Biogeochemical Investigations of a Full Scale Mussel Shell Bioreactor for the Treatment of Acid Mine Drainage (AMD), the Stockton Mine, New Zealand

Zach A. Diloreto
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Biogeochemical Investigations of a Full Scale Mussel Shell Bioreactor for the Treatment of Acid Mine Drainage (AMD), the Stockton Mine, New Zealand

By

Zachary A. DiLoreto

A Thesis
Submitted to the Faculty of Graduate Studies
Through the Great Lakes Institute for Environmental Research
In Partial Fulfillment of the Requirements for
The Degree of Master of Science
At the University of Windsor

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DECLARATION OF CO-AUTHORSHIP AND PREVIOUS PUBLICATION

I. Co-Authorship Declaration

I hereby declare that this thesis incorporates material that is result of joint research, as follows:

This thesis incorporates work in collaboration with Dr. Paul Weber, William Olds, Dr. James Pope, Dr. Dave Trumm, and Dr. SubbaRao Chaganti under the supervision of Dr. Chris Weisener and Dr. Dan Heath. Collaboration is covered in Chapter 2 of the thesis and all experimental designs, data interpretation and collection were carried out by the author. Co-authors contributed in creation of the physical bioreactor, as well as providing expertise in the writing process to prepare research properly for publication.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

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ABSTRACT

Acid mine drainage (AMD) impacted waters are a worldwide concern for the mining industry; both active and passive technologies are employed for their treatment. System design and biogeochemical investigations are presented here for a novel, fully operational, mussel shell bioreactor (MSB) used to treat low pH effluents elevated in Al, Fe, Ni, and Zn. This bioreactor is located within the Whirlwind catchment of the Stockton Coal Mine, on the West Coast of the South Island of New Zealand. The bioreactor raised the effluent pH from 3.4 to 8.3 while removing ~99% of the dissolved Al, and Fe and >90% Ni, Tl, and Zn. To understand the performance and functionality of the bioreactor a systematic approach was undertaken to investigate its bio-physico-chemical dynamics. This work describes a comprehensive investigation of the chemistry, microbiology, and functionality of this novel passive treatment approach and sheds light on performance for global technology transfer.
ACKNOWLEDGEMENTS

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LIST OF ACRONYMS

AMD: Acid Mine Drainage

SAP: Successive Alkalinity Producing System

HRT: Hydraulic Retention Time

MSB: Mussel Shell Bioreactor

PCA: Principal Component Analysis

CCA: Canonical Correspondence Analysis

PAF: Potentially Acid Forming

PRB: Permeable Reactive Barrier:

OLC: Open Limestone Channel

ALD: Anoxic Limestone Drain

SRB: Sulfate Reducing Bacteria

VFW: Vertical Flow Wetland

ANC: Acid Neutralization Capacity
Chapter 1: INTRODUCTION

1.1 Acid Mine Drainage: Scope of the Problem

Acid Mine Drainage (AMD) is a persistent issue and of concern for the international mining community. In the United States alone approximately 200,000 AMD sites exist and in Europe there are over 5000 km of AMD impacted watersheds some predating 1000 years (Hochella et al., 1999; Ließmann, 1992; Schippers et al. 2010; Baker and Banfield 2003). To further illustrate AMD as a global issue, Egiebor and Oni estimated that there are 15,000 ha of land in Canada contaminated by AMD; Harries (1997) reported 54 mine sites in Australia with significant amounts of potentially acid forming (PAF) waste and another 62 sites with minor amounts of PAF resulting in management costs of approximately $60 million per year. A large extent of AMD has also been documented in South Korea with 1000 abandoned metal mines (Cheong et al., 1998; Neculita et al., 2011), and 300 coal mines generating up to 48,000 tons day$^{-1}$ of AMD, affecting 153 km of streams (Ji et al., 2008; Neculita et al., 2011). Furthermore, in 2003 AMD had been observed at approximately 450 closed mines as reported by Japan's Oil Gas and Metals National Corporation (JOGMEC) (Koide et al. 2012). Clearly AMD remains a major issue facing the mining industry and its large extent is echoed in several additional studies such as Alcolea et al., (2012); Hengen et al., (2014); Nieto et al., (2013). This situation illustrates the need for continued research into creation and optimization of cost-effective treatment technologies.

1.2 Acid Mine Drainage: Causes and Reactions

Acid Mine Drainage (AMD) is the result of the oxidation of sulfide bearing minerals, mainly pyrite, within rock from overburden, tailings and high-walls. Oxidation of sulfides results
in the generation of an acidic metal and metalloid laden effluent, as well as a variety of additional products that are detrimental to receiving environments. These include oxyhydroxides, metal-bearing sulfates, oxides, as well as colloidal and adsorbed material (Bigham and Nordstrom, 2000; Jamboor et al., 2000; Evangelou and Zhang 1995; Benner et al., 1999). Sulfide oxidation, using pyrite as the main reactant, occurs in multiple steps as described in the following reactions (Nordstrom, 1982; Akcil and Koldas, 2006; Blowes et al., 2013):

Oxidation of pyrite through interaction with atmosphere and oxidative waters leading to the generation of ferrous iron, sulfate, and hydrogen.

\[(1) \ FeS_2(s) + \frac{7}{2} O_2 + H_2O \rightarrow Fe^{2+} + 2 SO_4^{2-} + 2H^+\]

Products from (1) will may result in a decrease in pH and provided the environment remains oxidative released ferrous iron will proceed to ferric iron through reaction (2)

\[(2) \ Fe^{2+} + \frac{1}{4} O_2 + H^+ \rightarrow Fe^{3+} + \frac{1}{2} H_2O\]

Commonly AMD effluent reaches pH ranges conducive to the formation of iron oxyhydroxides, such as ferrihydrite \((5Fe_2O_3\cdot9H_2O)\), as well as jarosite \((KFe^{3+}(OH)_6(SO_4)_2)\) leading to removal of ferric iron from solution and a subsequent lowering of pH (3).

\[(3) \ Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+\]

Through a combination of these reactions, the total reaction for AMD generation can be expressed as (4)

\[(4) \ FeS_2(s) + \frac{15}{4} O_2(aq) + \frac{7}{2} H_2O(aq) \rightarrow 2SO_4^{2-} + Fe(OH)_3(s) + 4H^+(aq)\]
Additionally it should be noted that any ferric iron not precipitated in reaction (2) can further increase the rate of pyrite oxidation through reaction (5)

\[
(5) \text{FeS}_2(s) + 14\text{Fe}^{3+} + 8\text{H}_2\text{O} \rightarrow \text{Fe}^{2+} + 2\text{SO}_4^{2-} + 16\text{H}^+
\]

Aside from pyrite, oxidation mechanisms for additional sulfidic phases include: pyrrhotite (Fe$_{1-x}$S), sphalerite (ZnS), galena (PbS), chalcopyrite (CuFeS$_2$), cinnebar (HgS), and arsenopyrite (FeAsS), and may affect AMD generation and have been reviewed by Blowes et al. (2013). Many of these minerals do not directly release protons upon oxidation, but rather through subsequent reactions, lessening their contribution to acid generation (Eby, 2004; Weisener, 2003; Blowes et al., 2013). However, their impact in terms of metal concentrations within AMD effluent may be considerable. The large variety of sulfidic phases, along with trace impurities in sulfides, and the size of economic mining operations, leads to a diverse range of AMD effluents. This range includes net acidic (pH ≈2) and net alkaline (pH ≈6) waters enriched in Ag, Al, As, Au, Cd, Co, Cu, Fe, Ni, Mg, Mn, Ni, Pb, Se, Tl, and Zn (Robb and Robinson 1995, Jambor et al. 2000, Akcil and Koldas 2006, Benner et al. 1999).

Although abiotic oxidation of sulfides is the principal cause of AMD, interactions with microorganisms can greatly enhance the oxidation rates of both sulfur and iron thus increasing the rate of AMD generation. Acid-soluble sulfides (e.g. ZnS) are susceptible to dissolution by the sulfuric acid that can be generated by microbes such as *Acidithiobacillus thiooxidans* as they oxidize elemental sulfur or reduced inorganic sulfur compounds (Blowes et al., 2013). Another competing reaction involves two aerchaecal and eight bacterial divisions that are known to accelerate AMD rates through metabolic oxidation and reduction of Fe accelerating the regeneration of Fe$^{3+}$ (Baker and Banfield, 2003; Edwards et al., 2000; Johnson and Hallberg 2000).
This can lead to the dissolution of acid-insoluble sulfides (e.g. FeS; FeS$_2$) through oxidation by ferric iron and is influenced by species such as *Leptospirillum* (Blowes et al., 2013).

The reactions responsible for AMD are also subject to different rates depending on a number of different parameters including, pH, microbial distribution and activity, waste pile permeability/flow rate through mine, temperature, surface area of exposed metal sulfide, oxygen content of water phase and gas phase, Fe$^{3+}$ regeneration rate/activity, time exposed to atmosphere, and energy required to start AMD processes. (Akcil and Koldas 2006, Blowes et al. 2003, Ritchie 1994). Understanding these parameters within an affected environment are key to mitigating the harmful effects of AMD effluent in the most effective and economic way.

