© prediction for intrinsically unfolded segment of the N[FZ]SBP+*mer*B variant, indicating the SBP segment is intrinsically unfolded.

3.3. Binding Assays

Solid binding peptides are a valuable tool for recombinant production of immobilized enzymes. The particular SBP used in the current approach is known to have a very high selective affinity for FZ, affording it great potential for use in a variety of contexts [21]. To evaluate the binding of our products to silica and zeolites, a binding assay was performed. Although the binding mechanism has not been fully elucidated, to assess the robustness of the N[FZ}SBP +merB:FZ conjugate, particle bound enzyme were subjected to repeated washing by 30s vortexing in wash buffer and the wash fractions analyzed for unbound enzyme.

As some native *E.coli* proteins are known to bind to silica, both N and C terminal products were purified by HIS-tag and exchange buffer treatments prior to binding. To ensure purity, silica and zeolite particles were prewashed in wash buffer and dried under N_2. No enzyme was detected for the C[FZ]SBP+*merB* variant after purification. This was not surprising given the lack of detection of the SBP conjugate with MS/MS, troublesome growth issues, and poorly resolved MW data for this variant. Considering the ongoing issues with the C terminal variant, we decided to concentrate on N[FZ]SBP+*merB*, and all following results are based on that construct.

Our initial use of 60Å silicas and CBV100 showed low or no binding capacity which was expected and is in line with previous research [17,21]. Type Y faujasite zeolite is known to have greater binding capacity for this SBP. Figure 5 and Figure 6 tend to support that notion. Further, we have used a truncated version of the SBP where only 3 repeats of the given amino sequence were used instead of the four used by Nygard *et.al.,* (2002) in their initial work. This was proposed by Sunna *et.al.,* (2013) and seems to be confirmed by our results.

Figure 6 indicates that the N[FZ]SBP+*mer*B variant was successfully bound to FZ type zeolite. The unbound fraction in lane 2 is likely due to loading issues where too much enzyme was mixed with particles and maximum binding capacity was breached. No attempt was taken to optimize binding amounts, but this would obviously be required in scale up. Similarly, the first two wash fractions indicate loss of enzyme, and we surmise this is due to overloading because, by wash 3 fraction, there is little or no enzyme

coming off. The eluted fraction clearly shows that the enzyme had previously been successfully bound to the FZ type zeolite, and so the fact there was no enzyme in the third wash fraction indicates what was bound was bound strongly.

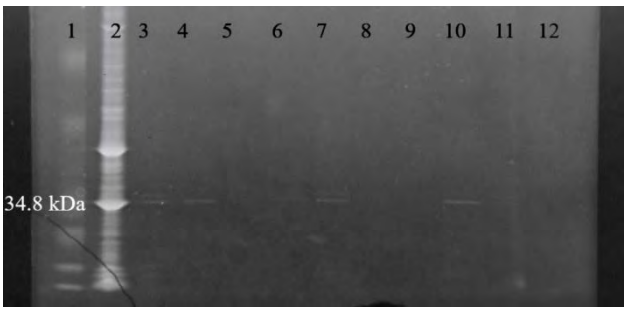


Figure 5. SDS-PAGE gel indicating SBP does not bind to Siliaflash or Sigma-Aldrich 60Å silica gel, or CBV100 zeolite. Lane 1. Ladder (Bio-rad broad range), Lane2. Crude extract, Lane 3. Ni purified, Lane 4. Unbound on Sigma-Aldrich gel, Lane 5. Combined wash fractions for previous, Lane 6. Eluted fraction, Lane 7. Unbound on CBV100 zeolite, Lane 8. Combined wash fractions for previous, Lane 9. Eluted fraction, Lane 10. Siliaflash gel unbound, Lane 11. Combined wash fractions for previous, Lane 12, Eluted fraction

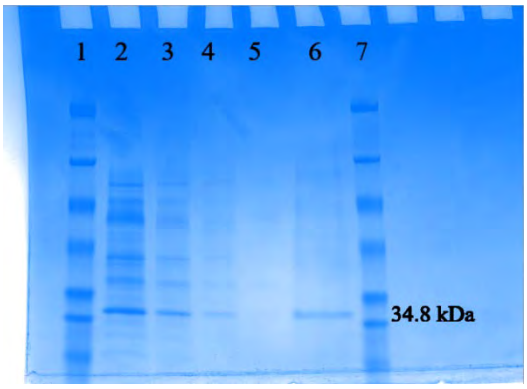


Figure 6. SDS-PAGE gel indicating SBP binds to Type Y Faujasite zeolite. Lane 1. Ladder (Bio-rad), Lane2. Unbound fraction, Lane 3. Wash fraction 1, Lane 4. Wash fraction 2, Lane 5. Wash fraction 3, Lane 6. Eluted fraction showing enzyme was bound, Lane 7. Repeated ladder

Our attempts to assess functionality of the enzyme were not conclusive at this stage. In particular, we are yet to quantify enzymatic activity using methylmercury compounds. In previous work by the author (unpublished) the companion *mer*A gene was designed and bound to silica in a similar fashion. There was evidence of retained functionality in that case. One issue with the *mer*A enzymatic reaction is that it requires $NADPH^+$ as a co-factor, making the functionality more difficult and expensive to replicate while the enzyme was immobilized on a solid substrate. However if a suitable co-factor analog can be found then this technology may be more practical. One can envision a co-localised merA;merB set up where both enzymes could be bound to the same particle providing two stage catalysis resulting in not only degradation but removal of MeHg from contaminated waters.

4. Conclusion

Successful expression and binding to a solid zeolite substrate for this novel recombinant enzyme paves the way for a methyl mercury filter to be designed and employed to remediate contaminated waters by degrading the compound to its much less toxic form. The benefits of binding with an amorphous peptide linker are that it offers advantages over other traditional binding methods in terms of orientation and steric hindrance problems that are often encountered. Even though we were unable at this stage to show the *mer*B derived lyase enzyme remained functional while bound, this technique has been used to bind enzymes that remained functional. Difficulties with the highly toxic nature of methylmercury precluded any further optimization at this preliminary stage. The method used in this work seems to be promising in that both *merB* and *merA* (unpublished) have now been bound to silica nanoparticles using this method, More research is required to address how to implement an efficient and cost effective way to degrade and remove MeHg from aqueous environments.

Acknowledgements

The authors wish to thank Christie Howard, PhD, and Brandon Purdy of University of Nevada, Reno (UNR) for their generous assistance with this work and Assoc. Dean David Shintani, Biotechnology and Natural Resources, UNR, for logistical support. Funding for this work was provided by the Australian Government Research Training Program (RTP), and Macquarie University, NSW, Australia (HDR funding initiative).

Competing Interests Statement

The authors whose names are listed on this manuscript certify that they have NO affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript, and that there are no conflicts of interest to disclose.

Abbreviations

Hg = mercury; MeHg = methylated mercury; SRB = sulfur reducing bacteria; FeRB = iron reducing bacteria; SBP = solid binding peptide; HgR = mercury resistant microbes; OD_{600} = optical density at λ600 nm, FZ = faujasite type zeolite.

References

[1] Frohne, T., Rinklebe, J., Langer, U., Du Laing, G., Mothes, S. and Wennrich, R., 2012. Biogeochemical factors affecting mercury methylation rate in two contaminated floodplain soils. *Biogeosciences*, *9*(1), pp.493-507.

[2] Gilmour, C.C., Henry, E.A. and Mitchell, R., 1992. Sulfate stimulation of mercury methylation in freshwater sediments. *Environmental Science & Technology*, *26*(11), pp.2281-2287.

[3] Kerin, E.J., Gilmour, C.C., Roden, E., Suzuki, M.T., Coates, J.D. and Mason, R.P., 2006. Mercury methylation by dissimilatory iron-reducing bacteria. *Applied and environmental microbiology*, *72*(12), pp.7919-7921.

[4] Morel, F.M., Kraepiel, A.M. and Amyot, M., 1998. The chemical cycle and bioaccumulation of mercury. *Annual review of ecology and systematics*, pp.543-566.

[5] Lyyra, S., Meagher, R.B., Kim, T., Heaton, A., Montello, P., Balish, R.S. and Merkle, S.A., 2007. Coupling two mercury resistance genes in Eastern cottonwood enhances the processing of organomercury. *Plant biotechnology journal*, *5*(2), pp.254-262.

[6] Basak, B. and Dey, A., 2015. Bioremediation Approaches for Recalcitrant Pollutants: Potentiality, Successes and Limitation. *Toxicity and Waste Management Using Bioremediation*, p.178.

[7] Boyd, E. and Barkay, T., 2012. The mercury resistance operon: from an origin in a geothermal environment to an efficient detoxification machine.*Frontiers in microbiology*, *3*, p.349.

[8] Fox, B. and Walsh, C.T., 1982. Mercuric reductase. Purification and characterization of a transposon-encoded flavoprotein containing an oxidation-reduction-active disulfide. *Journal of Biological Chemistry*, *257*(5), pp.2498-2503.

[9] Parks, J.M., Guo, H., Momany, C., Liang, L., Miller, S.M., Summers, A.O. and Smith, J.C., 2009. Mechanism of Hg− C Protonolysis in the Organomercurial Lyase MerB. *Journal of the American Chemical Society*, *131*(37), pp.13278-13285.

[10] Silva, P.J. and Rodrigues, V., 2015. Mechanistic pathways of mercury removal from the organomercurial lyase active site. *PeerJ*, *3*, pp 1127.

[11] Lafrance-Vanasse, J., Lefebvre, M., Di Lello, P., Sygusch, J. and Omichinski, J.G., 2009. Crystal structures of the organomercurial lyase merb in its free and mercury-bound forms insights into the mechanism of methylmercury degradation. *Journal of Biological Chemistry*, *284*(2), pp.938-944.

[12] Di Lello, P., Benison, G.C., Valafar, H., Pitts, K.E., Summers, A.O., Legault, P. and Omichinski, J.G., 2004. NMR structural studies reveal a novel protein fold for MerB, the organomercurial lyase involved in the bacterial mercury resistance system. *Biochemistry*, *43*(26), pp.8322-8332.

[13] Boopathy, R., 2000. Factors limiting bioremediation technologies. *Bioresource technology*, *74*(1), pp.63-67.

[14] Wingard, L.B., Katchalski-Katzir, E. and Goldstein, L. eds., 2014.*Immobilized enzyme principles: applied biochemistry and bioengineering* (Vol. 1). Elsevier.

[15] Care, A., Bergquist, P.L. and Sunna, A., 2015. Solid-binding peptides: smart tools for nanobiotechnology. *Trends in biotechnology*, *33*(5), pp.259-268.

[16] Patwardhan, S.V., Emami, F.S., Berry, R.J., Jones, S.E., Naik, R.R., Deschaume, O., Heinz, H. and Perry, C.C., 2012. Chemistry of aqueous silica nanoparticle surfaces and the mechanism of selective peptide adsorption. *Journal of the American Chemical Society*, *134*(14), pp.6244-6256.

[17] Sunna, A., Chi, F. and Bergquist, P.L., 2013. A linker peptide with high affinity towards silica-containing materials. *New biotechnology*, *30*(5), pp.485-492.

[18] Murtaza, I., Dutt, A. and Ali, A., 2002. Biomolecular Engineering of Escherichia coli Organo-mercurial Lyase Gene and its Expression, *Indian Jnl of Biotech*, Vol. 1, pp 117-120.

[19] Mertens, N., Remaut, E. and Fiers, W., 1995. Tight transcriptional control mechanism ensures stable high-level expression from T7 promoter-based expression plasmids. *Nature Biotechnology*, *13*(2), pp.175-179.

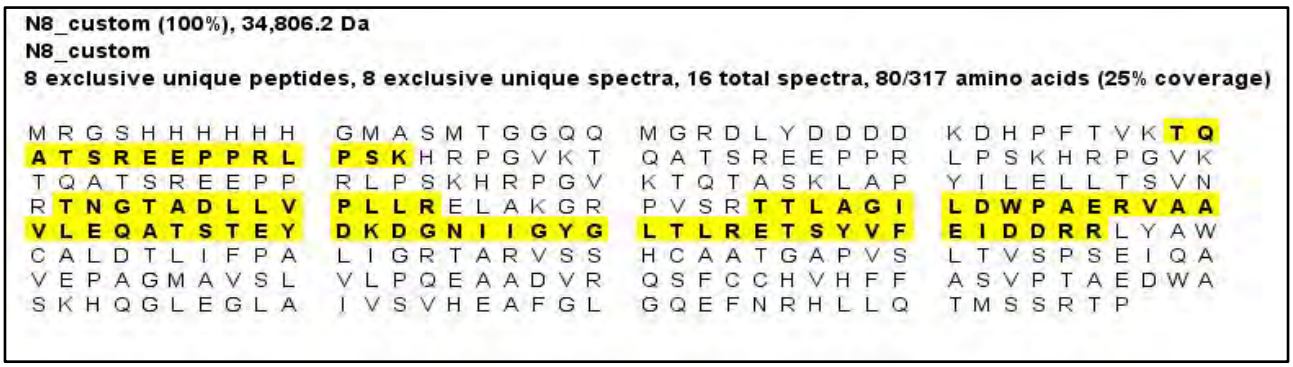
[20] Waugh, D.S., 2005. Making the most of affinity tags. *Trends in biotechnology*, *23*(6), pp.316-320.

[21] Nygaard, S., Wendelbo, R. and Brown, S., 2002. Surface-specific zeolite-binding proteins. *Advanced Materials*, *14*(24), pp. 1853-1856.

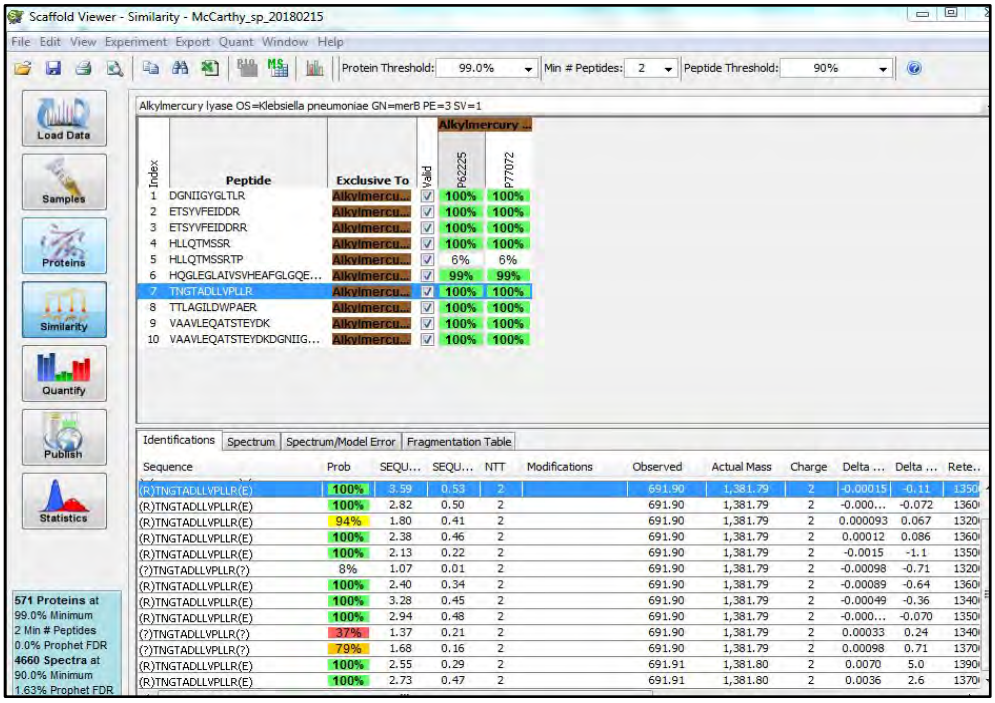
[22] Prilusky, J., Felder, C.E., Zeev-Ben-Mordehai, T., Rydberg, E.H., Man, O., Beckmann, J.S., Silman, I. and Sussman, J.L., 2005. FoldIndex©: a simple tool to predict whether a given protein sequence is intrinsically unfolded. Bioinformatics, 21(16), pp.3435-3438.

Supplementary data

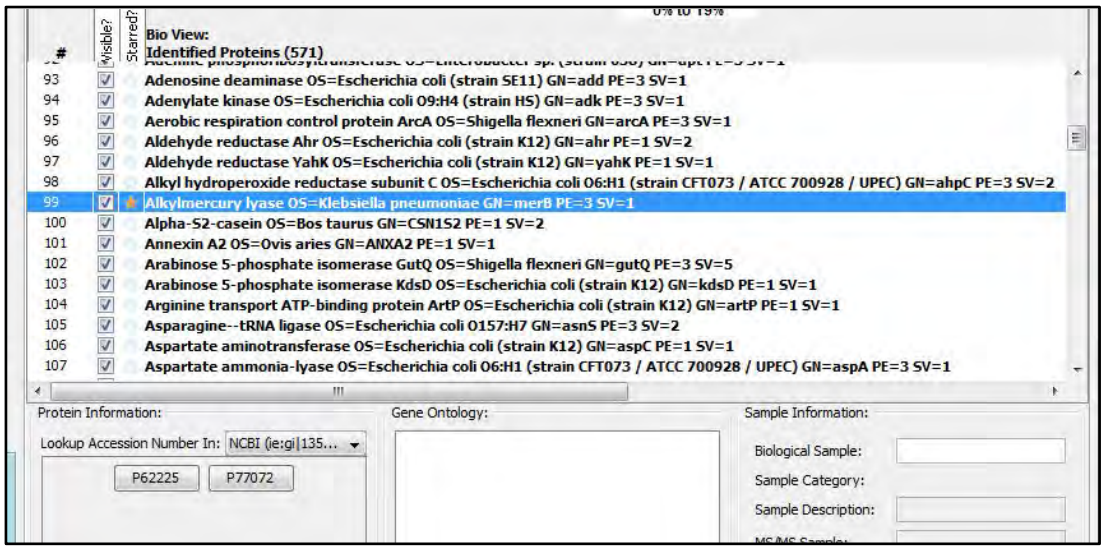
The following are for N terminal variant only.



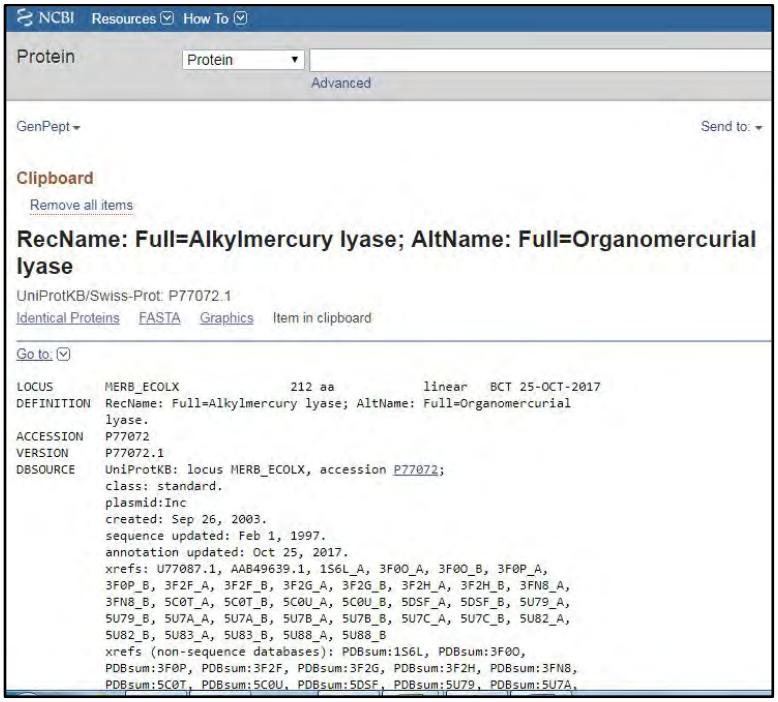
MS results following trypsin digest as per Scaffold v 4.8.4. Highlighted are peptide matches.



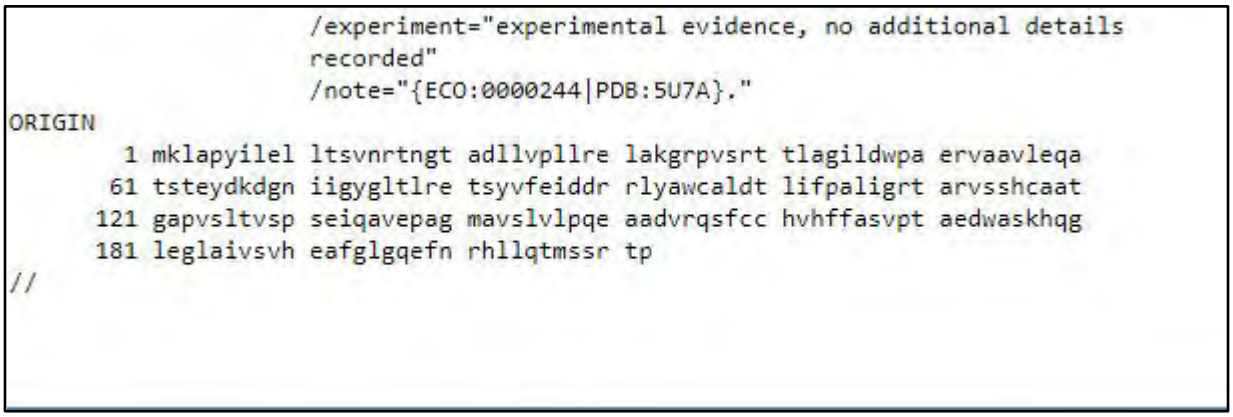
Scaffold output for second expression replicate (gel band excision). 10 unique peptide matches for P77072 (alkyl organomercurial lyase)



Scaffold output second replicate expression (gel excision) – mass spec results.



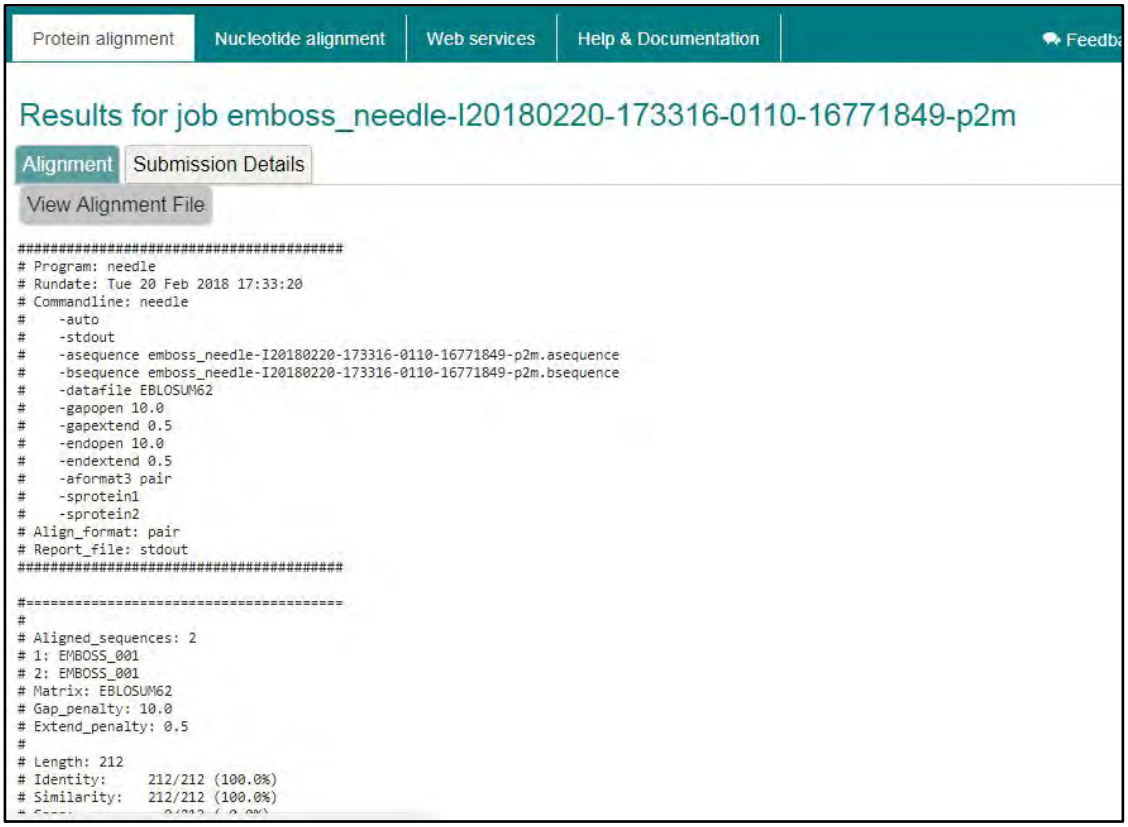
NCBI search results on accession number P77072



NCBI sequence confirmation on accession number P77072

			Database: McCarthy_N8_20171031.fasta (unknown version, 1 entries)									
			Digestion Enzyme: Trypsin									
			Max Missed Cleavages: 4									
			Probability Model:									
			McCarthy_N8_LM_20171026_custom_minpeptide4_NoRC_JustN8: Peptide Prophet with Delta Mass Correcti									
			McCarthy_1D_20180214_custom_minpeptide4_NoRC_JustN8: Peptide Prophet with Delta Mass Correction (									
Scaffold: Version: Scaffold_4.8.4												
		Modification Metadata Set: 2334 modifications										
			Source: C:\Program Files\Scaffold 4\parameters\unimod.xml									
			Comment:									
		Protein Grouping Strategy: Experiment-wide grouping with binary peptide-protein weights										
		Peptide Thresholds: 80.0% minimum										
		Protein Thresholds: 99.0% minimum and 2 peptides minimum										
		Peptide FDR: 7.4% (Prophet)										
		Protein FDR: 0.0% (Prophet)										
		GO Annotation Source(s):										
		Alternate ID Source(s):										
Experime	Biological	Biological sample n	MS/MS sa	Protein na	Protein ac	Database	Protein m	Protein id	Exclusive	Exclusive	Exclusive	Percentag	Percentage se
McCarthy_	Uncategor	N8	McCarthy_	N8_custor	N8_custor	McCarthy_	34,806.20	100.00%	8	8	16	9.70%	25.20%
McCarthy_	Uncategor	1Dband_Feb2018	McCarthy_	N8_custor	N8_custor	McCarthy_	34,806.20	100.00%	4	4	4	8.33%	16.40%
END OF FILE													

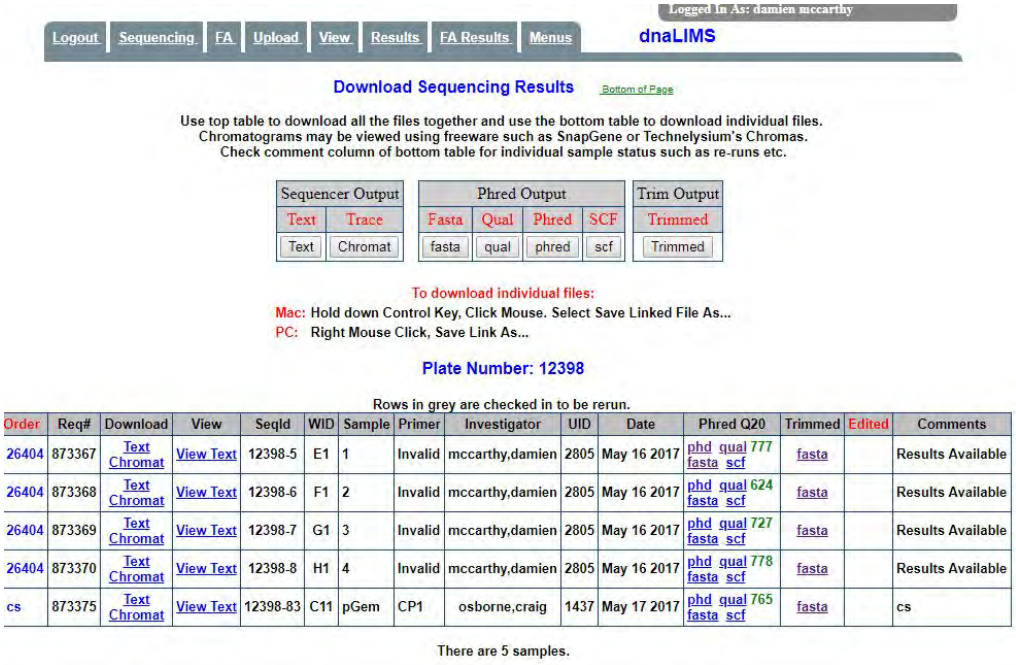
Excerpt from protein report indicating 100% id for both replicates



Emboss Needle pairwise sequence results P77072 amino seq vs designed construct (100% match 212 residues).

```
#=======================================
#
# Aligned_sequences: 2
# 1: EMBOSS_001
# 2: EMBOSS_001
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 212
# Identity:     212/212 (100.0%)
# Similarity:   212/212 (100.0%)
# Gaps:           0/212 ( 0.0%)
# Score: 1084.0
#
#
#=======================================

EMBOSS_001         1 MKLAPYILELLTSVNRTNGTADLLVPLLRELAKGRPVSRTTLAGILDWPA     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001         1 MKLAPYILELLTSVNRTNGTADLLVPLLRELAKGRPVSRTTLAGILDWPA     50

EMBOSS_001        51 ERVAAVLEQATSTEYDKDGNIIGYGLTLRETSYVFEIDDRRLYAWCALDT    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        51 ERVAAVLEQATSTEYDKDGNIIGYGLTLRETSYVFEIDDRRLYAWCALDT    100

EMBOSS_001       101 LIFPALIGRTARVSSHCAATGAPVSLTVSPSEIQAVEPAGMAVSLVLPQE    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       101 LIFPALIGRTARVSSHCAATGAPVSLTVSPSEIQAVEPAGMAVSLVLPQE    150

EMBOSS_001       151 AADVRQSFCCHVHFFASVPTAEDWASKHQGLEGLAIVSVHEAFGLGQEFN    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       151 AADVRQSFCCHVHFFASVPTAEDWASKHQGLEGLAIVSVHEAFGLGQEFN    200

EMBOSS_001       201 RHLLQTMSSRTP    212
                     ||||||||||||
EMBOSS_001       201 RHLLQTMSSRTP    212


#---------------------------------------
#---------------------------------------
```

Emboss Needle pairwise sequence results P77072 amino sequence vs designed construct confirming mass spec id match 100%.