### 1.3 Methods of Acid Mine Drainage Treatment

The high variability in characteristics, as well as detrimental impacts of AMD on the receiving environment, has led to the development of a number of different mitigation and remediation approaches. Mitigation strategies involve the prevention of sulfide oxidation through approaches such as physical barriers, subaqueous disposal, covering, chemical treatments to encapsulate sulfides, and bactericide to prevent or lessen microbially mediated iron and sulfur oxidation (Blowes et al., 2013). Due to the large reactive surface areas of point sources generating AMD, many mitigation strategies are not feasible as a sole means of AMD prevention. In addition to mitigation, treatment of AMD effluent before discharge offsite is common practice and often referred to as "Migration Control" (Johnson and Halberg, 2005). Overall, treatment of AMD effluent can be broadly divided into abiotic and biotic methods and further subdivided into active and passive systems. While the former description is apparent,
active and passive systems warrant further definition as they consist of a large group of different technologies.

The most prevailing methods of active AMD treatment are abiotic and involve the collection of effluent and addition of a chemical neutralizing agent such as lime, calcium carbonate, sodium carbonate, sodium hydroxide, and magnesium oxide/hydroxides resulting in the generation of a metal rich ferric hydroxide precipitate, referred to as AMD sludge (Johnson and Hallberg, 2005, Blowes et al., 2013). The sludge is then contained through flocculation, flotation or a combination of both and impounded (Da Silveria et al., 2009). Although effective, active treatment is expensive and not always feasible for closed operations and legacy sites where power is not available. Such situations favour the use of passive treatment systems as they are generally easy to implement, are reasonably cost-effective, require no power or other services, and have lower maintenance requirements than active treatment systems.

The advantages and shortcomings of varying passive treatment technologies have been reviewed by several studies including Johnson and Hallberg (2002 and 2005), Neculita et al. (2007), Ziemkiewicz et al. (2003), Skousen (1997), Skousen et al. (2000), Rose (2010), Gazea et al. (1996), and Watzlaf et al. (2004). Among these treatment systems are constructed wetlands, permeable reactive barriers (PRBs), open limestone channels (OLCs), anoxic limestone drains (ALDs), and bioreactors. There is no one technology that is best for passive AMD treatment and implementation is subject to site specific conditions based upon various effluent parameters such as; net acidity/alkalinity, DO, [Fe$^{3+}$], [Al$^{3+}$], and flow rate (Hedin et al., 1994; Skousen, 1997.)

Constructed wetlands are a long standing method of treatment implemented at various sites and are used because of their ability to reduce suspended sediment, remove metals and their
inherent buffering capacity. The main constituents of constructed wetlands are plants (*Typha* /cattails), microbes and limestone which promote increased pH and metal retention. Aside from classical neutralization, associated carbonate neutralization reactions, wetlands promote photosynthetic reactions which may also increase neutralization. One such example is conversion, through metabolic processes by microbes, of bicarbonate to hydroxyl ions in the following reaction (Johnson and Hallberg, 2005):

\[
(6) \quad 6\text{HCO}_3^- \quad (\text{aq.}) + 6\text{H}_2\text{O} \quad \rightarrow \quad \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2 + 6\text{OH}^-
\]

Although wetlands may be a favourable approach to passive AMD treatment there are drawbacks, such as the amount of land and cost required for installation, as well as unpredictability of the chemical nature of treated material.

Permeable reactive barriers (PRBs) are mixtures of reactive material placed within an excavated pit. These are down-gradient from mine sites are used to treat AMD affecting aquifers (Blowes et al., 2000; Blowes et al., 2013). Many PRBs employ reductive microbial metabolism to generate alkalinity and sequester metals within sulfides. PRBs are a highly effective for treatment of groundwater, but not applicable to surface treatment of AMD.

Open limestone channels (OLCs) and anoxic limestone drains (ALDs) are purely abiotic treatment systems designed to neutralize AMD effluent and promote metal precipitation. OLCs are drainage streams lined with crushed limestone, while ALDs are buried beds of limestone that promote anoxic conditions and neutralization. These systems are favoured for AMD that is lower in Fe$^{3+}$ and Al$^{3+}$ as a high abundance of these elements results in the precipitation of hydroxide phases. These hydroxide phases create a cement concretion within limestone in a process referred to as "armouring", which significantly reduces the neutralization capacity of the system.
and may result in clogging and eventual failure. Overall, while these methods are generally low cost and easily implemented on a large scale, their long-term performance is poor.

While many of these systems involve microbial pathways to promote alkalinity generation and sequester metals, these are a secondary design parameter and act in an ancillary manner. In comparison, bioreactors are specifically designed to promote these processes and built with optimal carbon sources, retention time, and geochemical conditions in mind. Of specific interest to passive AMD treatment are engineered bioreactors which capitalize on bacterial sulfate reduction pathways under chemically reducing conditions, and utilize a variety of sulfur reducing microbes (SRBs), and organic carbon sources. An example pathway is demonstrated in the following reactions for heterotrophic sulfate reduction (Stumm and Morgan, 1981).

\[(7) \text{CH}_2\text{O} + \text{SO}_4^{2-} \rightarrow \text{H}_2\text{S} + 2\text{HCO}_3^- \]

Organic carbon + sulfate \rightarrow hydrogen sulfide + bicarbonate

\[(8) \text{M}^{2+} + \text{H}_2\text{S} + 2\text{HCO}_3^- \rightarrow \text{MS} + 2\text{H}_2\text{O} + 2\text{CO}_2 \]

Divalent metal + hydrogen sulfide + bicarbonate \rightarrow metal sulfide + water + carbon dioxide

The subsequent alkalinity generating reactions provide conditions favourable for the cycling of sulfur (e.g. $\text{SO}_4 \leftrightarrow \text{H}_2\text{S}$) and the complexation of reduced metals (e.g. Fe(II), Zn(II) Mn(II), or As(III)).

Since their development, sulfur reducing bioreactors have operated with a variety of organic carbon sources (Kaksonen and Puhakka, 2007; Liamleam and Annachatre, 2007; Papirio et al., 2013). For example manures (cow, pig, goat, and buffalo), sawdust, rice straw, woodchips, sugarcane waste, mushroom compost and chitinous material have all been used with variable
levels of success (Chang et al., 2000; Gibert et al., 2004; Zagury et al., 2006; Daubert and Brennan, 2007; Robinson-Lora and Brennan, 2009; Choudhary and Sheoran, 2012; Song et al., 2012; Zhang and Wang, 2014). These organic substrates can be grouped into labile and recalcitrant carbon sources based on their ease of biodegradation. Bioreactor substrates containing composites of labile (e.g. manures) and recalcitrant carbon sources (e.g. chitin, cellulose) have been shown to achieve greater sulfate reduction rates than those with only a single carbon source (Zagury et al., 2006; Waybrant et al., 1998; Waybrant et al., 2002; Cocos et al., 2002; Neculita et al., 2007) suggesting substrates with a mix of carbon sources with different reactivity is optimal for use in these types of bioreactors. Many of these systems use a porous media, which can range from organic mulch blended with crushed limestone, or systems unique to this particular study that utilise weathered mussel shells (Sapsford and Watson, 2011; Sapsford, 2013; Lindsay et al., 2011; Macias et al., 2012; Strosnider et al., 2013; Hengen et al., 2014; Blowes et al., 2013; Zipper and Skousen, 2014). The latter provides exceptional permeability and reactive surface area with extensive neutralization capacity and ability to remove 99% of metals (McCauley et al., 2010), which has led to its use a main constituent of a mussel shell based bioreactor installed at the Stockton Mine, New Zealand.

1.4 Mussel Shell Bioreactors: Technological Progression

Chitinous waste materials have been investigated as an organic substrate for passive AMD treatment utilizing sulfate reduction processes (Daubert and Brennan, 2007; Robinson-Lora and Brennan, 2009a; Newcombe and Brennan 2009; Robinson-Lora and Brennan, 2009b). These studies demonstrated high alkalinity generation in comparison to other organic substrates of 25.2 mg CaCO$_3$ L$^{-1}$ d$^{-1}$and metal removal of Al, Fe, and Mn, coupled to sulfate reduction rates of 185 nmol ml$^{-1}$ day$^{-1}$. However, crab shell chitin based bioreactors have not been examined in a
field setting, are more expensive to implement than traditional substrates, and have been shown
to be most effective when amended with 30% spent mushroom compost rather than as a single
substrate (Grembi et al., 2015). A similar chitinous waste product has been examined for use in
bioreactors over the last several years.

Mussel shells contain up to 5-12wt% organic content and have a structure that consists of
sheets of amorphous CaCO₃ with interlamellar sheets of chitin in a "brick and mortar"
arrangement (Jacob et al., 2008; Kawaguchi and Watabe, 1993). They host both a labile and
recalcitrant carbon source containing residual meat and chitinous components. The remaining
88-95 wt% of mussel shell material consists of CaCO₃ which serves to generate alkalinity
making mussel shells an ideal substrate.