Logged In As: damien mccarthy

Logout | Sequencing | FA | Upload | View | Results | FA Results | Menus — dnaLIMS

Download Sequencing Results Bottom of Page

Use top table to download all the files together and use the bottom table to download individual files.
Chromatograms may be viewed using freeware such as SnapGene or Technelysium's Chromas.
Check comment column of bottom table for individual sample status such as re-runs etc.

Sequencer Output		Phred Output				Trim Output
Text	Trace	Fasta	Qual	Phred	SCF	Trimmed
Text	Chromat	fasta	qual	phred	scf	Trimmed

To download individual files:
Mac: Hold down Control Key, Click Mouse. Select Save Linked File As...
PC: Right Mouse Click, Save Link As...

Plate Number: 12398

Rows in grey are checked in to be rerun.

Order	Req#	Download	View	SeqId	WID	Sample	Primer	Investigator	UID	Date	Phred Q20	Trimmed	Edited	Comments
26404	873367	Text Chromat	View Text	12398-5	E1	1	Invalid	mccarthy,damien	2805	May 16 2017	phd qual 777 fasta scf	fasta		Results Available
26404	873368	Text Chromat	View Text	12398-6	F1	2	Invalid	mccarthy,damien	2805	May 16 2017	phd qual 624 fasta scf	fasta		Results Available
26404	873369	Text Chromat	View Text	12398-7	G1	3	Invalid	mccarthy,damien	2805	May 16 2017	phd qual 727 fasta scf	fasta		Results Available
26404	873370	Text Chromat	View Text	12398-8	H1	4	Invalid	mccarthy,damien	2805	May 16 2017	phd qual 778 fasta scf	fasta		Results Available
cs	873375	Text Chromat	View Text	12398-83	C11	pGem	CP1	osborne,craig	1437	May 17 2017	phd qual 765 fasta scf	fasta		cs

There are 5 samples.

Genomic centre results

GTGCATGCAAGGAGATGGCGCCCAACAGTCCCCCGGCCACGGGGCCTGCCACCATACCCACGCCGAAAC
AAGCGCTCATGAGCCCGAAGTGGCGAGCCCGATCTTCCCCATCGGTGATGTCGGCGATATAGGCGCCAGCA
ACCGCACCTGTGGCGCCGGTGATGCCGGCCACGATGCGTCCGGCGTAGAGGATCGAGATCTCGATCCCGCG
AAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAAC
TTTAAGAAGGAGATATACATATGCGGGGTTCTCATCATCATCATCATCATGGTATGGCTAGCATGACTGGTG
GACAGCAAATGGGTCGGGATCTGTACGACGATGACGATAAGGATCATCCCTTCACCATGGTTAAAACCCAG
GCAACCAGCCGTGAAGAACCGCCTCGTCTGCCGAGCAAACATCGTCCGGGTGTGAAAACACAGGCCACCTC
ACGCGAAGAACCTCCACGCCTGCCTTCAAAACACCGTCCTGGCGTAAAAACGCAGGCGACAAGTCGTGAG
GAACCTCCGCGTTTACCGTCTAAACATAGACCTGGGGTGAAAACCCAGACCGCAAGCAAACTGGCACCGTA
TATTCTGGAACTGCTGACCAGCGTTAATCGTACCAATGGCACAGCCGATCTGCTGGTTCCGCTGCTGCGTGA
ACTGGCAAAACGTCGTCCGGGTTAGCCGTACCACACTGGCAGTATTCTGGATTGGCCTGCAGACGTGTTGC
AG

Chapter 7 – Results and Discussion

7.0 Discussion

As the particular results have been discussed at length individually in the research papers enclosed herein, this section will instead attempt to synthesise those results into a cohesive narrative. Important findings will be highlighted that show the potential utility of the approach taken. The research can be summarised as proposing a mechanism to deliver inoculants for mobilising terrestrial mercury from biocatalytic emissions, a novel device to capture those emissions, and synthesis of a bound organo-mercurial lyase with potential for reducing methylmercury concentrations.

A bacterial strain (*P.veronii*) known to be somewhat tolerant to mercury from previous unpublished work by the author was bound to a natural bulk substrate (zeolite) via encapsulation in a xanthan gum based biopolymer and applied to mercury contaminated mine tailings. The current work affirmed suitability of this organism, in that matrix bound mercury was indeed actively converted to gaseous mercury. Of interest in the current experiments were the effect of inoculation and water treatments on the rates of Hg volatilisation, viability of the organism over time, and transport and storage of the bound organism. Besides GEM, also of interest was the effect (if any) on GOM emissions, as reactive mercury species are of major environmental concern.

Microbial bioremediation is not a novel concept, having been deployed successfully in many arenas for many pollutants, including mercury. However, this work represents the first known use of microbes to volatilise mercury from contaminated soils after immobilisation on a natural bulk substrate. The results generally mirror what is seen with bacterial inoculants for soil and sediment-based remediation of mercury via volatilisation. That is, there was an initial positive large spike in emissions post inoculation that quite rapidly reaches a peak and then tails off. This tailing off does not seem to be

from a rapid decline in population numbers, as viability measurements do not seem to indicate this is the issue. Increasing water amplified the spike, but did nothing to aid continued emission rates. One explanation may simply be a rapid decline in bioavailable mercury. In any case, the spike in these experiments was substantial, reaching at least 4 orders of magnitude above background levels, which if sustainable, could greatly reduce soil remediation times.

The success of the remediation of Minamata Bay sediments was largely due to proactive acid-mobilisation of the mercury together with inoculation with *mer* carrying microbes. Their initial measures suffered the same decline in volatilisation activity as seen in the research contained herein, but when combined with Hg mobilisation, their approach proved much more successful. That issue is beyond the scope of this work, but noteworthy in that it reinforces the fact remediation techniques will need to be determined by the characteristics of each site. This work has not undergone a field trial at scale at this stage, but these results do show that cells with mercury transformation capabilities can be bound to a solid bulk substrate, stored in ambient conditions in sealed containers, and transported with relative ease without consequential impact to viability and more importantly, with retained functionality. Of note is that the material used for the flux experiments was untreated tailings from a heap leaching operation, indicating any pre-treatment required might be minimal.

This solution represents a facile low cost and scalable way to deliver inoculants to Hg contaminated soils and other terrestrial sites such as mining operations. By inference, this solution is not limited to mercury remediation, and many other bacterial inoculants can be deployed in this manner, including potentially as microbial consortia. The problem with transporting sufficient microbial inoculants is greatly reduced if they can be stored and transported in a granular format that requires only tilling and watering to start the remediation going. Another potential use is for batch processing, whereby excavated

material could be inoculated off-site. This would reduce complexity at processing facilities, as the inoculant could readily be stored on site, and would assist in management of leakage from any mercury mobilising pre-treatments used.

One unexpected but noteworthy result was the reduction in GOM emissions using bacterial inoculants, and this is the first known instance of a bioremediation technique showing this result. This makes sense in that there is normally always trace levels of GOM that accompanies naturally occurring GEM emissions, and increased microbial conversion of $Hg^{2+} \rightarrow Hg^{0}$ seems to non-randomly reduce availability to escape as GOM. It was unclear what the microbial uptake and subsequent removal of Hg^{2+} as GEM would have on GOM emissions, as no published work was available for reactive mercury emissions during such treatments. Gaseous oxidized mercury emissions were therefore monitored simultaneously with GEM. It is not currently possible to differentiate in real time between mercury species, and so to assess total GOM, suitable selective filters were tested by digestion for GOM content at the completion of the trials. This is an important finding from an ecotoxicology perspective as GOM is more reactive and also more available for methylation to highly toxic MeHg compounds, and finding ways to reduce GOM can only have beneficial environmental consequences.

Several limitations to this experiment are noted due to time constraints in that no attempt was made to assess long term viability of organisms once applied to the contaminated matrix, and no attempt was made to assess bacterial community dynamics in the matrix. In fact, viability was measured separately from GEM flux. This is because viability was measured in sterile soils to reduce confounding influences, but it is important to evaluate inoculant viability as compared to desired functionality, in this case increased GEM emissions. Important information could emerge that would assist in optimising the process.

Further, no attempt was made to optimise a strain for the purpose, or to search for a strain with hyper-volatilising properties, although both these aspects could be explored in depth. The organism was simply chosen from a small but readily available selection of organisms, as mercury resistance in *P. veronii* had separately been established. However, the literature suggests a more robust approach would be to isolate an endemic strain from an area to be remediated, amplify this strain, and inoculate the site with that increased concentration. Binding said organism to a bulk substrate such as zeolite granules could potentially greatly simplify the logistics of this approach. Research into the binding capacity of a wide distribution of *mer* carrying organisms might provide a library that could be referenced by the practitioner when acquiring the endemic strain.

An important question seemingly not yet explored in the literature or in these experiments is whether simply increasing the abundance of bioavailable mercury might naturally produce increased numbers of volatilising microbes, negating the requirement for inoculants. This could have deleterious effects on surrounding biota until the mercury was remediated and equilibrium was achieved, but data in this regard may provide some basis for estimating the value of inoculants. The rate of remediation will largely depend on the turn-over rate from sequestered to bio-available mercury in any case, which is why mobilising techniques are used to speed up the process. This area could be explored in depth to parameterise at least some of these characteristics.

Previous research on immobilised cells for Hg transformation is limited to Hg intracellular incorporation and used alginate beads as both the encapsulation and delivery mechanism. Alginate bead encapsulation does have many benefits in that the encapsulation matrix can be manipulated or doped, and it also becomes the delivery product, negating attachment to a substrate. However, raw material costs are about 50% higher for alginate bead immobilising technology compared to the zeolite / xanthan gum biopolymer system in this research. Additionally, alginate bead synthesis and

encapsulation processes are simple but so is bio-functionalising zeolite, which is essentially an add and mix process, greatly simplifying scale up.

As far as immobilising *P. veronii* for the purpose of volatilising matrix bound mercury, it can be stated that proof of concept has been shown in that the cells were successfully bound to a zeolite bulk substrate using a xanthan gum based biopolymer, and cells remained sufficiently viable during storage, transportation and inoculation, and further, retained functionality as it relates to actively increasing GEM emissions to a level where remediation using this method becomes viable.

Of course, volatilising Hg by any method so it escapes only shifts the environmental burden to the atmosphere unless the emissions are captured in some manner. Mercury emissions in general is a highly active and fertile research area, as can be seen by the quantity of research material dedicated to the issue. Given the first series of results that showed inoculation of mine tailings with immobilised cells could substantially increase GEM emissions, it came to mind that an adapted geotextile might be employed for the purpose of capturing those emissions when applied at scale. Other substrates might have been investigated, but as coir is already widely used as a geotextile, is available in bulk at low cost, and is a renewable resource, it seemed an ideal substrate to investigate.

To capture GEM, a coir fibre substrate coated with a siloxane polymeric formulation that is infused with a metal halide was constructed, and shown to efficiently bind GEM in ambient conditions in a solid, stable and non-soluble form. This product could potentially be used in a variety of natural and industrial settings and represents a less expensive method than those currently employed, while additionally generating less secondary waste. The vexing issue of mercury emissions has been a topic of greater interest in the last decade or so as more data has come to light on the extent and complexity of the problem. While diffuse emissions from artisanal gold mining accounts

for the highest overall anthropogenic contribution to atmospheric mercury, little applied research has been devoted to addressing GEM capture in that context.

Compared to industrial point sources emission capture, such as from coal combustion, virtually no literature exists in the area of abatement of ASGM emissions. This is likely due to difficulties with scale and the diffuse nature of emissions, but equally because gaseous mercury has proved very difficult to capture. If emissions can be isolated and are free of other contaminants, mercury can be readily isolated by condensation, or through activated carbons. However, activated carbons are very expensive to manufacture. At the scales required for site remediation and similar which may require tons of carbon, this becomes economically problematic. Further, capture of diffuse emissions is difficult at logistically. However, a coir-based geotextile may provide an economical and scale-able solution, as it can be simply rolled out over the surface of an area. The product could conceivably also be adapted to suit many point source emissions.

Many other coatings could have been examined for the purpose, and this may prove a fruitful area of future research. However, for the purpose of proof of concept of a GEM capture device, such optimisation considerations were ignored in this body of work. The basic requirements were that the material was inexpensive, had fibre protective qualities, and provided a surface upon which GEM capture material might be securely affixed. An additional benefit would be if the attachment was reversible such that any bound mercury could be removed and isolated, which is why the three binding materials were chosen – there was no polymeric cross-linking required.

The results of the formulation characteristics showed that a poly-siloxane coating was the most desirable in relation to the above general characteristics. It is available in bulk at low cost and is inert. In comparison, being cellulosic, it was very quickly revealed through microbial contamination that CMC was not a good option, as part of the reason

for the coating was to protect the integrity of the geotextile, which could potentially be on site for years. CMC suffered from other poor handling characteristics as well and is rather insoluble, meaning obtaining a consistency amenable for coating fibres was problematic. While natural rubber had many positive characteristics, supply is an issue, and it can be difficult to work with due to changeable viscosity. A poly-siloxane coating seems to satisfy the desired characteristics, and importantly does not require cross linking so in terms of recovery of mercury, this means attachment to the substrate can be reversed. In other words, the coating can be redissolved. This means any solid mercury bound could be isolated through gravitation or other standard means, and theoretically recoverable as copper(I) tetra-iodide mercurate.

Unfortunately, the capture characteristics of the different GEM capture formulations proved difficult to measure and a thorough examination and comparison to biochar type versions was not possible. Instrument failure was an issue that requires a fresh approach to quantifying the capture characteristics, as contamination from excessive copper, silicone, or indeed a combination of both means a less direct method must be employed. Unfortunately, bound mercury was not able to be very accurately quantified such that mass balance considerations could not be attempted.

The GEM capture experiments proved much more successful in that accurate data was able to be achieved and replicated. Scale of the experiment meant that it was difficult to examine any higher filter tolerances, but this is the next obvious series of questions that one might ask. Halogens are known to react with mercury gas to varying degrees, but only iodine reacts strongly and also forms stable and virtually insoluble solids that will not readily re-volatilise, a strongly desirable characteristic given the current application. This is especially so for terrestrial settings, as the material would be exposed to the weather. Copper iodide is dangerous to aquatic life, so unreacted material would also have to be strongly bound to the matrix. This was not tested to any great extent except

through manual manipulation, but no loss was detected in these experiments when CuI was bound to silicone-based coating. In terms of environmental safety, this leaching off the substrate aspect would need further examination and or potential management.

The most relevant issue at hand in terms of capture of GEM is that no catalytic oxidation of the mercury was required for the adapted coir to work. It is recognised that activated carbon capture of mercury is greatly enhanced if the mercury is oxidised, either prior to contact or at the interfacial boundary, and desorption is problematic in an evolving environment such as is encountered in flue stack gasses. Capture of mercury in coal combustion is largely a happy coincidence from SOx and NOx reduction strategies via oxidation, but non-oxidised GEM largely remains uncaptured. This device might fill a substantial gap in mercury capture for existing coal fired power stations with little modification. For example, if situated downstream from traditional scrubbing devices, particulate and other chemical interference could be reduced. However, the technology described herein could be applied to ASGM and other gold mining activities with ease in its current form as it is known to capture GEM in ambient conditions.