Trials using mussel shells were first used to treat AMD at the Stockton Coal Mine in
2007 (Weber et al., 2008). This study assessed the effects of infiltrating rainwater through a
waste rock pile. Two piles of 250 tonnes of acid-forming overburden were placed above
lysimeters that were 4m x 10m x 0.3m. One pile was underlain by 10 tonnes of mussel shell
material, the other was a control pad. It was observed that the leachate from the mussel shell
padded overburden maintained a circum-neutral pH of 6.8 compared to a pH of 3.3 from the
control pad and that acidity was 1.9 mg L⁻¹ CaCO₃ and 350.2 mg L⁻¹ CaCO₃ respectively. These
preliminary experiments showed that dissolved Fe and Al concentrations were reduced to
background concentrations of 0.5 and 0.2 mg/L in the mussel shell leachate compared to elevated
concentrations of 8.5 and 54.7 mg/L in the control pad leachate. The study also noted that total
organic carbon (TOC) and carbonaceous biochemical oxygen demand (CBOD) were elevated in
the mussel shell pad compared to the control pad due to the residual biological tissue associated
with the mussel shells. These findings were encouraging, suggesting that the shells could be used
as a potential source of alkalinity generation and promote SRB activity, which was later proven in laboratory studies. Laboratory results from McCauley et al. (2008, 2009a, 2009b, 2010) showed that the alkalinity generation resulted in the removal of >0.8 moles of metal m$^{-3}$ day$^{-1}$, as well as achieving acidity removal rates of >66 g CaCO$_3$ m$^{-2}$ day$^{-1}$, which are comparable to classic vertical flow wetlands (VFWs) and SAPs using limestone as the sole alkalinity generating source.

In 2009 a field scale prototype mussel shell bioreactor (MSB) was installed to treat the Manchester Seep discharging from waste overburden. The Manchester Seep had a mean flow rate of 0.3Ls$^{-1}$ with a pH 2.8 and calculated acidity of 420 mgL$^{-1}$. The influent chemistry contained elevated metal concentrations (Al 49 mgL$^{-1}$; Fe 31 mgL$^{-1}$; Ni 2.47 mgL$^{-1}$; Zn 1.2 mgL$^{-1}$; Tl 4.6x10$^{-3}$ mgL$^{-1}$; and sulfate 795 mgL$^{-1}$) (Crombie et al., 2011). The MSB was constructed using Perna Canaliculus, which are green lipped mussels capable of growing up to 240 mm in length (Crombie et al., 2011; SITO, 2006;). Shells used in the pilot-scale reactor were taken from the seafood processing industry and were crushed to approximately 30 mm and contained 5-12 wt% meat. The MSB was a trapezoidal pit 2 m deep, 35 m long, 3-10 m wide with 60° angle sides. During operation it contained 160 tonnes (240 m$^3$) of mussel shell material and was saturated with a 100-200 mm water cap. Influent flowed through the reactor at a mean rate of 0.3 L s$^{-1}$ resulting in an HRT of ≈6 days. The prototype MSB was in operation for a total of 1,027 days, from June 2009 through March 2012, sequestering 99.7% of Al, %99.3 of Fe, 98.8% of Ni, 98.4% Tl and 99.3% of Zn, while maintaining a high neutralization capacity of the treated influent resulting in a shift in influent pH from 2.8 to 6.9 in the effluent. This success illustrated the viability of mussel shell based systems in the field.
Within the Manchester prototype MSB distinctive reactive layers formed consisting of a sediment layer up to a 330 mm depth, an ocherous precipitate layer from 330–350 mm, an aluminum layer sampled at two intervals (350-500mm and 500-600mm), as well as black precipitate up to 1100 mm in depth. These layers portrayed the development of a distinct geochemical gradient, which had been documented before in a similar substrate (Thomas and Romanek, 2002). Additionally, ZnS precipitates detected in reduced layers of the MSB exhibited a spherical colloform texture that are associated with bacteria, suggesting the presence of active sulfate-reducing bacteria (SRBs) within the MSB.

After several years of study, MSB technology was proven as a cost-effective AMD treatment method at the Stockton Mine. A full scale system was proposed to not only treat a larger AMD effluent, but also provide an opportunity to closely examine the influence of SRBs on MSB dynamics and optimize the technology. This led to the installation of a full scale system upon which this research is based.

1.5 Research Scope

MSB technology has proven to be a cost-effective means of passive treatment of AMD effluent with systems at all scales successfully buffering effluent to circum-neutral pH and sequestering, with high efficiency, problematic metals. However, to date much of the research has focused on optimizing construction and understanding the geochemical nature of precipitates within the MSB. As this technology moves to a full scale operation, further understanding of the biogeochemical mechanisms operating within the bioreactor is warranted. Additionally, many studies that have examined the use of SRBs to promote the generation of biogenic sulfides through metabolic processes (J.W.H et al. 1994; Elliott et al.1998; Girguis et al. 2005; Neculita
et al. 2007; Fang et al. 2013; Albuquerque et al. 2013) have been performed only using laboratory studies. The new full scale reactor provides a prime opportunity to study biogeochemical interactions within a field setting. Understanding these mechanisms in depth will allow for optimization of the system, as well as a determination of longevity, and considerations for multi-site implementation. The basis of the biogeochemical evaluation will consist of detailed geochemical measurements along with metagenomic data correlated at different depths within the bioreactor. By understanding how geochemical conditions and microbial community varies with depth, a detailed understanding of the mechanisms within the bioreactor can be obtained.

1.6 Hypothesis

In this thesis it is hypothesized that there will be a clear geochemical gradient with depth, progressing from oxidative conditions within the top portions of the MSB to reductive conditions at depth. This geochemical gradient will govern the behaviour of precipitates within characteristic layers that are known to form in MSB systems, as well as determine the microbial community present. I hypothesize that species within the microbial community will follow the same trend as the geochemical gradient with oxidative microbes in the top portion and reductive microbes in the bottom portion. It is expected that these organisms will play a major role in the neutralizing and metal sequestration potential of the MSB. This influence on elemental cycling will be due to metabolic pathways, particularly those involving Fe and S.

1.7 Research Objectives

This thesis consists of 3 chapters describing the geochemical nature of the MSB, a metagenomic and statistical analysis of the microbial community of the MSB, and recommendations for further research into MSB technology. The research objectives for chapter
2 will evaluate geochemical-microbial performance of the MSB, with regards to effluent treatment parameters and operational longevity of the MSB system. Additionally, chapter 2 will examine the influence of geochemical conditions on layer development and microbial diversity and provide insight into the microbial community operating within the MSB and its influence on metal cycling. It is predicted that the MSB will be dominated by specialist species, which occupy environmental niches. These niches will arise from the characteristic geochemical zones of the MSB.

Chapter 3 will discuss the implications of biogeochemical parameters for the continued operation and optimization of MSB technology. The findings from the previous chapter will be reviewed with an emphasis on information still required to fully understand this technology. Overall this research will provide pertinent knowledge for operators of the technology and consolidate the use of MSBs as a proven method of passive AMD treatment. Additionally, this study will provide insights into microbial influences in a field setting, which are currently lacking.
References


15.


18.


CHAPTER 2: BIOGEOCHEMICAL CHARACTERIZATION OF A FULL SCALE MUSSEL SHELL BIOREACTOR

2.1 Introduction

In order to provide pertinent operational data pertaining to performance, longevity and stability of MSB precipitates to users of MSB technology, there is a need to holistically understand the biogeochemical dynamics at all depths within the bioreactor. Understanding the biogeochemical nature of the MSB will also be of importance when evaluating the microbial community and its influence on MSB dynamics.

It is expected that the full scale MSB will perform similarly to previous smaller scale systems, successfully buffering pH to circumneutral values and removing metals with a high efficiency (up to 99%), as well as forming characteristic geochemical layers with depth. These layers, in order of increasing depth, will consist of a sediment layer, iron precipitate layer, aluminium layer, and reduced layer. It is expected that the sediment layer will be highly oxic, acidic, with high concentrations of iron, while the iron precipitate layer will be suboxic and circumneutral with high concentrations of iron. Beneath the iron precipitate layer, the aluminum layer will be sub-oxic/anoxic and neutral, with iron depleted, but high concentrations of aluminum and trace metals. The reduced layer will be anoxic and neutral with depletion of iron and aluminum, but increased trace metal concentration and the presence of sulfides. As the majority of the precipitated phases are expected to be iron and aluminum oxyhydroxides, conditions that result in the instability of these phases will cause the greatest release of metals when subjected to selective extractions.
The influence of microbes is ubiquitous across many environments, both moderate and extreme. This influence stems from their ability to catalyze many reactions be it nitrogen fixation, ammonification, or even methanogenesis through their metabolic activity. To fully understand the microbial influence on MSB dynamics a holistic approach is needed. This requires the geochemical data to assess which redox couples are favourable, as well as the activity of species with respect to aerobes, anaerobes, or facultative organisms along the depth profile within the MSB. Additionally, metagenomic data is needed to identify which species are present and actively contributing to metal cycling. While these two aspects will provide information regarding which processes are occurring, to optimize and understand what the effect will be in an operational system, rates of cycling of elements of interest are needed. With information regarding organisms present, their likely metabolic pathways, and the rate at which they can influence element cycling, proper manipulations of certain factors can be proposed to optimize MSB technology. This may include any number of modifications including, but not limited to: the addition of more organic carbon, or a specific carbon source; pretreatment to remove elements problematic to microbial metabolism, such as aluminum; up-flow configuration versus down-flow configuration; and even inoculation with specific microbes. Additionally, behaviour of MSB sludge under varying environmental conditions can be determined, which is a key factor in determining disposal once MSB material is exhausted.
2.2 Methods