While there remain questions over suitability for particular industries, constrained only from a lack of data, it seems coir adapted in this manner to capture GEM emissions is a very promising technology. It can be potentially used at scale for gold mining operations, soil remediation, and to reduce ambient concentrations in ASGM operations with very direct health benefits to those workers and can also potentially be deployed in a range of high mercury emitting industries.

Having addressed both terrestrial and atmospheric compartments with potential technologies for mercury reduction, the final aspect of this work relates to attempts to reduce methylmercury in the aquatic compartment using an immobilised organo-mercurial lyase. Immobilised enzyme technology is well advanced and many industrial applications make use of this approach, with an emerging emphasis in the literature on

biocatalysis. Where site remediation also involves methyl mercury, for example in wetlands, it is envisioned that a combinatorial approach could be taken using immobilised cells and the coir mat as previously described, and immobilised enzymes to both de-methylate and reduce methylmercury production rates.

Previous work by the author used a mercuric reductase bound to zeolite nano-particles, with some indirect evidence to show this product remained functional while bound (McCarthy, 2015). The reader is directed to page 55 of that study to review the assay results. The data is not presented here as the author feels that data is not reliable, specifically because there remains a question over why the oxidation gradient was not smooth over time, and there were no replicates due to time constraints and a lack of available enzyme. However, as dissolved mercuric ions in aqueous environments can be readily captured efficiently by many existing cheaper alternatives, and this method requires expensive NADPH as a co-factor, this approach was not thought worth exploring. However, that work did raise the question of whether the methyl-mercury degrading enzymes of the *mer*B carrying organisms could also be bound, as these do not require co-factors, potentially simplifying deployment. This may lead to other applications such as biosensorics detectors, discussed in the future direction section following this discussion.

This research is the first known attempt to create a biomimetic device to degrade methylmercury. Specifically, a recombinant gene was designed as to make a dual role polypeptide, in essence an organo-mercurial lyase that also binds to silica via addition of an SBP sequence that directionally tethers the enzyme to a solid substrate. The synthetic DNA was successfully expressed by commercially available strains of *E.coli,* and further, was shown to successfully bind to the substrate, in this case faujasite zeolite particles. Unfortunately, enzymatic functionality and optimum operating parameters remain as unsolved at this stage.

Expression was difficult under standard isopropyl β-D-1-thiogalactopyranoside (IPTG) induction, and it was found lactose induction was more suitable. This may have something to do with the intrinsic toxicity of the lyase to the organism, and lactose induction seemed to allow for more robust and repeatable expression, perhaps due to the growth stage of the colony at induction. However changing IPTG induction timing points did not resolve the issue of expression using that reagent, and the cause of that problem remains a mystery. Regardless, robust expression was achieved through the alternate induction method without a complicated protocol.

The produced enzyme was readily purified by standard gravimetric methods and bound to the solid substrate. This was as expected as far as the SBP binding is concerned, as this fact is well characterised in the literature. However, this is the first time an organo-mercurial lyase has been tethered in this way. Functionality testing was hampered in part due to a lack of literature on suitable investigative methods. The protocol used in this research did not prove successful, but of note is that the approach substituted methyl-mercury. Safety issues and a lack of MeHg expertise rendered experimental work on enzyme functionality difficult, and due to logistical and time constraints, it was not possible to outsource this set of experiments at this stage

Currently, while it is known methyl-mercury is extremely toxic, the approach is to try and simply remove mercury from a site more generally in hope of reducing methylation, and while this certainly needs doing, existing mercury is subject to methylation. Natural demethylation does occur, through both abiotic and biotic pathways. It is rather challenging to manipulate the abiotic factors involved in demethylation (such as sunlight), and it is hoped that augmenting environmental demethylation rates by deploying biomimetic tools for this purpose could reduce the environmental burden of MeHg species. At any rate, natural production of methyl mercury from contaminated areas is biogenic and depends on several complex environmental factors, but seemingly

only minimally related to mercury loading. Two sites with differing aquatic chemistry and micro-ecology will produce different levels of MeHg given similar mercury levels.

Many successful bench scale trials have shown how engineered bio-molecular solutions can be employed to treat aqueous phase mercury. Natural microbial solutions may involve binding, encapsulating, chemically transforming, harnessing toxicity, phase changes, active transport and more. Similarly, bio-molecular solutions rely on these evolutionary inspirations as a way of targeting Hg, most often as Hg^{2+}, that being the most commonly found ionic state. While methylmercury only accounts for about 1-2% of overall mercury, there still seems a lack of literature on treating methylmercury contamination, given its extreme toxicity. There is a substantial body of work on the effects of methylmercury (MeHg) from a health and environmental health perspective, as well as on molecular structural aspects, biomolecular mechanisms, production and degradation fluxes, geographic distribution and other factors influencing potential harm. However, except for total removal of mercury as a source which is not practical in many areas, there exists a gap in *solutions* targeting a reduction in methylated mercury (MeHg), often occurring as the cation CH_3Hg^+. This research begins investigations into whether bio-functionalising a solid substrate for deployment to sites is a viable plan.

Working with MeHg is notoriously problematic due to extreme toxicity, and the subsequent imposts and expertise required may be why there is a seeming lack of literature on remedial approaches. In any event, biomolecular research aimed at mercury remediation generally shows promise but importantly, suffers from limited demonstrable scale up successes at this stage. As stated, methylmercury is highly toxic, with even minute amounts of dimethyl-mercury in particular being extremely dangerous, a point highlighted by the 1997 accidental death of Karen Wetterhan, renowned Professor of Chemistry at Dartmouth College, New Hampshire, USA.

Alternatives could be attempted for comparative purposes. For example as the main route to *in situ* methylation of mercury is known to largely depend on the existence of sulphur reducing bacteria (SRB) in the system, one could take a broad brush approach and inhibit sulphur reduction, for example through the addition of molybdate to sediments. This would reduce the SRB population size, potentially reducing methylation. Molybdate is any salt in which the anion contains molybdenum and oxygen, usually in the form MoO_4^{2-}. Methylmercury production is known to be highest in anoxic sediments, in particular at the anoxic / oxic boundary, usually located near the sediment surface, meaning a sulphur reducing inhibitor could readily be deployed to reduce MeHg production. This may be very much more practical than removing mercury in many cases. Molybdenum (Mo) acute poisoning has not been demonstrated in humans, although rat LD_{50} rates of 180 mg kg^{-1} were seen for some Mo compounds. The US National Academy of Medicine (NAM) sets the upper limit (UL) at 2000 µg Mo day^{-1}. One can envision management of such an approach to limiting MeHg primary production such that any Mo leaching into drinking water systems from the affected catchment could be kept well under that limit.

However, such a broad brush approach is not without problems, the most significant being the impact across the entire system from inhibiting sulphur reduction. These effects are impossible to predict entirely accurately even with a detailed knowledge of relevant site specific characteristics, particularly those related to MeHg production. Such disruptions to microbial populations requires a thorough understanding of potential outcomes. This is not just good management practice, in the case of MeHg production, because of its acute toxicity, understanding intervention impacts to the environment is non-trivial because one may inadvertently create the conditions for *increased* MeHg production, for example, through suppression of methanogens. Furthermore,

management of other important abiotic factors such as water temperature and DOC would prove impossible or at least too difficult to manage in practice.

Much research has been undertaken in understanding the DNA sequence, physical structure and mechanics of the lyase molecule, as well as phylogenetic distribution of the *mer*B gene, but little research has been undertaken in applying this knowledge to active solutions to degrade methyl mercury. Given the problems and complexity associated with altering benthic community structure in an open system, or manipulation of abiotic factors, it is probably not feasible to try and resolve net mercury methylation problems by these approaches. However, one may take a more direct approach to actively decrease methyl-mercury compounds, drawing inspiration from microbial solutions by utilizing immobilised enzyme technology. More research is required to better assess whether the enzyme remains functional while bound, and under what physico-chemical conditions such functionality is maintained.

Given the complexity of mercury related environmental problems, from its changeable chemical state, to abiotic and biotic environmental conditions, and the many ways the biogeochemical cycle affects distribution and impacts of this heavy metal, it is critical to take steps to not only reduce new production and emissions but to actively remediate existing problems. This research has contributed something in each environmental compartment, and should be seen as a cohesive approach with the aim of adding more tools for the remediation specialist to draw upon when it comes to mercury remediation.

In summary, this research has contributed important findings and insight into the complex area of remediation of mercury pollution. Microbial cells previously immobilised and stored were shown to exhibit continued high mercury volatilising capability when applied to contaminated mine material, and further, such emissions could be captured effectively without catalytic oxidation using a novel approach derived from renewable resources. For

methylmercury treatments, an immobilised enzymatic approach shows promise in that using synthetic biology, an enzyme that de-methylates mercury could be tethered to a readily deployable substrate.

7.1 Conclusion

The complexity and costs of remediation are significant, much higher than any resale value for recovered mercury. Thus, remediation requires not only effective solutions that are safe and robust, it also needs low-cost technologies as key to successful remediation at the scales required. This body of work has attempted to show proof of concept for several novel low cost and scalable solutions to solve some problems often encountered, and in that sense has been largely successful. More specifically, to evaluate the overall contribution of this research, the following restatement of aims together with a brief research evaluation is provided to assist the reader map out research outcomes compared to those stated aims.

I. To show proof of concept for binding Pseudomonas veronii cells to zeolite by encapsulation in a biodegradable biopolymer

This research shows that *P.veronii* cells can in fact be successfully bound to a solid substrate, that being zeolite particles. A xanthan gum-based biopolymer was made from inexpensive and readily available products using a simple add and mix procedure. Loss of cells during the binding procedure was kept to a reasonable level ensuring a sufficient concentration was bound to allow for a final product with the catalytic power to actively remove mercury from untreated contaminated mine tailings. The biopolymer ingredients are biodegradable, available in bulk at low cost, as is the substrate, and no specialised equipment was required to achieve binding. It should be noted no attempt was made to find an optimised strain, as in practise the strain will depend on the site-specific circumstances of the remediation. However, *Pseudomonas* strains are ubiquitous in soil, carry limited risk to humans, and are known to harbor the *mer*A gene responsible for catalysis of mercury, and importantly, can be cultured with relative ease.

II. *To examine the viability of those immobilised cells during storage*

Weekly assessment of viability over an extended period of storage of bio-functionalised zeolite showed that bound cells retained sufficient viability. After an initial decline during the first few weeks, the bound population stabilized at about 10^6 cfu mL^{-1}, and subsequently remained stable around this level during the course of assessment of four months. The binding process is exothermic which is damaging to cells, but viability was not affected to the extent where it was considered a major obstacle. This stabilised viable level represents sufficient concentration of cells to achieve active catalysis of divalent mercury to GEM. No special requirements are necessary for storage except to keep the material dry and to keep contamination of the biopolymer to a minimum (for example from air borne fungi and similar). Further, the material was transported internationally and showed no deleterious signs (for example, from the conditions in the cargo hold of an airplane), and cells retained functionality after such transportation.

III. *To evaluate the effectiveness of zeolite-bound cells as an inoculant in removing mercury from contaminated mine tailings*

Biofunctionalised zeolite particles were added to untreated mine tailings that were heavily contaminated with mercury. The results showed active catalysis that produced two results; a significant increase in mercury volatilisation, and a reduction in oxidised mercury emissions. GEM emissions at their peak were at least 10^4 above background levels, and this strongly suggests a dramatic reduction could be achieved in soil remediation time. It remains unclear whether the cells remain viable for sufficient time once material is inoculated to achieve full remediation, as a peak in catalytic activity is rapidly followed by steady decline. It was impossible to test viability once inoculation had occurred as the system was open and thus confounding factors could not be sufficiently

controlled. Similarly, no speciation characteristics of the mercury were monitored for tailings bound fractions. These issues are explored in the final part of this chapter. Natural zeolites are an inexpensive substrate on which to base immobilised techniques. Such bio-functionalisation may offer a readily transportable, storable and easy to use platform technology that is highly scalable.

IV. To design a synthetic gene that could be cloned into E. coli cells for production of an enzyme with methylmercury transformation capabilities

A synthetic gene coding for a fusion enzyme containing an organo-mercurial lyase and an SBP linker that could bind to zeolite particles was designed *in silico*. The DNA synthesis was done commercially, and subsequently expressed in standard *E. coli* strains. Cells were successfully transformed using standard biomolecular techniques and confirmed using antibiotic resistance and DNA sequencing. Expression was confirmed using standard protein gel electrophoresis and mass spectrometry of the purified enzyme. Production of this novel enzyme was achieved using standard laboratory techniques suggesting this may be a practical solution to employ this enzyme for the purpose of methylmercury treatment.

V. To evaluate that organo-mercurial lyases ability to bind to zeolite particles

The novel enzyme was purified and successfully bound in a directional and reversible manner to faujasite zeolite granules. Binding assays showed the enzyme could be readily bound to zeolite using a simple add and mix technique. In theory, these granules could be used to construct fixed bed reactors of varying configuration, although functionality remains undetermined at this stage. The reactors could potentially be deployed in areas

prone to methyl-mercury production such as wetlands, either as stand-alone technology used in conjunction with other remediation strategies.

VI. *To examine the biomimetic particles for methylmercury transformation functionality*

This aim has not been achieved as yet. This is largely due to a lack of clear protocols for establishing experimental parameters in measuring functionality and the lack of proper technical expertise in dealing with such a highly toxic substance as methylmercury. Unfortunately, the assays attempted were not conclusive and results could not be replicated. A methylmercury analogue was used, but it is envisioned that a suitably established and qualified laboratory for measuring methylmercury transformation could be engaged to examine the particles for functionality against methylmercuy species.

VII. *To develop an alternative to activated charcoals to capture gaseous mercury emissions without prior catalytic oxidation or other physico-chemical treatment*

Gaseous elemental mercury was successfully captured without prior physico-chemical alteration required. A coir fibre-based substrate was coated with silicon that had copper iodide crystals embedded on the surface. A plume of GEM was passed through an enclosed filter packed with the functionalised substrate while mercury concentrations of the plume were monitored. The substrate exhibited excellent GEM capture characteristics. This is an inexpensive alternative to activated carbons that shows excellent promise as it can be readily scaled, is made from simple and renewable resources, and requires only minimum post processing. A wide variety of applications are envisaged for this technology. Much research has been directed at finding suitable catalytic technologies for capture of GEM, however this technology does away with that complication.

To summarise, the complexity of the environmental problems caused by mercury stem from the intrinsic characteristics of this heavy metal, evolved mechanisms of biota to deal with this pressure, and other abiotic factors that aid cycling through each environmental compartment. This requires a multi-lateral approach, but also presents opportunities to exploit these very factors in an endeavour to seriously address the problem of mercury pollution. This research has contributed much to the practitioners' tool kit for mercury remediation solutions.

7.2 Future Directions

This body of work has brought to light some further areas requiring investigation. This is either to extend the work where proof of concept has been established or to cover areas where the results have been less than optimal. The following is added as a future road map of desired research required to fill the afore mentioned gaps.

Bio-functional zeolite

Successful proof of concept for *P. veronii* bound to zeolite particles via entrapment in a biopolymer has shown that, in theory, this could be an ideal way of transporting large volumes of inoculants to mercury remediation sites. While bacterial community dynamics were not studied as part of this work, this aspect would need consideration for scale up. Bacteria do not work in isolation but rather work as communities, so an understanding of this aspect would be beneficial in designing the remedial works. For example, it is important to know how well inoculants survive in an open system. The viability studied in this work was limited to pots of pre-sterilised soil to ascertain ongoing viability of the inoculant, but in practise this could not be done. For this reason, research into environmental DNA (eDNA) during remediation would help practitioners assess the bacterial community health and dynamics.

Further, as a pronounced GEM emission spike was noted after which time the rate of mercury volatilisation decreased, it would be very useful to know the reasons for this. This could be a result of inoculant population collapse, or some other factor affecting the community dynamics, such as the watering regime employed to achieve re-animation of cells once applied to the contaminated material. This may also be from a lack of nutrients

available, as no pre-conditioning of the material was attempted. eDNA studies may shed light on whether the inoculant was sufficiently viable.

Alternatively, the rapid decline of the GEM emission rate could simply be a result of lower bio-available mercury in the system from active removal via GEM. This is a reasonable assumption given divalent mercury is highly reactive. A detailed mercury speciation investigation is warranted on soil being remediated, as this would inform practitioners the best option for increasing bio-available mercury for active uptake and expulsion as GEM by the inoculant. It is difficult to assess the reasons for the spike in emissions without investigating these two aspects, that is, both bacterial community dynamics and mercury speciation dynamics.

Another important aspect requiring investigation following this proof of concept for the technique would be to assess an optimal strain for the purpose. Literature strongly suggests the best way forward is to enhance populations of indigenous strains capable of catalysing mercury in this manner. A library of such strains could be established for differing geographical locations and environmental niches for ready use by practitioners. Alternatively, a hyper-volatilising strain could be sought that was robust enough to accommodate a variety of environmental circumstances. This may require increased conditioning of the site prior to inoculation to ensure the strain had conditions where it remained viable. Either way, optimising the strain for the purpose of mercury removal from the matrix may have large impacts on the time taken for remediation, and therefore economic aspects of the remediation plan.

Additionally, if one uses phyto-remediation to clean matrices of mercury, one is left with bulk contaminated biomass. This has proved difficult to process, but one unexplored aspect is composting this material using *mer*A containing strains of bacteria. The material could then be turned into useful secondary products while removing mercury at the same time.

Gaseous mercury capture

The primary reason for constructing a mat was to overlay soils and larger areas such as heap leaching beds to capture emitted gaseous mercury. Proof of concept was shown for this technique in that the GEM was captured in ambient conditions without the need for catalytic pre-treatment of the gas. However, the matting could be configured into a variety of shapes depending on application such as tubes, cones, loose bags etc.

The application of the technology to other sources of GEM, such as from cement manufacture or coal combustion, requires further investigation. The reason is the complexity of the chemistry and thermal characteristics of such environments are obviously quite different and dynamic, and further, other material such as ash particulates could impede the efficiency of the substrate. As such, this aspect requires a detailed examination of the relevant aspects which may include thermal and chemistry, as well as physical aspects such as the impact of plume velocity and impurities such as ashes.

The active ingredient is stable up to high temperatures, however the coir fibre substrate may be susceptible to combustion at elevated temperatures. An alternative substrate could be found, such as glass fibre, however an understanding of the thermal parameters effecting the technology as it stands would inform how best to employ the technology in these other industries. Additionally, co-contaminants such as sulphur could have deleterious effects on the filter, and these aspects would need to be examined in more detail.

Other aspects that require some investigation are strength of binding of the active ingredient to mitigate against leaching of the material back into the environment, and how best to achieve coverage of the substrate with the active ingredient to maximise surface area binding. It is known that the basic constituent, copper iodide, is stable and virtually insoluble, as is the product, copper (i) tetra iodide mercurate. This was an important

consideration in choosing this material, as copper iodide is very toxic to aquatic life, so it was important that in inclement weather episodes, the material was not leaching from the substrate by being solubilised. Further, investigations could be performed to optimise coating and find best surface area coverage by EDS or ICPMS to ensure the correct ratio of materials is applied.

It is also important to ascertain the mercury binding capacity of the material. It was impossible to test this at bench scale at the concentrations and plume velocities tested as stoichiometry suggested the experiment would need to be run continually over several months at least. However, the experimental set up could be scaled up such that the mercury passing through the system was greatly increased, which could inform breakthrough parameters. For commercialisation purposes, this aspect would be critical. Operators need to know the volume of mercury that could be captured per unit of material to assess the amount of matting required for remediation of bulk surfaces, or how often filters need to be replaced when employed in industrial settings.

Additionally, it would be useful to know how readily the mercury could be isolated after capture. In theory this is straight forward as the binding material could be redissolved and the matting cleaned in solvent, with mercury remaining in the re-cured silicon. There is a small but important market for copper(I) tetra-iodide mercurate and it would be useful to know whether the material could be economically retrieved from the filter for re-sale depending on volumes and business priorities of the end users.

Finally, for applications such as in facial masks, it would be critical to know binding stability and efficiency to ensure user safety. The materials may need to be optimised, for example with an alternative to poly-siloxanes for binding purposes, or indeed to embed the crystals in existing material such masks are currently made of, reducing manufacturing unknowns.

While the enzyme as designed was successfully cloned and expressed into laboratory strains of *E.coli* suggesting mass production may well be feasible, functionality has not be proven at this stage. Binding to a solid substrate was also shown to be feasible, however ongoing bound fractions would need to be assessed over a prolonged period to ascertain any leaching of the enzyme off the substrate.

Functionality is obviously the critical factor requiring ongoing investigation. This would be best done in a dedicated methyl mercury laboratory by highly trained professionals with expertise in methylmercury due to its extreme toxicity. The particles could be produced as per the research described herein, then the functionality testing could be outsourced. A suitable protocol would need to be found. The author could find only one protocol that tested the functionality of an organomercurial lyase, and that was a plant-based experiment where an analog to methylmercury was tested. To show actual proof of functionality, methylmercury itself would need to be assessed.

Further, given successful functionality tests, several other factors require a certain level of investigation to move the technology forward. For example, is faujasite zeolite the best substrate to use. There is a family of SBP's that could be employed that expand the binding substrate beyond silica-based materials. This is important for efficient binding as well as to explore any deleterious effects over time such as leaching off the substrate.

Given *mer*B carrying strains are capable of both degrading methylmercury and catalysing the produced divalent mercury to GEM, it might be more practical to attempt to permanently bind *mer*B strains to a solid substrate and deploy the technology that way. There are many benefits to using immobilized enzymes, however this must be compared to immobilizing whole cells that have already been shown to be able to demethylate methylmercury species. Binding living cells can be achieved in many ways, many more

than can be canvassed in this conversation, but a series of experiments testing functionality and efficiency of this approach would be prudent.

Finally, these proposed research results must be interpreted with regard to existing cycles of both produced methyl mercury and natural transformation in systems, such as via photo-oxidation. For the purposes of environmental remediation, this is a critical factor as it is pointless deploying the technology if natural degradation rates are higher than that achieved by the particles. Besides environmental purposes, the technology could be employed in medical settings for acute poisoning, however this would require substantial testing for both efficacy and safety, including clinical trials and similar from suitably qualified institutions.

The research contained herein has produced some exciting results that expand the knowledge base in several areas of mercury remediation. Proof of concept has been shown for binding bacterial inoculants on a solid substrate for mercury remediation. Proof of concept has also been shown for direct capture of GEM without the need for prior catalysis. These two technological advances increase the knowledge and tools for both remediation and environmental specialists for dealing with both new and legacy mercury pollution. The concept of immobilising an enzyme for methylmercury transformation shows promise but is yet to be proven as a feasible technique for degrading this highly toxic material. It is envisioned that research will continue in these areas as outlined.

This concludes this dissertation, and I wish to thank the reader for taking the opportunity to consider this of this body of work and hopefully to avail themselves of some of these important findings.

Bibliography

- Achternbosch, M., Bräutigam, K.R., Hartlieb, N., Kupsch, C., Richers, U., Stemmermann, P. and Gleis, M., 2003. *Heavy metals in cement and concrete resulting from the co-incineration of wastes in cement kilns with regard to the legitimacy of waste utilisation*. Karlsruhe: Forschungszentrum Karlsruhe GmbH.
- Adams, G.O., Fufeyin, P.T., Okoro, S.E. and Ehinomen, I., 2015. Bioremediation, biostimulation and bioaugmention: a review. *International Journal of Environmental Bioremediation & Biodegradation*, 3(1), pp.28-39.
- Agnan, Y., Le Dantec, T., Moore, C.W., Edwards, G.C. and Obrist, D., 2015. New constraints on terrestrial surface–atmosphere fluxes of gaseous elemental mercury using a global database. *Environmental science & technology*, 50(2), pp.507-524.
- Amin, A. and Latif, Z., 2017. Cloning, Expression, Isotope Labeling, and Purification of Transmembrane Protein *Mer*F from Mercury Resistant Enterobacter sp. AZ-15 for NMR Studies. *Frontiers in microbiology*, 8, p.1250.
- Andreani, T., Silva, A.M. and Souto, E.B., 2015. Silica-based matrices: State of the art and new perspectives for therapeutic drug delivery. *Biotechnology and applied biochemistry*, 62(6), pp.754-764.
- Angel, R.J., Cressey, G. and Criddle, A.J., 1990. Edgarbaileyite, Hg6Si2O7; The crystal structure of the first mercury silicate. *American Mineralogist*, 75(9-10), pp.1192-1196.
- Arechederra, R. and Minteer, S.D., 2008. Organelle-based biofuel cells: Immobilized mitochondria on carbon paper electrodes. *Electrochimica Acta*, 53(23), pp.6698-6703.
- Aylett, B.J., 1975. *The chemistry of zinc, cadmium and mercury* (Vol. 18). Pergamon Press.
- Bae, W., Wu, C.H., Kostal, J., Mulchandani, A. and Chen, W., 2003. Enhanced mercury biosorption by bacterial cells with surface-displayed *Mer*R. *Applied and environmental microbiology*, 69(6), pp.3176-3180.
- Barkay, T. and Wagner-Döbler, I., 2005. Microbial transformations of mercury: potentials, challenges, and achievements in controlling mercury toxicity in the environment. *Advances in applied microbiology*, 57, pp.1-52.
- Barkay, T., Miller, S.M. and Summers, A.O., 2003. Bacterial mercury resistance from atoms to ecosystems. *FEMS microbiology reviews*, 27(2-3), pp.355-384.

- Basu, N., Scheuhammer, A.M., Rouvinen-Watt, K., Evans, R.D., Grochowina, N. and Chan, L.H., 2008. The effects of mercury on muscarinic cholinergic receptor subtypes (M1 and M2) in captive mink. *Neurotoxicology*, *29*(2), pp.328-334.
- Basu, P., 1999. Combustion of coal in circulating fluidized-bed boilers: a review. *Chemical Engineering Science*, 54(22), pp.5547-5557.
- Bernhoft, R.A., 2012. Mercury toxicity and treatment: a review of the literature. Journal of environmental and public health, 2012.
- Bieby Voijant Tangahu, Siti Rozaimah Sheikh Abdullah, Hassan Basri, Mushrifah Idris, Nurina Anuar, Muhammad Mukhlisin (2011) A Review on Heavy Metals (As, Pb, and Hg) Uptake by Plants through Phytoremediation. *International Journal of Chemical Engineering*, v. 2011, Article ID 939161.
- Bizily, S., Rugh, C., Meagher, R. (2000) Phytodetoxification of hazardous organomercurials by genetically engineered plants. *Nature Biotechnology*, v. 18, pp. 213 – 217.
- Bizily, S., Rugh, C., Summers, A., Meagher, B. (1999) Phytoremediation of methylmercury pollution: merB expression in Arabidopsis thaliana confers resistance to organomercurials. *Proceedings of the National Academy of Sciences of the United States of America*, v. 96, iss. 12, pp. 6808-6813.
- Blumenroth, P. and Wagner-Döbler, I., 1998. Survival of inoculants in polluted sediments: effect of strain origin and carbon source competition. *Microbial ecology*, 35(3-4), pp.279-288.
- Bogdanova, E., Minakhin, L., Bass, I., Volodin, A., Hobman, J.L. and Nikiforov, V., 2001. Class II broad-spectrum mercury resistance transposons in Gram-positive bacteria from natural environments. *Research in microbiology*, 152(5), pp.503-514.
- Bogdanova, E.S., Bass, I.A., Minakhin, L.S., Petrova, M.A., Mindlin, S.Z., Volodin, A.A., Kalyaeva, E.S., Tiedje, J.M., Hobman, J.L., Brown, N.L. and Nikiforov, V.G., 1998. Horizontal spread of mer operons among Gram-positive bacteria in natural environments. *Microbiology*, 144(3), pp.609-620.
- Borin, G.P., de Melo, R.R., Crespim, E., Sato, H.H. and Contesini, F.J., 2018. *An Overview on Polymer Gels Applied to Enzyme and Cell Immobilization. In Polymer Gels*. (pp. 63-86). Springer, Singapore.
- Borole, A. and Hamilton, C. (2011) US Patent US7998724 B2: Using acidithiobacillus, leptospirillium and/or sulfolobus as bioreactor for removing mercury from fossil fuels; viewed 20/4/2018 < http://www.google.com/patents/US7998724 >

- Bostock, J., Riley, H., 1855, *The Natural History of Pliny, Vol 4*, Henry Bohn, York St, Covent Garden, England.
- Boswell, C.D., Dick, R.E., Eccles, H. and Macaskie, L.E., 2001. Phosphate uptake and release by Acinetobacter johnsonii in continuous culture and coupling of phosphate release to heavy metal accumulation. *Journal of industrial microbiology and biotechnology*, 26(6), pp.333-340.
- Böttcher, H., Soltmann, U., Mertig, M. and Pompe, W., 2004. Biocers: ceramics with incorporated microorganisms for biocatalytic, biosorptive and functional materials development. *Journal of Materials Chemistry*, 14(14), pp.2176-2188.
- Boyd, E. and Barkay, T., 2012. The mercury resistance operon: from an origin in a geothermal environment to an efficient detoxification machine. *Frontiers in microbiology*, 3, p.349.
- Boyd, E. and Barkay, T., 2012. The mercury resistance operon: from an origin in a geothermal environment to an efficient detoxification machine. *Frontiers in microbiology*, 3, p.349.
- Bramwell, G., Wilson, F. and Faunce, T., 2018. Mercury Pollution from Coal-Fired Power Plants: A Critical Analysis of the Australian Regulatory Response to Public Health Risks. *Journal of law and medicine*, 26(2), pp.480-487.
- Brim, H., McFarlan, S.C., Fredrickson, J.K., Minton, K.W., Zhai, M., Wackett, L.P. and Daly, M.J., 2000. Engineering Deinococcus radiodurans for metal remediation in radioactive mixed waste environments. *Nature biotechnology*, 18(1), p.85.
- Brim, H., Venkateswaran, A., Kostandarithes, H.M., Fredrickson, J.K. and Daly, M.J., 2003. Engineering Deinococcus geothermalis for bioremediation of high-temperature radioactive waste environments*. Applied and environmental microbiology*, 69(8), pp.4575-4582.
- Brunke, M., Deckwer, W.D., Frischmuth, A., Horn, J.M., Lünsdorf, H., Rhode, M., Röhricht, M., Timmis, K.N. and Weppen, P., 1993. Microbial retention of mercury from waste streams in a laboratory column containing merA gene bacteria. *FEMS microbiology reviews*, 11(1-3), pp.145-152.
- Camurcuoğlu, D., 2015, *The Wall Paintings of Çatalhöyük (Turkey): Materials, Technologies and Artists,* Unpublished PhD thesis, University College London.
- Carvalho, C.M., Chew, E.H., Hashemy, S.I., Lu, J. and Holmgren, A., 2008. Inhibition of the human thioredoxin system a molecular mechanism of mercury toxicity. *Journal of Biological Chemistry*, *283*(18), pp.11913-11923.