2.2.1 Site Description

The Brunner Coal Measures (BCM) occur on the West Coast of New Zealand and are currently mined by several companies including Solid Energy NZ Ltd at Stockton Mine [Figure 2.1]. These coal measures release AMD due to their high sulfide content in the waste and overburden coupled with high rain fall (∼7000 mm y⁻¹) and an annual average temperature of about 8°C. The BCM commonly contains up to 1 wt% sulfur and the overlying marine mudstones contain up to 5 wt% pyrite (Pope et al., 2010; Weber et al., 2004; Weisener and Weber, 2010). These materials result in the formation of acidic AMD effluents which are elevated in Fe, Al, Zn, Ni, Mn ± As, Cd, Cu, Pb, & Tl (Pope et al., 2010b; McCauley et al., 2008; McCauley et al., 2009a; McCauley et al., 2009b; McCauley et al., 2010; Pope and Trumm, 2015).
Figure 2.1: Location of Stockton Mine (Blue Box), West Coast, South Island, New Zealand indicated by drop marker. Coordinates 41.66 °S, 171.881 °E. (McCauley et al., 2010)
2.2.2 Bioreactor Design

The MSB system consists of 3 cells; a sediment retention pond, the bioreactor and an outflow channel [Figure 2.2]. With a trapezoidal design the bioreactor measures 32m x 20m at the top tapering down 1.2m vertically to 24m x 12m at the bottom and is saturated with 200mm of water cover. The MSB was filled with 362 T (~1t m$^{-3}$ density) of mussel shell waste product with a pore volume of 192m$^3$. The drainage network contains 6 lengths of megaflo drainage pipe wrapped in filter cloth with PVC capped ends to prevent clogging. These pipes were arranged in a rib like pattern and are connected to a central PVC pipe drain which flows out a riser into a final settling cell before discharge. The MSB was drained and sampled in May 2013 (8 months operational) and again in June 2014 (20 months operational). Samples were collected for geochemical and biological analyses. The samples were collected using a 4x4m spatial grid pattern [Figure 2.3]. At each location, samples were collected as a function of depth into the MSB system and in response to layering of the system [Figure 2.4] (Crombie et al., 2011; Diloreto et al., 2016). The depth measurements for each layer were taken during the two sampling periods, as well as the mean depths used in data analysis, are presented in [Table 2.1].
Figure 2.2: Three celled bioreactor system for treatment of the whirlwind seep on the Stockton Plateau. The first cell (1) is the sediment settling pond to reduced sediment loads within the MSB. The second cell (2) houses all chemical reactions as it contains the mussel shell material and drainage system. The final cell (3) is a second settling pond to allow for aeration and residual sediment settling before discharge.
Figure 2.3: Sample collection grid schematic at 8 and 20 months within the MSB. The 8 Month sampling period consisted of points A, B, and C while 20 month sampling consisted of points G, H, I and J. Bacterial samples for DNA were taken from D, E and F, and porewater was sampled from these points as the reactor was drained. All sampling points were taken in undisturbed areas.
**Figure 2.4:** Cross-sectional profile of the MSB shows, from right to left: Ocherous sediment layer, dark varved sediment, orange iron precipitate, black/silver aluminium layer, reduced and unreacted layer.
Table 2.1: Depths for sampling at 18 and 20 months

<table>
<thead>
<tr>
<th>Layer</th>
<th>Depth (mm) 8 Months</th>
<th>Depth (mm) 20 Months</th>
<th>Mean Depth (mm) 8 Months</th>
<th>Mean Depth (mm) 20 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allochthonous Sediment</td>
<td>0-10</td>
<td>0-22</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Iron Precipitate</td>
<td>10-22</td>
<td>22-38</td>
<td>16</td>
<td>30</td>
</tr>
<tr>
<td>Aluminum</td>
<td>22-52</td>
<td>28-80</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>Reduced</td>
<td>&gt;52</td>
<td>&gt;80</td>
<td>100</td>
<td>130</td>
</tr>
</tbody>
</table>
2.2.3 Water Chemistry and Selective Extractions

Influent and effluent water samples were collected on a bimonthly basis from 2012 –2014 and analyzed for pH, total metals, sulfate, nitrogen, and phosphorous (Hill Laboratories, New Zealand) with data collection ongoing. While the MSB drained pore-water was collected using Rhizon samplers (Rhizosphere Research Products) and frozen on dry ice. Pore-water pH and Eh was measured using an Orion 8102BN and 01301MD probes (Thermo Scientific). Selective extractions were used to evaluate metal partitioning between different phases within the system. The selective extractions targeted several phases including; water soluble, bio-available, reducible, carbonate and amorphous, amorphous oxyhydroxides and sulfides and strong acid extractable phases; details on the methods and reagents as per Diloreto et al. (2016) [Appendix Table A1]. Extractions performed on the 8 and 20 month samples were done in triplicate and analyzed using a Perkin Elmer ICP-OES, and a 700 series Agilent 720-ES ICP-OES system respectively.

2.2.4 Acid Neutralization Capacity and Bioreactor Infiltration Performance

Acid neutralization capacity (ANC) was performed on the 8 and 20 month samples using a modified test (IWRI and EGI, 2002; Sobek et al, 1978) [Appendix A2]. The MSB material depletion rate was evaluated by measuring layer growth and profile migration between 8 and 20 months. System performance is impacted by sediment accumulation and was evaluated by coupling infiltration rate with meteorological and flow data using an omnilog WT-HR water level and temperature data logger by Intech Instruments. Infiltrometer measurements were collected using a double ring infiltrometer with 60 cm outer ring and 30 cm inner ring. Data from a compliance monitoring site downstream of the MSB was used to assist in longevity estimates.
2.2.5 Metagenomic Library Preparation and Data Analyses

Samples for metagenomic analysis were collected and frozen in liquid nitrogen at -180 °C in the field. Samples collected in the field were subsequently stored in -80 °C freezer in the laboratory until DNA extraction. DNA extractions were performed using MoBioPower-Soil DNA isolation kit. The PCR reactions were carried out in two stages (PCR1 and PCR2). PCR1 was done to amplify the targeted region of 16s rRNA gene for both archaea and bacteria, while PCR2 was done to attached the barcodes to individual samples. Primer details are mentioned in [Table 2.2]. The thermocycling profile for PCR1 were as follows: initial denaturation for 5 min. at 95°C; 34 cycles of 15 sec. at 94°C; 15 sec. at 55/48°C (bacteria/archaea); and 30 sec. at 72°C; final extension for 1 min at 72°C. The amplicon products were purified using AMPure bead purification, following the manufacturer’s protocol. A second PCR was then performed for barcoding each of the samples (PCR2), using a unique barcode for each sample as the forward primer and a universal reverse primer referred to as UniB-P1 [Table 2.2]. The thermocycling profile for PCR2 were as follows: initial denaturation for 5 min. at 95°C; 7 cycles of 15 sec. at 94°C; 15 sec. at 60 °C (bacteria and archaea); and 30 sec. at 72°C; final extension for 1 min at 72°C. These PCR2 products were pooled and subjected to a slow gel electrophoresis using TAE buffer and the desired product was obtained by band excision. Excised bands were purified using Qiagen Gel Extraction kit following the manufacturer’s instructions. Purified pooled library DNA concentration and purity were determined by using Agilent 2100 Bioanalyzer. The samples were diluted to 25 ng/µL and sequenced using the Ion Torrent Personal Genome Machine (Life Technologies). Further metagenomics data was processed using the UPARSE algorithm (Edgar, 2013) by using the default parameters. The representative sequence for each Operational Taxonomic Units (OUT) was selected using most abundant method for assigning
taxonomy using RDP Classifier program with minimum 80% confidence level (Wang et al. 2007).

Further for the statistical analyses (Principle component analyses (PCA), Canonical Correspondence Analysis (CCA), simper, and microbial diversity index’s) PAST software (version 3.0) was used (Hammer et al. 2001). Nine environmental factors including depth (location of samples collected in mm), Eh (mV), pH, ANC (kg H₂SO₄ t⁻¹), and metal concentration for Fe (mg kg⁻¹), Al (mg kg⁻¹), Ni (mg kg⁻¹), Zn (mg kg⁻¹) were included for the PCA analyses. For CCA analyses the top 20 dominant genera of bacteria were included in addition to the nine environmental parameters. A Brays-Curtis similarity index was used for the CCA. Simper analyses were performed between the chemical zones (e.g. Allochthonous sediment, iron oxide and chemically reduced layers). The alpha diversity was estimated through Shannon H index and Chao 1.

2.2.6 Microbial Enrichments and Activity

Sulfur reduction and iron oxidation rates were determined from bacterial enrichments collected and preserved from the bioreactor. An autoclaved sample of material collected from the bioreactor served as a control to compare abiotic Fe and S rates. To determine the rate of iron oxidation three 100ml glass crimp top vials were filled with 80 ml of Wolfe's media, 1 ml of Wolfe's vitamin solution, 1 ml of Wolfe's mineral solution, (Emerson and Moyer, 2002) and inoculated with 5g of material from the iron precipitate layer of the MSB. Two ml of sterile 100mM FeCl₂ solution was added prior to the first measuring time. Iron(II) and Iron(III) concentrations were determined at 1 day intervals over a 10 day period using the ferrozine method (Viollier et al., 2000). Absorbance was measured at 562nm on a Genesys 20
spectrophotometer. Differences in Fe (II) and Fe (III) concentrations as a function of time were determined and normalized. Sulfate reduction was performed in triplicate using 100ml glass crimp top vials flushed with nitrogen and inoculated with sediment from the MSB using 80ml of Postgate media C (Postgate, 1979). Samples were collected over a ten day period at 1 day intervals. Due to the high concentration of sulfate present in the samples an AQUAfast 4000 colorimeter was used to track decreases in sulfate concentrations in the vials. The Hydrogen sulfide production was measured simultaneously during this period using a H2S-500microsensor which has a HS detection limit of < 20nM (Unisense). Normalized bacterial cell counts were obtained using a haemocytometer and a Leica CTR fluorescent light microscope.

2.3 Results and Discussion

2.3.1 Hydrological Conditions

The hydrologic gradient of the MSB system follows a vertical flow path with an estimated flow capacity of 1-6 Ls⁻¹ and a theoretical residence time ranging from 2.2 to 0.37 days. The influent treated by the MSB is derived from the Whirlwind Seep, which has a pH of 3.3 with elevated metals (e.g. Al 15.7 mgL⁻¹; Fe 1.9 mgL⁻¹; Ni 0.07 mgL⁻¹; Tl 7.9x10⁻⁴ mgL⁻¹; Zn 0.26 231mgL⁻¹) and sulfate 172.6 mgL⁻¹; Flow 1-6Ls⁻¹; Acidity 71.5 mg CaCO₃ L⁻¹. During the first 20 months of MSB operation the influent pH was successfully neutralized producing an effluent pH of 7.9 [Table 2.3]. The treatment of metals was evident with ~99% removal efficiency achieved for all metals of concern, including Al, Fe, Ni, and Zn [Table 2.3]. Similar metal removal performance were observed by Crombie et al. (2011) with more acidic and trace element rich AMD. Standard limestone oxic and anoxic drains that have been used to neutralize
Table 2.2: Metagenomic targets and primers used in PCR cycle. Lower-case areas are the linker zones of the Primers. XXXX are representative of barcodes 10-12 base pairs in length.

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCR1</strong></td>
<td></td>
</tr>
<tr>
<td>Bacterial 16S V5/V6</td>
<td>UniA+V5F acctgcctgccgATTAGATACCCNGGTAG</td>
</tr>
<tr>
<td></td>
<td>UniB+V6R acgccaccgagcCGACAGCCATGCANCACT</td>
</tr>
<tr>
<td>Archaeal 16S A785/A921</td>
<td>UniA+785F acctgcctgccgGGATTAGATACCCSGG</td>
</tr>
<tr>
<td></td>
<td>UniB+921R acgccaccgagcCCCCGCAAATTCCTTTAAGTTTC</td>
</tr>
<tr>
<td><strong>PCR2</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P1+UniB CCTCTCTATGGGCAGTCGGTGATacgccaccgagc</td>
</tr>
<tr>
<td></td>
<td>A+Barcode+UniA CCATCTCATCCTGCCTGTCTCCGACTCAGXXXX XXGATacctgcctgccg</td>
</tr>
</tbody>
</table>
Table 2.3: Influent/effluent chemistry and removal efficiencies

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Influent (mg L(^{-1}))</th>
<th>Effluent (mg L(^{-1}))</th>
<th>Removal Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.2±3.5</td>
<td>7.6-8.3</td>
<td></td>
</tr>
<tr>
<td>Al</td>
<td>15 (±4.5)</td>
<td>0.03 (±0.01)</td>
<td>99</td>
</tr>
<tr>
<td>Fe</td>
<td>1.9 (±0.5)</td>
<td>0.10 (±0.01)</td>
<td>99</td>
</tr>
<tr>
<td>Zn</td>
<td>0.26 (±0.05)</td>
<td>0.01 (±0.02)</td>
<td>97</td>
</tr>
<tr>
<td>Ni</td>
<td>0.10 (±0.01)</td>
<td>0.01 (±0.01)</td>
<td>90</td>
</tr>
<tr>
<td>SO(_4)</td>
<td>172 (±36)</td>
<td>158 (±37)</td>
<td>-</td>
</tr>
<tr>
<td>P</td>
<td>0.01 (±0.01)</td>
<td>0.03 (±0.02)</td>
<td>-</td>
</tr>
<tr>
<td>Total N</td>
<td>0.04 (±0.03)</td>
<td>0.71 (±1.3)</td>
<td>-</td>
</tr>
</tbody>
</table>
acidity consistently generate alkalinity resulting in a pH between 6 and 7 (Cortina et al., 2003; Castillo et al., 2012). In part this is due to combination of limestone reaction kinetics and reactive surface area which reaches equilibrium quickly. By comparison the MSB generates more alkalinity due to its higher reactive surface area and is able neutralize the effluent to pH ~7.9. Additional alkalinity generation likely occurs through secondary enhanced biological reactions generated through microbial sulfate reduction. This hypothesis is corroborated as average sulfate concentrations appear reduced by ~14 mg L\(^{-1}\) upon comparison of the influent to effluent chemistry. Nutrient concentrations of N and P in the influent AMD are below detection and increase to 0.71 and 0.03 in the effluent respectively. While these nutrients are essential to microbial community function and have even been proposed as a limiting factor of growth (Waybrant et al., 2002), release of these nutrients in excess, especially nitrogen, has been a concern raised by use of chitinous substrate bioreactors, due to its potentially detrimental effects on receiving environments (Robinson-Lora 2009; Grembi et al., 2015). Grembi et al. (2015) reported significant NH\(_4^+\) generation likely associated with fermentation of protein. NH\(_4^+\) concentrations generated were on the order of 28.4-32.9 mg N L\(^{-1}\), that decreased over time to zero generation after 60 days. These concentrations were well above the 2.6 mg N L\(^{-1}\) criteria for 1 hour acute exposure for freshwater aquatic life set by the EPA (U.S. EPA 2013) and the 1-5 mg N L\(^{-1}\) (site specific) compliance limit for primary industries over long term discharge (ANZECC). Currently, total nitrogen output by mussel shell material is significantly lower than this and phosphorus output is comparable to pristine tropical waters in Australia at 0.014 mg L\(^{-1}\) (Tsatsaros et al., 2013).
2.3.2 MSB Longevity Estimates

Calcite and its polymorph aragonite are the neutralizing mineral(s) of traditional limestone treatments and these systems are susceptible to armouring and clogging due to the precipitation of Al and Fe hydroxides (Rose, 2006), making prevention of clogging or armouring a primary design aspect in numerous systems (Skousen et al., 2000; Watzlaf et al., 2004; McCauley, 2010; Keppler and McCleary, 1997). Armouring can result in a reduction in limestone dissolution efficiency by up to 50% (Skousen et al., 2000) and in the worst cases can lead to failure (McCauley, 2010; Keppler and McCleary 1997). Failure of passive treatment can be extremely costly especially if it occurs early, 2-10 years (Rose, 2006), within passive systems commonly designed to last an average of 20 years (Ziemkiewicz et al., 2003). In contrast, although there has been a decrease in hydrological efficiency over the 2-3 year operational period of this MSB, it has not been related to armouring or resulted in failure of the system. The material has a high porosity, reactive surface area, and hydraulic conductivity minimizing secondary precipitation locally. In terms of providing an operational longevity estimate for MSB technology two factors need to be considered. The first is the rate of reduction in hydraulic conductivity of MSB due to sediment accumulation and cementation with Fe precipitates. This can be evaluated through a monitoring program using an infiltrometer and calculating sediment accumulation versus time. The second is depletion of the shell material itself, which can be evaluated in terms of growth of the aluminium layer (Crombie et al., 2011). Flow rate based on infiltrometer and omnilog data are shown in [Figure 2.5] from October, 2012 until July, 2015. Maximum infiltration rates steadily decrease from about 6L s$^{-1}$ to 2 L s$^{-1}$ over this period. Based on average flow rates from April 2013, 2014, and 2015 a yearly decrease of roughly 1 L s$^{-1}$ year$^{-1}$ is observed. Relatively low cost maintenance can be completed to remove
Figure 2.5 (top): Discharge rate over time from the MSB in L s⁻¹. Discharge rate shows a decrease of roughly 1 L s⁻¹ year⁻¹ corresponding to sediment accumulation atop the reactor. (bottom): pH measurements from environmental monitoring site S4 showing pre and post MSB values. There is a marked increase in pH after MSB installation. This site receives additional, untreated acidic effluent making buffering waters released from the MSB vital to maintaining acceptable pH values at the site.
the sediment and precipitate off the top of the reactor to promote longevity. At Stockton this process would be required once per year, but the frequency of this process will be site and design specific. It should be noted that a decrease in flow through the MSB does not affect effluent water chemistry, and the improved quality of treated effluent remains relatively unchanged under reduced flow conditions. However, at the Stockton site, as flow decreases the volume of AMD effluent treated also decreases, and more AMD bypasses the MSB through the spillway [Figure 2.2]. Increased AMD bypassing the system is evident in data from compliance monitoring site S4 downstream from the settling pond [Figure 2.5] where water quality decreases slightly with time.