- Cassidy, M.B., Lee, H. and Trevors, J.T., 1996. Environmental applications of immobilized microbial cells: a review. *Journal of Industrial Microbiology*, 16(2), pp.79-101.
- Cattani, I., Zhang, H., Beone, G., Del Re, A., Boccelli, R., Trevisan, M. (2009) The role of natural purified humic acids in modifying mercury accessibility in water and soil. *Journal of Environmental Quality.* V. 38, pp. 493–501.
- Chang, T.C. and Yen, J.H., 2006. On-site mercury-contaminated soils remediation by using thermal desorption technology. *Journal of Hazardous Materials*, *128*(2-3), pp.208-217.
- Chang, T.C. and Yen, J.H., 2006. On-site mercury-contaminated soils remediation by using thermal desorption technology. *Journal of Hazardous Materials*, *128*(2-3), pp.208-217.
- Chattopadhyay, S., Fimmen, R.L., Yates, B.J., Lal, V. and Randall, P., 2012. Phytoremediation of mercury-and methyl mercury-contaminated sediments by water hyacinth (Eichhornia crassipes). *International journal of phytoremediation*, 14(2), pp.142-161.
- Chen, S. and Wilson, D., (1997), Genetic engineering of bacteria and their potential use in mercury remediation. *Biodegradation*, v. 8, pp. 97-103.
- Chernyak, Y.I., Itskovich, V.B., Baduev, B.K. and Borovskii, G.B., 2012. Dependence of blood levels of HSP70 and HSP90 on genotypes of HSP70, GSTT1, and GSTM1 gene polymorphism in individuals chronically exposed to mercury. *Bulletin of experimental biology and medicine*, *154*(1), pp.68-72.
- Clarkson, T.W., 1997. The toxicology of mercury. *Critical reviews in clinical laboratory sciences*, 34(4), pp.369-403.
- Clarkson, T.W., Vyas, J.B. and Ballatori, N., 2007. Mechanisms of mercury disposition in the body. *American journal of industrial medicine*, 50(10), pp.757-764.
- Cohen, M.D., Draxler, R.R., Artz, R.S., Blanchard, P., Gustin, M.S., Han, Y.J., Holsen, T.M., Jaffe, D.A., Kelley, P., Lei, H. and Loughner, C.P., 2016. Modeling the global atmospheric transport and deposition of mercury to the Great Lakes. *Elementa: Sciience of the Anthroprocene*, *4*.
- Cox, C.D., Shoesmith, M.A. and Ghosh, M.M., 1996. Electrokinetic remediation of mercury-contaminated soils using iodine/iodide lixiviant. *Environmental Science & Technology*, *30*(6), pp.1933-1938.
- Cyr, B., PPG Industries Inc, 1993. *Process for recovery of metallic mercury from contaminated mercury-containing soil*. U.S. Patent 5,244,492.

- Datta, S., Christena, L.R. and Rajaram, Y.R.S., 2013. Enzyme immobilization: an overview on techniques and support materials. *3 Biotech*, *3*(1), pp.1-9.
- De Andrés, F., Walter, I., Tenorio, J. (2007) Revegetation of abandoned agricultural land amended with biosolids. *Science of the total environment*, v. 378, pp. 81–83.
- De Souza, M., Huang, C., Chee, N., Terry, N. (1999) Rhizosphere bacteria enhance the accumulation of selenium and mercury in wetlands plants. *Planta,* v. 209, pp. 259–263, pmid:10436229 doi: 10.1007/s00425005.
- Deng, X., & Wilson, D. (2001) Bioaccumulation of mercury from wastewater by genetically engineered Escherichia coli. *Applied microbiology and biotechnology*, v. 56, iss. 1-2, pp. 276-279.
- Dermont, G., Bergeron, M., Mercier, G. and Richer-Laflèche, M., 2008. Soil washing for metal removal: a review of physical/chemical technologies and field applications. *Journal of hazardous materials*, *152*(1), pp.1-31.
- Distefano, M.D., Au, K.G. and Walsh, C.T., 1989. Mutagenesis of the redox-active disulfide in mercuric ion reductase: catalysis by mutant enzymes restricted to flavin redox chemistry. *Biochemistry*, 28(3), pp.1168-1183.
- Donatello, S., Fernández-Jiménez, A. and Palomo, A., 2012. An assessment of Mercury immobilisation in alkali activated fly ash (AAFA) cements. *Journal of hazardous materials*, *213*, pp.207-215.
- Dony Chacko, M., Ying-Ning, H., Gicaraya Gicana, R., Gincy, M., Mei-Chieh, C., Chieh-Chen, H. (2015) A rhizosphere-associated symbiont, Photobacterium spp. strain MELD1, and its targeted synergistic activity for phytoprotection against mercury. *PLOS*, DOI: 10.1371
- Dranga, B.A., Lazar, L. and Koeser, H., 2012. Oxidation catalysts for elemental mercury in flue gases—a review. *Catalysts*, 2(1), pp.139-170.
- Dronen, L.C., Moore, A.E., Kozliak, E.I. and Seames, W.S., 2004. An assessment of acid wash and bioleaching pre-treating options to remove mercury from coal. *Fuel*, 83(2), pp.181-186.
- Echeverria, D., Woods, J.S., Heyer, N.J., Martin, M.D., Rohlman, D.S., Farin, F.M. and Li, T., 2010. The association between serotonin transporter gene promotor polymorphism (5-HTTLPR) and elemental mercury exposure on mood and behavior in humans. *Journal of Toxicology and Environmental Health, Part A*, *73*(15), pp.1003-1020.
- Echeverria, D., Woods, J.S., Heyer, N.J., Rohlman, D., Farin, F.M., Li, T. and Garabedian, C.E., 2006. The association between a genetic polymorphism of

coproporphyrinogen oxidase, dental mercury exposure and neurobehavioral response in humans. *Neurotoxicology and teratology*, *28*(1), pp.39-48.

- El-Azab, N.E.E., El-Mahalaway, A.M. and Sabry, D., 2018. Effect of methyl mercury on the cerebellar cortex of rats and the possible neuroprotective role of mesenchymal stem cells conditioned medium. histological and immunohistochemical study. *Stem Cell Res Ther*, *8*(430), p.2.
- EPA, S., 1997. *Mercury Study Report to Congress. Volume II: An Inventory of Anthropogenic Mercury Emissions in the United States*. EPA-452/R-97-004. Research Triangle Park, NC: US Environmental Protection Agency, Office of Air Quality Planning & Standards and Office of Research and Development.
- European Commission. Directive 2010/75/EU of the European Parliament and of the Council on industrial emissions (integrated pollution prevention and control). *Official Journal of the Euopean Union*, 2010, L334, 17–117.
- Falciglia, P.P., Malarbì, D., Greco, V. and Vagliasindi, F.G., 2017. Surfactant and MGDA enhanced–Electrokinetic treatment for the simultaneous removal of mercury and PAHs from marine sediments. *Separation and Purification Technology*, 175, pp.330-339.
- Finkelman, R.B., 1994. Modes of occurrence of potentially hazardous elements in coal: levels of confidence*. Fuel processing technology*, 39(1-3), pp.21-34.
- Fomina, M. and Gadd, G.M., 2003. Metal sorption by biomass of melanin-producing fungi grown in clay-containing medium. Journal of Chemical Technology & Biotechnology: International Research in Process. *Environmental & Clean Technology*, 78(1), pp.23-34.
- Fox, B. and Walsh, C.T., 1982. Mercuric reductase. Purification and characterization of a transposon-encoded flavoprotein containing an oxidation-reduction-active disulfide. *Journal of Biological Chemistry*, 257(5), pp.2498-2503.
- Fox, B. and Walsh, C.T., 1982. Mercuric reductase. Purification and characterization of a transposon-encoded flavoprotein containing an oxidation-reduction-active disulfide. *Journal of Biological Chemistry*, 257(5), pp.2498-2503.
- Frohne, T., Rinklebe, J., Langer, U., Laing, G., Mothes, S., Wennrich, R. (2012) Biogeochemical factors affecting mercury methylation rate in two contaminated floodplain soils. *Biogeosciences*, v. 9, pp. 493-507.
- Fukuda, N., Takaoka, M., Doumoto, S., Oshita, K., Morisawa, S. and Mizuno, T., 2011. Mercury emission and behavior in primary ferrous metal production. *Atmospheric environment*, 45(22), pp.3685-3691.

- Gadd, G.M., 2010. Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology*, 156(3), pp.609-643.
- Galbreath, K.C. and Zygarlicke, C.J., 1996. Mercury speciation in coal combustion and gasification flue gases. *Environmental science & technology*, 30(8), pp.2421-2426.
- Galbreath, K.C. and Zygarlicke, C.J., 2000. Mercury transformations in coal combustion flue gas. *Fuel Processing Technology*, 65, pp.289-310.
- Ghorishi, B. and Gullett, B.K., 1998. *Fixed-Bed Control of Mecury; Role of Acid Gases and a Comparison between Carbon-Based, Calcium-Based, and Coal Fly Ash Sorbents*.
- Gianfreda, L. and Rao, M.A., 2004. Potential of extra cellular enzymes in remediation of polluted soils: a review. *Enzyme and microbial technology*, 35(4), pp.339-354.
- Gochfeld, M., 2003. Cases of mercury exposure, bioavailability, and absorption. *Ecotoxicology and environmental safety*, *56*(1), pp.174-179.
- Goodarzi, F. and Goodarzi, N.N., 2004. Mercury in Western Canadian subbituminous coal—a weighted average study to evaluate potential mercury reduction by selective mining. *International Journal of Coal Geology*, 58(4), pp.251-259.
- Guven, D.E. and Akinci, G., 2013. Effect of sediment size on bioleaching of heavy metals from contaminated sediments of Izmir Inner Bay. *Journal of Environmental Sciences*, 25(9), pp.1784-1794.
- Ha, E., Basu, N., Bose-O'Reilly, S., Dórea, J.G., McSorley, E., Sakamoto, M. and Chan, H.M., 2017. Current progress on understanding the impact of mercury on human health. *Environmental research*, *152*, pp.419-433.
- Hall, B., Schager, P. and Lindqvist, O., 1991. Chemical reactions of mercury in combustion flue gases. *Water Air & Soil Pollution*, 56(1), pp.3-14.
- Hall, B., Schager, P. and Lindqvist, O., 1991. Chemical reactions of mercury in combustion flue gases. *Water Air & Soil Pollution*, 56(1), pp.3-14.
- Hamlett, N.V., Landale, E.C., Davis, B.H. and Summers, A.O., 1992. Roles of the Tn21 merT, merP, and merC gene products in mercury resistance and mercury binding. *Journal of bacteriology*, 174(20), pp.6377-6385.
- Harada, M., 1995. Minamata disease: methylmercury poisoning in Japan caused by environmental pollution. *Critical reviews in toxicology*, *25*(1), pp.1-24.

- Hart, M.C., Elliott, G.N., Osborn, A.M., Ritchie, D.A. and Strike, P., 1998. Diversity amongst Bacillus merA genes amplified from mercury resistant isolates and directly from mercury polluted soil. *FEMS microbiology ecology*, 27(1), pp.73-84.
- He, K., Jian, S., Xian, F., Czalo, M., Marton, L . (2001) Differential mercury volatilization by tobacco organs expressing a modified bacterial merA gene. *Cell Research*, v. 11, iss. 3, pp. 231-236.
- Heaton, A., Rugh, C., Kim, T., Wang, N., Meagher, R. (2009) Toward detoxifying mercury-polluted aquatic sediments with rice genetically engineered for mercury resistance. *Environmental Toxicology and Chemistry*, v. 22, iss. 12, pp. 2940–2947.
- Hills, L.M. and Stevenson, R.W., 2006. *Mercury and lead content in raw materials*. Portland Cement Association.
- Hower, J.C., Robl, T.L., Anderson, C., Thomas, G.A., Sakulpitakphon, T., Mardon, S.M. and Clark, W.L., 2005. Characteristics of coal combustion products (CCP's) from Kentucky power plants, with emphasis on mercury content. *Fuel*, 84(11), pp.1338-1350
- Huang, C.F., Hsu, C.J., Liu, S.H. and Lin-Shiau, S.Y., 2008. Neurotoxicological mechanism of methylmercury induced by low-dose and long-term exposure in mice: oxidative stress and down-regulated Na+/K+-ATPase involved. *Toxicology Letters*, *176*(3), pp.188-197.
- Ingo, G.M., Guida, G., Angelini, E., Di Carlo, G., Mezzi, A. and Padeletti, G., 2013. Ancient mercury-based plating methods: combined use of surface analytical techniques for the study of manufacturing process and degradation phenomena. *Accounts of chemical research*, 46(11), pp.2365-2375.
- Iohara, K., Iiyama, R., Nakamura, K., Silver, S., Sakai, M., Takeshita, M. and Furukawa, K., 2001. The mer operon of a mercury-resistant Pseudoalteromonas haloplanktis strain isolated from Minamata Bay, Japan. Applied microbiology and biotechnology, 56(5-6), pp.736-741.
- Johansen, V.C. and Hawkins, G.J., 2003. Mercury speciation in cement kilns: A literature review. Res. Dev. Serial, (2567).
- Juntawang, C., Rongsayamanont, C. and Khan, E., 2017. Entrapped cells-based-anaerobic membrane bioreactor treating domestic wastewater: Performances, fouling, and bacterial community structure. Chemosphere, 187, pp.147-155.
- Kairies, C.L., Schroeder, K.T. and Cardone, C.R., 2006. Mercury in gypsum produced from flue gas desulfurization. Fuel, 85(17-18), pp.2530-2536.