The rate of exhaustion of shell material can be determined through the average growth of the reactive profile within the MSB. Failure would occur by extrapolating the average growth rate overtime until the aluminium layer reaches the drainage network depth at 1.2 m. Currently, within this system there is not enough data to make an accurate estimation of failure due to shell depletion.

2.3.3 Pore Water and Solid Phase Characterization

A summary of the ANC profiles, extractable Fe and Al distribution, pH and Eh from the bioreactor are provided in [Figure 2.6]. Both pH and Eh [Figure 2.6a] measurements show dramatic changes along the vertical depth suggesting a defined redox gradient. This is supported by the physical appearance of distinct geochemical zones of precipitation observed in [Figure 2.4]. The ANC (kg H\textsubscript{2}SO\textsubscript{4} t\textsuperscript{-1}) [Figure 2.6b], as well as the distribution of Fe and Al within the vertical transect is shown in [Figure 2.6c and 2.6d]. The sediment layer (0 – 10 mm) has an Eh
Figure 2.6: Geochemical conditions with respect to the vertical profile of the MSB for 8 and 20 month sampling periods. A: Eh (mV), and pH measurements. B. Acid neutralization capacity (ANC) in kg H$_2$SO$_4$ t$^{-1}$ of material. C: HCl extractable aluminum concentrations mg kg$^{-1}$ of material. D: HCl extractable iron concentrations mg kg$^{-1}$ of material.
of +80 to +120mV and a measured pH of 3.6 to 4. This location correlates to ANC values of <5 kg H$_2$SO$_4$ t$^{-1}$ for ANC potential within the top sediment horizon. The allochthonous sediment is both oxidized and acidic with little, if any, capacity to neutralize incoming AMD effluent. The subsequent ocherous layer is dominated by iron oxyhydroxides that extended from 10 to 22 mm depth and shows a rapid change in both pore water Eh and pH. Eh decreases from +45 to 26mV and pH increasing from 3.6 to 5.3. The ANC capacity within this layer increases to ~100 kg H$_2$SO$_4$ t$^{-1}$. The hydrolytic reactions involving iron, inferred by the abundance of Fe precipitates, within this layer are very characteristic of iron hydrolysis reactions that lead to its insolubility as pH increases above ~3.5. Below this reactive iron layer there is a zone from 22 to 52 mm deep dominated by white precipitates, which have been identified as amorphous aluminum hydroxide. Eh potentials continue to decrease from +26 mV to more reducing conditions ranging from -33 to -50 mV followed by a subsequent pH increase from 5.2 to 7 in the measured porewater. The ANC values collected from this layer is ~700 kg H$_2$SO$_4$ t$^{-1}$. The aluminum layer is characterized as a moderately reducing, circumneutral environment with high acid neutralization capacity. The bottom layer which extends from ~52 mm to 1200 mm (the base of the bioreactor) represents the chemically reduced mussel shell matrix this is based on the Eh and observed sulfide precipitation. Pore water collected from within this layer shows low Eh values of <-55 mV with alkaline pH ranging from 7.1 to 8.3. The measured ANC values are increased to 800 kg H$_2$SO$_4$t$^{-1}$. The shell layer represents a reduced environment with circum-neutral pH and a significant capacity to neutralize incoming acidic effluent. The hydrolysis and redox reactions, which occur in the bioreactor, are controlled by a series of abiotic chemical reactions and biologically catalyzed reactions. This results in a sequence of mineralogical phases consisting of iron and aluminum hydroxides, within their respective layers, progressing to sulfides within the reduced
layer. A conservative chemical extraction using 0.5M HCl shows the total extractable iron and aluminum as a function of depth in the MSB [Figure 2.6c]. It is clear from this that a strong correlation between high extractable Fe and Al exists between the corresponding geochemical environments in the MSB. This interpretation is confirmed by additional extractions [Appendix Figures A1; A2].

2.3.4 Trace Metal Behaviour, Principal Component Analysis (PCA), Microbial Diversity

The stability of trace elements incorporated into secondary mineral precipitates within the reaction profile of the MSB were investigated using a series of selective chemical extractions. These chemical extractions specifically target metals associated with water soluble, organically bound phases, phases susceptible to chemical reduction, as well as amorphous oxyhydroxide and sulfide phases. Changes in trace metal partitioning (e.g. Zn, Ni, and Tl) through the vertical profile of the MSB were strongly correlated with the water soluble, reducible phases, and amorphous oxyhydroxide/sulfide extractions and are shown in [Figure 2.7]. This has been observed in earlier investigations (Diloreto et al. 2016). Water Soluble Zn, Ni and Tl species were less than 10 mg kg\(^{-1}\) throughout the vertical profile [Figure 2.7A1.; 2.7A2.]. Zn, Ni and Tl were strongly associated with reducible phases, as well as amorphous oxyhydroxides and sulfides [Figure 2.7B1.; 2.7B2.; 2.7C1.; 2.7C2.]. The Zn, Ni and Tl associated with reducible phases are initially low in concentration ranging from 4 mg kg\(^{-1}\) at 8 months but then increase several orders of magnitude to 170 mg kg\(^{-1}\) for Ni, 5 mg kg\(^{-1}\) for Tl, and 353 mg kg\(^{-1}\) Zn at 20 months. A similar trend is observed with trace metals associated with amorphous oxyhydroxide and sulfidic phases. Tl was not detected. At 8 months concentrations of Ni and Zn were 38 mg kg\(^{-1}\) and 226 mg kg\(^{-1}\) respectively. At 20 months Ni increases to 45 mg kg\(^{-1}\) and concentrations of
Figure 2.7: Extractable trace metal concentrations in mg kg\(^{-1}\) of material, nickel, thallium and zinc, along the vertical profile of the MSB. Extraction targets include water soluble (A), reducible (B), as well as amorphous oxyhydroxides and sulfides (C). Measurements for extractions at 8 months are presented on the left half of each graph (A1, B1, C1), while measurements at 20 months are presented on the right half (A2, B2, C2). A visual representation of MSB layering can be found on the left hand side of each extraction series.
Zn double to 417 mg kg\(^{-1}\). The proportion of extractable Zn and Ni strongly correlate within the aluminium and reduced layers suggesting either complexation within the newly formed metal hydroxides and/or metal sulfides. In both the sediment and iron precipitate layers, trace metal deportment will be a function of competing sorption, and co-precipitation with Al and Fe oxyhydroxides. Iron oxide precipitates form at pH values >3, and Al hydroxides form at pH 4.5-5.5 where hydrolysis is the driving reaction consuming hydrogen (Bigham et al., 1996; Bigham and Nordstrom, 2000). Trace metals such as Ni, Zn, and Tl have been observed to partition into these phases (Gadde and Laitinen, 1974; Lee et al., 2002; Martinez and McBride, 1998; Shokes and Moller, 1999; Tessier et al., 1985; Doner and Ege, 2005). Based on the vertical flow of the MSB, following aluminium hydrolysis and precipitation, conditions shift from reduced to more reduced. This creates a third trace metal reservoir (e.g. sulfide hosting precipitates) (Diloreto et al., 2016). This shift is evident by the high amount of extractable trace metals associated with more aggressive extractions, specifically those targeting sulfide phases (Diloreto et al. 2016). Sulfides are able to incorporate trace metals such as Tl, Zn, Cd, As, and Ni within their crystal structure (Álvarez-Ayuso et al., 2013; Cook et al., 2009; Fu and Wang, 2011; Lewis, 2010). Additional extraction data for bio-available, carbonate and strong acid associated trace metals are shown in [Appendix Figure A3]. Upon examination no changes in deportment were observed under these extraction conditions.

Principal component analysis (PCA) [Figure 2.8], using the spatial geochemical measurements collected for the MSB system confirm the 3 distinct zones. PC1 explains 92.5 % variation associated with high loading of Al and S compounds compared to PC2 which explains 6% of the variance for high loading of Fe and S. To examine the significance of the principal components row-wise bootstrapping at 1000 repetitions (Peres-Neto et al., 2003), as well as
evaluation of eigenvalues using a random model (Jackson 1993) was conducted. These tests showed that only PC1 was significant and all other principal components may be ignored. Thus the characteristic geochemical zones present in the bioreactor are defined by Al and S concentration and behaviour. It should be noted that while the sediment layer forms its own distinct grouping there is overlap between the iron precipitate and reduced layers. This data is indicative of the geochemical behaviour of the MSB. It highlights the oxic nature of the upper portions of the MSB, which result in little to no precipitation of aluminum or sulfur phases, and the reduced nature of the MSB with depth resulting in precipitation of Al and S. To understand the influence of geochemical conditions on the principal microbes observed a canonical correspondence analysis (CCA) was performed [Figure 2.9]. The CCA incorporated 9 different environmental factors (Depth (mm), Eh (mV), pH, ANC (kg H2SO4t−1), Fe (mg kg−1), Al (mg kg−1), Ni (mg kg−1), Zn (mg kg−1)) and their respective microbiology in a matrix for the MSB. Both CCA1 and CCA2 were responsible for 99 % of the observed variance within the microbial distributions within the MSB. The CCA analyses describe a strong correlation between the microbial component and the developing geochemical environment within the MSB. Loading scores for CCA 1 show that differentiation is strongly positively correlated by Eh (0.63) and strongly negatively correlated with Al (-0.89). Additionally, the validity of each component was evaluated using eigen and p values. Analysis indicated that CCA1 is statistically significant with an eigenvalue of 0.1394 and p value of 0.001 at 999 permutations. CCA2 was much less significant with an eigenvalue of 0.001669 and p value of 0.017 at 999 permutations.

To confirm whether a similarity between microbial communities within these layers exists, an additional SIMPER analysis was performed. The SIMPER analysis yielded 63% dissimilarity in community structure between the allochthonous sediment and iron oxide
precipitate layers, and 74% dissimilarity between the chemically reduced layer respectively. There was only 23% dissimilarity in community structure between the lower iron oxide and reduced layers. This suggests that a direct relationship may exist between the bacterial community development and the geochemical conditions within the MSB.
Figure 2.8: Principal Component Analysis (PCA) of geochemical spatial data of the MSB. Ellipses represent sample groupings of each of the characteristic geochemical layers within the MSB.
Figure 2.9: Canonical Correspondence Analysis (CCA) of the microbial and geochemical data. Ellipses represent sample groupings of each of the characteristic geochemical layers within the MSB.
It is clear that within the geochemical zones that develop within the MSB an influence is exerted contributing to the variation in the microbial diversity. The Shannon H index values [Table 2.4] for the different layers show little differentiation, suggesting a similar amount of species richness and evenness. However, the Chao1 index shows a different trend with higher values in the sediment layer (52.34) progressing with depth to lower values in the iron (31.11) and increasing again within the reduced layers (44.11). The lower values calculated with the Chao1 index suggest that there are less distinct species with depth approaching the aluminum zone, which acts as a restrictive layer. Once Al has been precipitated diversity increases again within lower portions of the MSB that favor specialist organisms.
Table 2.4: Shannon H and Chao 1 diversity indexes comparing biodiversity and evenness.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Depth</th>
<th>Shannon H</th>
<th>Chao1</th>
<th>Average Reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allochthonous Sediment Layer</td>
<td>0-11 mm</td>
<td>1.91</td>
<td>52.34</td>
<td>9,513</td>
</tr>
<tr>
<td>Iron Precipitate Layer</td>
<td>11-40 mm</td>
<td>2.02</td>
<td>31.11</td>
<td>13,256</td>
</tr>
<tr>
<td>Reduced Layer</td>
<td>62-1655 mm</td>
<td>2.03</td>
<td>44.11</td>
<td>10,059</td>
</tr>
</tbody>
</table>
2.3.5 Sulfur and Iron Activity Rates

The rate of sulfur reduction determined for the MSB is similar to rates reported in other studies from constructed and natural environments with some exceptions [Table 2.5]. Based on SRB enrichments collected from the bioreactor sulfur reduction rates of 260 ± 60 nmol ml\(^{-1}\) day\(^{-1}\) were achieved. The sulfur reduction rate in the MSB is lower compared to other bioreactors where conditions were optimized (Montoya et al., 2013; Sanchez-Andrea et al., 2014). Specifically those that are based on influencing factors such as pH, temperature, bioreactor construction and tailoring the carbon source to the bacterial community. A comprehensive comparison of these varying factors is presented in Sanchez-Andrea et al. (2014) with rates ranging from 300 nmol ml\(^{-1}\) d\(^{-1}\) in an uncontrolled system similar to the MSB (Hiibel et al., 2011), to 9264 nmol ml\(^{-1}\) d\(^{-1}\) in a system controlling all bioreactor parameters (Montoya et al., 2013). While these controlled systems may achieve higher sulfate reduction rates, the parameters are strictly maintained making them unrealistic for use at full scale in a field setting where the microbial community is exposed to fluctuating conditions. Sulfate reduction rates from more natural systems are more applicable but are subject high variance. High variance in these systems is illustrated in studies such as Vile and Wieder (1993), which examined 5 constructed wetlands with varying sulfate reduction rates of 0-854 nmol ml\(^{-1}\) d\(^{-1}\); Roden and Wetzel (1996) observed rates of 54 ±4 nmol ml\(^{-1}\) d\(^{-1}\) within natural wetlands; Oil sands material showed rates of 50-232
Table 2.5: Comparison of sulfur reduction rates for natural and passive treatment environments in nmol ml$^{-1}$ day$^{-1}$.

<table>
<thead>
<tr>
<th>Sulfate Reduction Rate</th>
<th>System Description</th>
<th>Purpose</th>
<th>Scale</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>260 ± 63 nmol ml$^{-1}$ day$^{-1}$</td>
<td>Mussel shell bioreactor.</td>
<td>Passive mine treatment</td>
<td>Field scale/enrichment</td>
<td>This study</td>
</tr>
<tr>
<td>185 nmol ml$^{-1}$ day$^{-1}$</td>
<td>Crab chitin bioreactor.</td>
<td>Passive mine treatment</td>
<td>Laboratory scale/enrichment</td>
<td>Robinson-Lora and Brennan, 2009$^a$</td>
</tr>
<tr>
<td>300 nmol ml$^{-1}$ day$^{-1}$</td>
<td>Woodchip Bioreactor</td>
<td>Passive mine treatment</td>
<td>Field scale</td>
<td>Hiibel et al., 2011; Sánchez-Andrea et al., 2014</td>
</tr>
<tr>
<td>9264 nmol ml$^{-1}$ day$^{-1}$</td>
<td>Granular Sludge bioreactor. <em>inoculated culture</em></td>
<td>Passive mine treatment</td>
<td>Laboratory scale/enrichment</td>
<td>Montoya et al. 2013; Sánchez-Andrea et al., 2014</td>
</tr>
<tr>
<td>0-854 nmol ml$^{-1}$ day$^{-1}$</td>
<td>5 Constructed wetlands with varying substrates.</td>
<td>Passive mine treatment</td>
<td>Field scale</td>
<td>Vile and Wieder, 1993</td>
</tr>
<tr>
<td>50-232 nmol ml$^{-1}$ day$^{-1}$</td>
<td>OSPM</td>
<td>Carbon source treatment</td>
<td>TP* culture</td>
<td>Stasik and Wendy-Potthoff, 2013</td>
</tr>
<tr>
<td>0-90 nmol ml$^{-1}$ day$^{-1}$</td>
<td>OSPM</td>
<td>End pit lake reclamation</td>
<td>TP* culture enrichment</td>
<td>Stasik et al., 2014</td>
</tr>
<tr>
<td>2.5-1568 nmol ml$^{-1}$ day$^{-1}$</td>
<td>Natural Appalachian peatlands</td>
<td>End pit lake reclamation</td>
<td>Natural system</td>
<td>Wieder et al., 1990</td>
</tr>
<tr>
<td>0.2-1883 nmol ml$^{-1}$ day$^{-1}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>54 ± 4 nmol ml$^{-1}$ day$^{-1}$</td>
<td>Natural freshwater wetland, Alabama.</td>
<td>Fe(III) and CH$_4$ production in sediments.</td>
<td>Natural system</td>
<td>Roden and Wetzel, 1996</td>
</tr>
</tbody>
</table>
nmol ml⁻¹ d⁻¹ (Stasik and Wendy-Potthoff, 2013) and 0-90 nmol ml⁻¹ d⁻¹ (Stasik et al., 2014); and natural peatlands with recorded rates of 0.2-1883 nmol ml⁻¹ d⁻¹ (Weider et al., 1990).

Additionally, fluctuating conditions within natural systems have a profound effect on the microbial community, which in turn will affect the pathways and ultimately rates of sulfate reduction. The rate of iron oxidation from this and other studies are presented in [Table 2.6]. Iron oxidation rates were determined from enrichments collected from the MSB. Enrichments of iron oxidizing bacteria were only stimulated after being amended with a source of nitrate. This in itself illustrates the complex chemical pathway contributing to iron oxidation after DO is consumed in the MSB. Iron oxidation in the presence of bacteria capable of using nitrate as a terminal electron acceptor is controlling the rates of iron oxidation by several orders of magnitude. This reaction is possible in most anaerobic environments through the following reaction (Straub et al., 1996; Blothe and Roden, 2009):

\[ 5\text{Fe}^{2+} + \text{NO}_3^- + 12\text{H}_2\text{O} \rightarrow 5\text{F(OH)}_3 + 0.5\text{N}_2 + 9\text{H}^+ \]

Based on the MSB enrichments the determined iron oxidation rate is 9600 nmol ml⁻¹ day⁻¹. This rate is comparable to systems where neutrophillic iron oxidizers have been enriched in the laboratory (James and Ferris, 2004; Neubauer et al., 2002; Klueglein and Kappler, 2013). In contrast natural environments such as those resulting in bacteriogenic iron oxide precipitates (BIOS) and biofilms (James and Ferris, 2004), can exhibit iron oxidation rates several orders of magnitude higher of 33, 100 nmol ml⁻¹ d⁻¹, and 67, 600 nmol ml⁻¹ d⁻¹ both downstream of and at the biofilms respectively. However this is due to the efficiency of the organism relying on O₂ as a terminal electron acceptor versus N. In other bioreactors that have been optimized for temperature pH and use specific iron axenic cultures consisting of *Acidithiobacillus ferooxidans*
Table 2.6: Iron oxidation rates in comparison to this study in nmol ml d\(^{-1}\).