- Kelly, C.A. and Rudd, J.W., 2018. Transport of mercury on the finest particles results in high sediment concentrations in the absence of significant ongoing sources. *Science of The Total Environment*, *637*, pp.1471-1479.
- Kentucky University, 2019. Coal for electricity generation. Kentucky Geological Survey. http://www.uky.edu/KGS/coal/coal-for-elec.php accessed on line 20 Jan 2019.
- Khan, F.I., Husain, T. and Hejazi, R., 2004. An overview and analysis of site remediation technologies. *Journal of environmental management*, *71*(2), pp.95-122.
- Kierstan, M. and Bucke, C., 2000. The immobilization of microbial cells, subcellular organelles, and enzymes in calcium alginate gels. *Biotechnology and Bioengineering*, 67(6), pp.726-736.
- Kim, M.K. and Zoh, K.D., 2012. Fate and transport of mercury in environmental media and human exposure. *Journal of Preventive Medicine and Public Health*, *45*(6), p.335.
- Kiyono, M. and Pan-Hou, H., 1999. The merG gene product is involved in phenylmercury resistance in Pseudomonas strain K-62. *Journal of bacteriology*, 181(3), pp.726-730.
- Kiyono, M., Omura, T., Fujimori, H. and Pan-Hou, H., 1995. Lack of involvement of merT and merP in methylmercury transport in mercury resistant Pseudomonas K-62*. FEMS microbiology letters*, 128(3), pp.301-306.
- Kōmura, I. and Izaki, K., 1971. Mechanism of Mercuric Chloride Resistance in Microorganisms: I. Vaporization of a Mercury Compound from Mercuric Chloride by Multiple Drug Resistant Strains of Escherichia coli. *The Journal of Biochemistry*, 70(6), pp.885-893.
- Kostal, J., Mulchandani, A., Gropp, K. E., & Chen, W. (2003) A temperature responsive biopolymer for mercury remediation, *Environmental science & technology*, v. 37, iss. 19, pp. 4457-4462.
- Kucharski, R., Zielonka, U., Sas-Nowosielska, A., Kuperberg, J.M., Worsztynowicz, A. and Szdzuj, J., 2005. A method of mercury removal from topsoil using low-thermal application. *Environmental Monitoring and Assessment*, *104*(1-3), pp.341-351.
- Külaots, I., Hurt, R.H. and Suuberg, E.M., 2004. Size distribution of unburned carbon in coal fly ash and its implications. *Fuel*, 83(2), pp.223-230.

- Külaots, I., Hurt, R.H. and Suuberg, E.M., 2004. Size distribution of unburned carbon in coal fly ash and its implications. *Fuel*, 83(2), pp.223-230.

- Kunkel, A.M., Seibert, J.J., Elliott, L.J., Kelley, R., Katz, L.E. and Pope, G.A., 2006. Remediation of elemental mercury using in situ thermal desorption (ISTD). *Environmental science & technology*, *40*(7), pp.2384-2389.
- Lahaye, J., 1998. The chemistry of carbon surfaces. Fuel, 77(6), pp.543-547.
- Larsen, M.B., Schmidt, I., Paone, P., Salmento, J., Petersen, A. and Jorgensen, A.W., 2007. *Mercury in cement production-a literature review*. FLSmidth internal report.
- Laudal, D.L., Galbreath, K.C. and Zygarlicke, C.J., 1996, August. Experimental Investigation of Mercury Speciation in Coal Combustion Flue Gases. *In Fourth international Conference on Mercury as a Global Pollutant*. Hamburg, Germany.
- Ledwidge, R., Patel, B., Dong, A., Fiedler, D., Falkowski, M., Zelikova, J., Summers, A.O., Pai, E.F. and Miller, S.M., 2005. NmerA, the metal binding domain of mercuric ion reductase, removes Hg2+ from proteins, delivers it to the catalytic core, and protects cells under glutathione-depleted conditions. *Biochemistry*, 44(34), pp.11402-11416.
- Lee, C.W., Srivastava, R.K., Ghorishi, S.B., Hastings, T.W. and Stevens, F.M., 2003, May. Study of speciation of mercury under simulated SCR NOx emission control conditions. *In Combined Power Plant Air Pollutant Control Mega Symposium*, Washington, DC.
- Lee, J.Y., Ju, Y., Keener, T.C. and Varma, R.S., 2006. Development of cost-effective noncarbon sorbents for Hg0 removal from coal-fired power plants. *Environmental science & technology*, 40(8), pp.2714-2720.
- Lodenius, M. and Braunschweiler, H., 1986. Volatilisation of heavy metals from a refuse dump. *The Science of the Total Environment*, *57*, pp.253-255.
- Li, G., Wu, Q., Xu, L., Wen, M., Liu, K., Tang, Y., Zou, J., Wang, F., Wang, Y. and Wang, S., 2019. A Review on Adsorption Technologies for Mercury Emission Control. *Bulletin of environmental contamination and toxicology*, *103*(1), pp.155-162.
- Lian, P., Guo, H.B., Riccardi, D., Dong, A., Parks, J.M., Xu, Q., Pai, E.F., Miller, S.M., Wei, D.Q., Smith, J.C. and Guo, H., 2014. X-ray structure of a Hg2+ complex of mercuric reductase (MerA) and quantum mechanical/molecular mechanical study of Hg2+ transfer between the C-terminal and buried catalytic site cysteine pairs. *Biochemistry*, 53(46), pp.7211-7222.
- Liao, L., Selim, H.M. and DeLaune, R.D., 2009. Mercury adsorption-desorption and transport in soils. *Journal of environmental quality*, *38*(4), pp.1608-1616.

- Liu, D., Li, C., Wu, J. and Liu, Y., 2019. Novel carbon-based sorbents for elemental mercury removal from gas streams: a review. *Chemical Engineering Journal*, p.123514.
- Licata, A., Schüttenhelm, W. and Klein, M., 2000, May. Mercury control for MWCs using the sodium tetrasulfide process*. In 8th Annual North American Waste-to-Energy Conference* (pp. 22-24).
- Liebert, C. A., Hall, R. M. & Summers, A. O. (1999). Transposon Tn21, flagship of the floating genome. *Microbiology and molecular biology review*, v. 63, pp. 507–522.
- Liebert, C.A., Wireman, J., Smith, T. and Summers, A.O., 1997. Phylogeny of mercury resistance (mer) operons of gram-negative bacteria isolated from the fecal flora of primates. *Applied and Environmental Microbiology*, 63(3), pp.1066-1076.
- Liu, S., Zhang, F., Chen, J., Sun, G. (2010) Arsenic removal from contaminated soil via biovolatilization by genetically engineered bacteria under laboratory conditions. *Journal of environmental sciences*, v. 23, iss. 9, pp. 1544–1550.
- Magos, L. and Clarkson, T.W., 2006. Overview of the clinical toxicity of mercury. *Annals of clinical biochemistry*, 43(4), pp.257-268.
- Mattiasson, B., 2018. Immobilization methods*. In Immobilized cells and organelles* (pp. 3-26). CRC Press.
- Mathema, V.B., Thakuri, B.C. and Sillanpää, M., 2011. Bacterial mer operon-mediated detoxification of mercurial compounds: a short review. *Archives of microbiology*, *193*(12), pp.837-844.
- McCarthy, D., Edwards, G.C., Gustin, M.S., Care, A., Miller, M.B. and Sunna, A., 2017. An innovative approach to bioremediation of mercury contaminated soils from industrial mining operations. *Chemosphere*, 184, pp.694-699.
- McCarthy, D.N. and Edwards, G.C., 2018. Immobilized organo-mercurial lyase on zeolite using a solid binding peptide. *International Journal of Environmental Bioremediation & Biodegradation*. Vol. 6, No. 1, 2018, pp 8-17. http://pubs.sciepub.com/ijebb/6/1/2
- McCarthy, D. (2015) "*Immobilised cells and enzymes to decontaminate mercury polluted soil: a graduate thesis in applied biomolecular science presented to the Higher Research Degree Office in the Faculty of Science and Engineering, Macquarie University, Australia, Department of Chemistry and Biomolecular Sciences, in partial fulfulment of the requirements for the degree of Master of Research*."

- Meagher, R. (2000) Phytoremediation of toxic elemental and organic pollutants. *Current opinion in plant biology*, v. 3, iss. 2, pp. 153–162.
- Meij, R., 1991. The fate of mercury in coal-fired power plants and the influence of wet flue-gas desulphurization. *Water Air & Soil Pollution*, 56(1), pp.21-33.
- Miller, S.M., Ballou, D.P., Massey, V., Williams, C.H. and Walsh, C.T., 1986. Two-electron reduced mercuric reductase binds Hg (II) to the active site dithiol but does not catalyze Hg (II) reduction. *Journal of Biological Chemistry*, 261(18), pp.8081-8084.
- Mohamad, N.R., Marzuki, N.H.C., Buang, N.A., Huyop, F. and Wahab, R.A., 2015. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnology & Biotechnological Equipment*, 29(2), pp.205-220.
- Moo-Young, M., Bols, N.C., Overgaard, S.E. and Scharer, J.M., University of Waterloo, 1992*. Immobilization of biologically active material in capsules prepared from a water-soluble polymer and chitosan acetate*. U.S. Patent 5,116,747.
- Mulligan, C.N., Yong, R.N. and Gibbs, B.F., 2001. An evaluation of technologies for the heavy metal remediation of dredged sediments. *Journal of hazardous materials*, *85*(1-2), pp.145-163.
- Murugesan, A. , Maheswari, S., Bagirath, G. (2008) Biosorption of cadmium by live and immobilized cells of Spirulina platensis*. International journal of environment resources*, v. 2, iss. 3, pp. 307-312.
- Nakamura, K., Hagimine, M., Sakai, M. and Furukawa, K., 1999. Removal of mercury from mercury-contaminated sediments using a combined method of chemical leaching and volatilization of mercury by bacteria. *Biodegradation*, 10(6), pp.443-447.
- Narita, M., Chiba, K., Nishizawa, H., Ishii, H., Huang, C.C., Kawabata, Z.I., Silver, S. and Endo, G., 2003. Diversity of mercury resistance determinants among Bacillus strains isolated from sediment of Minamata Bay. *FEMS microbiology letters*, 223(1), pp.73-82.
- Navarro, A., Cañadas, I., Martinez, D., Rodriguez, J. and Mendoza, J.L., 2009. Application of solar thermal desorption to remediation of mercury-contaminated soils. *Solar Energy*, *83*(8), pp.1405-1414.
- Nguyen, V.K., Lee, M.H., Park, H.J. and Lee, J.U., 2015. Bioleaching of arsenic and heavy metals from mine tailings by pure and mixed cultures of Acidithiobacillus spp. *Journal of Industrial and Engineering Chemistry*, 21, pp.451-458.

- Niksa, S. and Fujiwara, N., 2005. A predictive mechanism for mercury oxidation on selective catalytic reduction catalysts under coal-derived flue gas. *Journal of the Air & Waste Management Association*, 55(12), pp.1866-1875.
- Norn, S., Permin, H., Kruse, E. and Kruse, P.R., 2008. Mercury--a major agent in the history of medicine and alchemy. *Dansk medicinhistorisk arbog*, 36, pp.21-40.
- Nsimba, E.B., 2013. *Development of a biophysical system based on bentonite, zeolite and micro-organisms for remediating gold mine wastewaters and tailings ponds* (Doctoral dissertation).
- Nucifora, G., Silver, S. and Misra, T.K., 1989. Down regulation of the mercury resistance operon by the most promoter-distal gene merD. *Molecular and General Genetics* MGG, 220(1), pp.69-72.
- Nunal, S., Mae, S., Santander-de-leon, S., Bacolod, E., Koyama, J., Uno, S., Hidaka, M., Yoshikawa, T., Maeda, H. (2014) Bioremediation of heavily oil-polluted seawater by a bacterial consortium immobilized in cocopeat and rice hull powder. *Biocontrol science*, v 19, iss. 1, pp. 11-22.
- Okino, S., Iwasaki, K., Yagi, O., Tanaka, H. (2000) Development of a biological mercury removal-recovery system. *Biotechnology Letters*, v. 22, pp. 783–788.
- Olsen, D., 2018 https://emedicine.medscape.com/article/1175560-overview
- OPCW, 2019. Chemical Weapons Convention, Annex on Chemicals, Schedule 3, Organisation for the Prohibition of Chemical Weapons, <https://www.opcw.org/chemical-weapons-convention/annexes/annex-chemicals/schedule-3> accessed on line 1 Feb 2019.
- Osborn, A.M., Bruce, K.D., Strike, P. and Ritchie, D.A., 1997. Distribution, diversity and evolution of the bacterial mercury resistance (mer) operon. *FEMS Microbiology Reviews*, 19(4), pp.239-262.
- Pan, H.Y., Minet, R.G., Benson, S.W. and Tsotsis, T.T., 1994. Process for converting hydrogen chloride to chlorine. *Industrial & engineering chemistry research*, 33(12), pp.2996-3003.
- Park, S.E., Chang, J.S. and Lee, K.W. eds., 2004. *Carbon Dioxide Utilization for Global Sustainability*: Proceedings of the 7th International Conference on Carbon Dioxide Utilization, Seoul, Korea, October 12-16, 2003 (Vol. 153). Elsevier.
- Parks, J., Johs, A., Podar, M., Bridou, R., Hurt, R., Smith, S., Tomanicek, S., Qian, Y., Brown, S., Brandt, C., Palumbo, A., Smith, J., Wall, J., Elias, D., Liang, L. (2013) The genetic basis for bacterial mercury methylation. *Science*, v. 339, pp. 1332-1335.

- Parks, J.M., Guo, H., Momany, C., Liang, L., Miller, S.M., Summers, A.O. and Smith, J.C., 2009. Mechanism of Hg− C Protonolysis in the Organomercurial Lyase MerB. *Journal of the American Chemical Society*, 131(37), pp.13278-13285.
- Patel, R., Kumari, M. and Khan, A.B., 2014. Recent advances in the applications of ionic liquids in protein stability and activity: a review. *Applied biochemistry and biotechnology*, 172(8), pp.3701-3720.
- Pavlish, J.H., Sondreal, E.A., Mann, M.D., Olson, E.S., Galbreath, K.C., Laudal, D.L. and Benson, S.A., 2003. Status review of mercury control options for coal-fired power plants. *Fuel processing technology*, 82(2-3), pp.89-165.
- Presto, A. and Granite, EJ., 2006. Survey of catalysis for oxidation of mercury in flue gas. *Environmental Science & Technology*, 40 (18), 5601-5609 DOI: 10.1021/es060504i
- Qu, L.Y., Fu, S.Z., Liu, L., An, Y.M. and Li, M., 2004. A study on the soil improvement polluted by mercury. *Journal of Guizhou Normal University (Natural Sciences)*, *22*, pp.49-51.
- Ralston, N.V. and Raymond, L.J., 2010. Dietary selenium's protective effects against methylmercury toxicity. *Toxicology*, *278*(1), pp.112-123.
- Ramond, J.B., Berthe, T., Duran, R. and Petit, F., 2009. Comparative effects of mercury contamination and wastewater effluent input on Gram-negative merA gene abundance in mudflats of an anthropized estuary (Seine, France): a microcosm approach. *Research in Microbiology*, *160*(1), pp.10-18.
- Rathore, S., Desai, P.M., Liew, C.V., Chan, L.W. and Heng, P.W.S., 2013. Microencapsulation of microbial cells. *Journal of Food Engineering*, 116(2), pp.369-381.
- Reddy, K.R., Chaparro, C. and Saichek, R.E., 2003. Iodide-enhanced electrokinetic remediation of mercury-contaminated soils. *Journal of Environmental Engineering*, *129*(12), pp.1137-1148.
- Renninger, N., McMahon, K.D., Knopp, R., Nitsche, H., Clark, D.S. and Keasling, J.D., 2001. Uranyl precipitation by biomass from an enhanced biological phosphorus removal reactor. *Biodegradation*, 12(6), pp.401-410.
- Renzoni, R., Ullrich, C., Belboom, S. and GERMAIN, A., 2010. *Mercury in the cement industry*. Université de Liège. Independently commissioned by CEMBUREAU-CSI.

- Richards, J., Goshaw, D., Speer, D. and Holder, T., 2008. *Air emissions data summary for Portland cement pyroprocessing operations firing tire-derived fuels*. PCA R&D Serial, (3050), pp.60077-1083.
- Robinson, B., Anderson, C., Dickinson, N. (2015) Phytoextraction: Where's the action. *Journal of Geochemical Exploration*, v. 151, pp. 34–40.
- Roy, A., Eaton, H.C., Cartledge, F.K., and Tittlebaum, M.E., 1991. Solidification / Stabilizing of a heavy metal sludge by a Portland cement / fly ash binding mixture. *Journal of hazardous materials*. http://doi.org/10.1089/hwm.1991.8.33
- Rugh, C., Senecoff, J., Meagher, R., Merkle, S. (1998) Development of transgenic yellow poplar for mercury phytoremediation. *Nature Biotechnology*, v. 16, pp. 925 – 928.
- Ruiz, O., Hussein, H., Terry, N., Daniell, H. (2003) Phytoremediation of Organomercurial Compounds via chloroplast genetic engineering. *Plant Physiology*, v. 132, iss. 3, pp. 1344-1352.
- Ruta, L., Paraschivescu, C., Matache, M., Avramescu, S. and Farcasanu, I.C., 2010. Removing heavy metals from synthetic effluents using "kamikaze" Saccharomyces cerevisiae cells. *Applied microbiology and biotechnology*, 85(3), pp.763-771.
- S. Song, S., Selin, S., Soerensen, A., Angot, H., Artz, R., Brooks, S., Brunke, E., Conley, G., Dommergue, A., Ebinghaus, R., Holsen, T., Ja, D., Kang, S., Kelley, P., Luke, W., Magand, O., Marumoto, K., Pfa, K., Ren, X., Sheu, G., Slemr, F., Warneke, T., Weigelt, A., Weiss-Penzias, P., Wip, D., Zhang, Q, (2015) Top-down constraints on atmospheric mercury emissions and implications for global biogeochemical cycling. *Atmospheric Chemistry and Physics Discussions*., v.15, pp. 5269–5325
- Salame, I.I. and Bandosz, T.J., 2001. Surface chemistry of activated carbons: combining the results of temperature-programmed desorption, Boehm, and potentiometric titrations. *Journal of colloid and interface science*, 240(1), pp.252-258.
- Saouter, E., Gillman, M., Turner, R. and Barkay, T., 1995. Development and field validation of a microcosm to simulate the mercury cycle in a contaminated pond. *Environmental Toxicology and Chemistry: An International Journal*, 14(1), pp.69-77.
- Sauge-Merle, S., Cuiné, S., Carrier, P., Lecomte-Pradines, C., Luu, D.T. and Peltier, G., 2003. Enhanced toxic metal accumulation in engineered bacterial cells expressing Arabidopsis thaliana phytochelatin synthase. *Applied and Environmental Microbiology*, 69(1), pp.490-494.

- Schelert, J., Dixit, V., Hoang, V., Simbahan, J., Drozda, M. and Blum, P., 2004. Occurrence and characterization of mercury resistance in the hyperthermophilic archaeon Sulfolobus solfataricus by use of gene disruption. *Journal of Bacteriology*, 186(2), pp.427-437.
- Schuster, E., 1991. The behavior of mercury in the soil with special emphasis on complexation and adsorption processes-a review of the literature. *Water Air & Soil Pollution*, *56*(1), pp.667-680.
- Schroeder, W.H. and Munthe, J., 1998. Atmospheric mercury—an overview. *Atmospheric environment*, *32*(5), pp.809-822.
- Selin, N.E., 2009. Global biogeochemical cycling of mercury: a review. *Annual Review of Environment and Resources*, 34, pp.43-63.
- Senior, C.L., Morency, J.R., Huffman, G.P., Huggins, F.E., Shah, N., Peterson, T., Shadman, F. and Wu, B., 1998. Interactions between vapor-phase mercury and coal fly ash under simulated utility power plant flue gas conditions. *In Proceedings of the 1998 91st Annual Meeting & Exposition of the Air & Waste Management Association*. Air & Waste Management Assoc.
- Senior, C.L., Sarofim, A.F., Zeng, T., Helble, J.J. and Mamani-Paco, R., 2000. Gas-phase transformations of mercury in coal-fired power plants*. Fuel Processing Technology*, 63(2-3), pp.197-213.
- Serrano, D., Vlassopoulos, D., Bessinger, B., O'Day, P. (2012) Immobilization of Hg(II) by coprecipitation in sulfate-cement systems, *Environ. Sci. Technol,* v. 46, pp. 6767−6775.
- Shen, Z., Zhang, J., Qu, L., Dong, Z., Zheng, S. and Wang, W., 2009. A modified EK method with an I−/I 2 lixiviant assisted and approaching cathodes to remedy mercury contaminated field soils. *Environmental geology*, 57(6), pp.1399-1407.
- Sierra, C., Menéndez-Aguado, J.M., Afif, E., Carrero, M. and Gallego, J.R., 2011. Feasibility study on the use of soil washing to remediate the As–Hg contamination at an ancient mining and metallurgy area. *Journal of hazardous materials*, *196*, pp.93-100.
- Sims, R.C., 1990. Soil remediation techniques at uncontrolled hazardous waste sites. *Journal of the Air & Waste Management Association*, *40*(5), pp.704-732.
- Sinhaa, A., Pantb, K., Kharea, S., (2012) Studies on mercury bioremediation by alginate immobilized mercury tolerant Bacillus cereus cells, International biodeterioration & biodegradation, v. 71, pp. 1–8.

- Skurnik, D., Ruimy, R., Ready, D., Ruppe, E., Bernede-Bauduin, C., Djossou, F., Guillemot, D., Pier, G.B. and Andremont, A., 2010. Is exposure to mercury a driving force for the carriage of antibiotic resistance genes?. *Journal of medical microbiology*, 59(7), pp.804-807.
- Slemr, F., Brunke, E.G., Ebinghaus, R. and Kuss, J., 2011. Worldwide trend of atmospheric mercury since 1995. *Atmospheric Chemistry and Physics*, 11(10), pp.4779-4787.
- Smith, D.H., 1967. R factors mediate resistance to mercury, nickel, and cobalt. *Science*, 156(3778), pp.1114-1116.
- Soltmann, U., Böttcher, H., Koch, D. and Grathwohl, G., 2003. Freeze gelation: a new option for the production of biological ceramic composites (biocers). *Materials Letters*, 57(19), pp.2861-2865.
- Sone, Y., Uraguchi, S., Takanezawa, Y., Nakamura, R., Pan-Hou, H. and Kiyono, M., 2017(a). A Novel Role of MerC in Methylmercury Transport and Phytoremediation of Methylmercury Contamination. *Biological and Pharmaceutical Bulletin*, 40(7), pp.1125-1128.
- Sone, Y., Uraguchi, S., Takanezawa, Y., Nakamura, R., Pan-Hou, H. and Kiyono, M., 2017(b). Cysteine and histidine residues are involved in Escherichia coli Tn21 MerE methylmercury transport. *FEBS open bio*, 7(12), pp.1994-1999.
- Spengler, G., Molnár, A., Schelz, Z., Amaral, L., Sharples, D. and Molnár, J., 2006. The mechanism of plasmid curing in bacteria. *Current drug targets*, 7(7), pp.823-841.
- Stelting, S., Burns, R.G., Sunna, A., Visnovsky, G. and Bunt, C.R., 2012. Immobilization of Pseudomonas sp. strain ADP: a stable inoculant for the bioremediation of atrazine. *Applied Clay Science*, 64, pp.90-93.
- Stelting, S.A., Burns, R.G., Sunna, A. and Bunt, C.R., 2014. Survival in sterile soil and atrazine degradation of Pseudomonas sp. strain ADP immobilized on zeolite. *Bioremediation Journal*, 18(4), pp.309-316.
- Summers, A.O., 1992. Untwist and shout: a heavy metal-responsive transcriptional regulator. *Journal of Bacteriology*, 174(10), p.3097.
- Svensson, M., 2006. *Mercury immobilization: a requirement for permanent disposal of mercury waste in Sweden* (Doctoral dissertation, Örebro universitetsbibliotek).
- Takeuchi, T., Morikawa, N., Matsumoto, H. and Shiraishi, Y., 1962. A pathological study of Minamata disease in Japan. *Acta Neuropathologica*, *2*(1), pp.40-57.

- Torres-Salas, P., del Monte-Martinez, A., Cutiño-Avila, B., Rodriguez-Colinas, B., Alcalde, M., Ballesteros, A.O. and Plou, F.J., 2011. Immobilized biocatalysts: Novel approaches and tools for binding enzymes to supports. *Advanced Materials*, *23*(44), pp.5275-5282.
- UN Environment, 2017. *Global mercury supply, trade and demand*. United Nations Environment Programme. Chemicals and Health Branch. Geneva, Switzerland
- UNEP Global Mercury Assessment (2013) *Sources, Emissions, Releases and Environmental Transport,* accessed online 12/11/2018 http://www.unep.org/PDF/PressReleases/GlobalMercuryAssessment2013.pdf
- USEPA (1997b) *Engineering bulletin: technology alternatives for the remediation of soils contaminated with As, Cd, Cr, Hg, and Pb.* U.S. EPA Report EPA/540/S-97/500
- USEPA (2007a) *Treatment technologies for mercury in soil, waste, and water*. U.S EPA Report EPA-542-R-07-003.
- USEPA, 2011. *Mercury and Air Toxics Standards*, <https://www.epa.gov/mats/epa-announces-mercury-and-air-toxics-standards-mats-power-plants-rules-and-fact-sheets> accessed on line 19 Jan, 2019.
- Vahter, M.E., Mottet, N.K., Friberg, L.T., Lind, S.B., Charleston, J.S. and Burbacher, T.M., 1995. Demethylation of methyl mercury in different brain sites of Macaca-fascicularis monkeys during long-term subclinical methyl mercury exposure. *Toxicology and applied pharmacology*, 134(2), pp.273-284.
- Van Elsas, J.D. and Heijnen, C.E., 1990. Methods for the introduction of bacteria into soil: a review. *Biology and Fertility of Soils*, 10(2), pp.127-133.
- Vassileva, C.G. and Vassilev, S.V., 2006. Behaviour of inorganic matter during heating of Bulgarian coals: 2. Subbituminous and bituminous coals. *Fuel Processing Technology*, 87(12), pp.1095-1116.
- Villar-Salvador, P., Valladares, F., Domínguez-Lerena, S., Ruiz-Díez, B., Fernández-Pascual, M., Delgado, A. and Peñuelas, J.L., 2008. Functional traits related to seedling performance in the Mediterranean leguminous shrub Retama sphaerocarpa: insights from a provenance, fertilization, and rhizobial inoculation study. *Environmental and experimental botany*, 64(2), pp.145-154.
- Wahba, H.M., Stevenson, M.J., Mansour, A., Sygusch, J., Wilcox, D.E. and Omichinski, J.G., 2017. Structural and Biochemical Characterization of Organotin and Organolead Compounds Binding to the Organomercurial Lyase MerB Provide New Insights into Its Mechanism of Carbon–Metal Bond Cleavage. *Journal of the American Chemical Society*, 139(2), pp.910-921.F

- Wakefield, J.C. and Maynard, R.L., 2012. *Combustion toxicology. Essentials of Toxicology for Health Protection*: A Handbook for Field Professionals, p.227.
- Wang, J., Feng, X., Anderson, C.W., Xing, Y. and Shang, L., 2012. Remediation of mercury contaminated sites–a review. *Journal of hazardous materials*, *221*, pp.1-18.
- Wang, X., Du, Y., Luo, J., Yang, J., Wang, W. and Kennedy, J.F., 2009. A novel biopolymer/rectorite nanocomposite with antimicrobial activity. *Carbohydrate Polymers*, 77(3), pp.449-456.
- Wireman, J., Liebert, C.A., Smith, T. and Summers, A.O., 1997. Association of mercury resistance with antibiotic resistance in the gram-negative fecal bacteria of primates. *Applied and environmental microbiology*, 63(11), pp.4494-4503.
- World Health Organization, 2016. Guidance for identifying populations at risk from mercury exposure. *Mercury publications*.
- Xu, J., Bravo, A., Lagerkvist, A., Bertilsson, S., Sjoblom, R., Kumpiene, J. (2015) Sources and remediation techniques for mercury contaminated soil. *Environmental International*, v. 74, pp. 42-53.
- Xu, J., Bravo, A.G., Lagerkvist, A., Bertilsson, S., Sjöblom, R. and Kumpiene, J., 2015. Sources and remediation techniques for mercury contaminated soil. *Environment International*, *74*, pp.42-53.
- Xu, W., Hussain, A. and Liu, Y., 2018. A review on modification methods of adsorbents for elemental mercury from flue gas. *Chemical Engineering Journal*, *346*, pp.692-711.
- Yamashita, T., Ando, Y., Sakashita, N., Hirayama, K., Tanaka, Y., Tashima, K., Uchino, M. and Ando, M., 1997. Role of nitric oxide in the cerebellar degeneration during methylmercury intoxication. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1334*(2-3), pp.303-311.
- Yang, H., Xu, Z., Fan, M., Bland, A.E. and Judkins, R.R., 2007. Adsorbents for capturing mercury in coal-fired boiler flue gas. *Journal of Hazardous Materials*, 146(1-2), pp.1-11.
- Yang, Z., Shi, W., Yang, W., Liang, L., Yao, W., Chai, L., Gao, S. and Liao, Q., 2018. Combination of bioleaching by gross bacterial biosurfactants and flocculation: A potential remediation for the heavy metal contaminated soils. *Chemosphere*, 206, pp.83-91.
- Zhang, J. and Bishop, P.L., 2002. Stabilization/solidification (S/S) of mercury-containing wastes using reactivated carbon and Portland cement. *Journal of hazardous materials*, *92*(2), pp.199-212.

- Zhao, L.L. and Rochelle, G.T., 1998. Mercury absorption in aqueous oxidants catalyzed by mercury (II). *Industrial & engineering chemistry research*, 37(2), pp.380-387.
- Zheng, W., Liang, L. and Gu, B., 2012. Mercury reduction and oxidation by reduced natural organic matter in anoxic environments*. Environmental science & technology*, 46(1), pp.292-299.
- Zheng, Y., 2012. *Mercury Removal from Cement Plants by Sorbent Injection upstream of a Pulse Jet Fabric Filter*. Graduate Schools Yearbook 2010, p.205.
- Zheng, Y., Jensen, A.D., Windelin, C. and Jensen, F., 2012. Review of technologies for mercury removal from flue gas from cement production processes. *Progress in Energy and Combustion Science*, 38(5), pp.599-629.
- Zhuang, Y. and Miller, S.J., 2006. Impact of supplemental firing of tire-derived fuel (TDF) on mercury species and mercury capture with the advanced hybrid filter in a western subbituminous coal flue gas. *Energy & fuels*, 20(3), pp.1039-1043.
- Zou, S.P., Huang, J.W., Xue, Y.P. and Zheng, Y.G., 2018. Highly efficient production of 1-cyanocyclohexaneacetic acid by cross-linked cell aggregates (CLCAs) of recombinant E. coli harboring nitrilase gene. Process Biochemistry, 65, pp.93-99.

[i] * Or biochar and similar