<table>
<thead>
<tr>
<th>Iron Oxidation Rate</th>
<th>System Description</th>
<th>Purpose</th>
<th>Scale</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>9600 nmol ml(^{-1}) day(^{-1})</td>
<td>Mussel shell bioreactor Nitrate TEA</td>
<td>Passive AMD treatment</td>
<td>Full scale/enrichment</td>
<td>This study</td>
</tr>
<tr>
<td>1,320,000 nmol ml(^{-1}) day(^{-1})</td>
<td>Packed bed reactor *O(_2) TEA</td>
<td>Fe (II) Cycling</td>
<td>Laboratory scale/axenic isolate</td>
<td>Long et al., 2003</td>
</tr>
<tr>
<td>12,100 nmol ml(^{-1}) day(^{-1})</td>
<td>Pulse feed bioreactor *O(_2) TEA</td>
<td>Fe(II) cycling in wetland rhizosphere.</td>
<td>Laboratory scale/enrichment</td>
<td>Neubauer et al., 2002</td>
</tr>
<tr>
<td>48-12,800 nmol ml(^{-1}) day(^{-1})</td>
<td>Continuous feed bioreactor *O(_2) TEA</td>
<td>Fe(II) cycling in wetland rhizosphere.</td>
<td>Laboratory scale/enrichment</td>
<td>Neubauer et al., 2002</td>
</tr>
<tr>
<td>5100 nmol ml(^{-1}) day(^{-1})</td>
<td>Culture enrichments- <em>Acidovorax spp.</em> Nitrate TEA</td>
<td>Fe(II) oxidation in cultures of <em>Acidovorax sp.</em></td>
<td>Laboratory scale/axenic isolate</td>
<td>Klueglein and Kappler, 2013</td>
</tr>
<tr>
<td>12, 300 nmol ml(^{-1}) day(^{-1})</td>
<td>Natural Wetland *O(_2) TEA</td>
<td>Bacteriogenic iron oxide (BIOS)</td>
<td>Laboratory scale/enrichment</td>
<td>James and Ferris, 2004</td>
</tr>
<tr>
<td>67,600 nmol ml(^{-1}) day(^{-1})</td>
<td>Natural wetland In-situ measurement at source of BIOS</td>
<td>Bacteriogenic iron oxide (BIOS)</td>
<td>Natural system</td>
<td>James and Ferris, 2004</td>
</tr>
</tbody>
</table>
can achieve rates of up to $1.32 \times 10^6$ nmol ml$^{-1}$d$^{-1}$. These rates are based on the organism's ability to couple iron oxidation to O$_2$, a strongly favoured thermodynamic reaction over nitrate (Bethke et al. 2011). In the context of the MSB the lower iron oxidation rates measured are representative of this relationship (Bethke et al., 2011). It is also possible that iron hydroxide phases present may be prone to bacterial dissolution facilitating and controlling the release of Fe. Iron cycling within the suboxic zones of the MSB could be influenced by neutrophillic iron oxidizers observed (e.g. Gallionella furringea, Leptothrix spp., Acidovorax sp. strain BoFeN, and Sideroxydans lithotrophicus ES-1) and discussed later (Miot et al., 2009; Druschel et al., 2008; Emerson and Moyer, 1997; Hedrich et al., 2011).

### 2.3.6 Microbial Community Composition

Based on the present taxonomy of the microbial community it is clear that a defined microbial community has developed in relation to the chemical environment within the MSB. This consists of euryarchaeota, fircmutes and proteobacteria and bacteroidetes [Figure 3.0]. DNA extracted from the aluminium layer was poor quality and fragmented so they were excluded from further analysis. As aluminium is known to inhibit the development of microbial communities, it constitutes a toxic ion during microbial metabolism with low bioavailability and no known biological function (Pina and Cervantes, 1996). This possibly contributes to the lack of extractable DNA from this layer. Bacteroidetes comprise the majority of the microbial community with an average of 80% abundance in all sampled layers of the MSB. Of this, 60% of bacteroidetes consists of flavobacterium, which are common to temperate and cold freshwater and soil environments (Bernardet and Bowman, 2006). Based on the taxonomic identification, certain species of flavobacterium, such as *Flavobacterium johnsoniae*, can secrete chitinase resulting in the breakdown of chitin and other complex polysaccharides (McBride and Zhu, 55.
Figure 3.0 (left): Trimmed relative abundance data at the domain and phylum level highlighting archaea, firmicutes, and proteobacteria associated with each characteristic layer as a function of pH. (right): Percent abundance of classes of proteobacteria associated with each characteristic layer as a function of pH. The Al oxide layer had no extractable, quality DNA, thus is excluded.
2013; Kharade and McBride, 2014) and in this case provide a mechanism for the mobilization of recalcitrant carbon contained within MSB material. Certain species of flavobacterium also generate H$_2$S indicating the potential for sulfate reduction (Van Trappen et al., 2004). The large fraction of bacteroidetes and their ability to fill varying environmental roles likely result in them having a significant impact on mechanisms occurring within the MSB. Proteobacteria species responsible for iron and sulfur cycling commonly detected in AMD environments are absent (e.g. Acidithiobacillus, Leptospirillum, Gallionella, Desulfobacter) suggesting a low diversity for these particular organisms within the MSB system. Metagenomic analyses using OTU comparisons show that the dominant acid tolerant species identified in the MSB profile was Acidovorax spp. Although this is a common genus, it should be noted that some species related to this genus are capable of metabolizing iron by coupling iron oxidation in the presence of nitrate (Straub et al., 2004; Weber et al., 2006; Pantke et al., 2011; Klueglein and Kappler, 2013). The dominant iron metabolizing species in the MSB were observed in the iron oxyhydroxide layer and consisted of Sideroxydans lithotrophicus. This species is also capable of iron oxidation using nitrate as a terminal electron acceptor (Blothe and Roden, 2009). This observation was also corroborated during the iron enrichment experiments in this study. In this case bacterial enrichments did not grow in the presence of oxygen as a possible terminal electron acceptor and were considered to be obligate anaerobes. However, when nitrate was added to the same bacterial enrichment a high growth rate was observed. No iron metabolizing bacteria were detected within the deeper profiles of the reactor including the aluminum oxide layer and the underlying reduced shell layers. A combination of both SRB enrichments and metagenomic investigation confirm the presence Desulfotomacculum acetoxidans (1-5% of total community. The main fermentation product of chitinous material has been shown to be acetate (Robinson and
Brennan, 2009), which is a primary metabolic requirement of *Desulfotomaculum acetoxidans*. This bacteria is a known spore forming SRB bacteria, which is more resistant to extreme environmental change (e.g. periods of desiccation and fluctuating oxic conditions) (Castro et al., 2000). The ability to form spores explains the presence of persistent SRBs (*D. acetoxidans*) within all layers of the MSB with the exception of the aluminum layer. Increased abundance of archaea (e.g. methanogens represented by *Methanosaeta concilii*, and *Methanolinea tarda*) was detected below the aluminum reaction zones within what is termed the "reduced" shell layer. These organisms control carbon dioxide and methane cycling associated with the further decay of residual organic matter associated with the mussel shells. *Methanosaeta concilii* uses acetic acid as its sole source of energy (Patel and Sprott, 1990), which is a product of degradation of chitinous material by hydrolytic reactions under anoxic conditions (Hock, 1940). Microbial composition in relation to the vertical geochemical profile correlation was shown in [Figure 3.1]. In part this diversity is being controlled chemically through competing hydrolytic reactions (e.g. Fe and Al) within the redox profile of the MSB. It is likely the dominant neutrophillic iron oxidizers present thrive under these conditions catalyzing abiotic iron oxidation (Neubauer et al., 2002, Weber et al., 2006, Druschel et al., 2008). Overall there is strong evidence for a system dominated by chitin degradation through fermentation and hydrolysis, leading to an acetate driven microbial community, exploited by niche species within each reactive layer. However, to confirm this, the relative function and activity of the organisms present should be investigated further. One possibility would be to use metatranscriptomics approach to determine and quantify gene regulation (e.g. mRNA) occurring within the operational cycle of the MSB.
**Figure 3.1:** Venn diagram of OTU generated genera designations based on characteristic layers. Each genus was assigned a proposed environmental function based on species and geochemical environment with designations as follows: ¹Iron oxidizer, ²iron reducer, ³denitrifier, ⁴ammonifier, ⁵sulfate reducer, ⁶sulfur oxidizer, ⁷methanogen.

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59.
References


61.


CHAPTER 3: SUMMARY AND CONCLUSIONS

3.1 Implications

MSB technology is a proven efficient and cost-effective means for the treatment of the Whirlwind effluent generated at the Stockton site and possibly other AMD impacted waters. MSB technology makes use of mussel shells, a novel organic substrate for a full scale SRB that is beneficial in terms of its abiotic and biotic properties. Material is also obtained at low cost and as a waste product, repurposing what would otherwise be landfill material. This allows MSB technology to be considered green and, as significant amounts of mussel-shell waste is generated by the fishing industry annually, renewable. Additionally mussel shell material functions as a stand-alone substrate and requires no inoculation to develop a beneficial microbial community and provides substantial amounts of bio-available carbon to maintain the microbial community.

Currently, the geochemical dynamics of MSB technology are well understood. Mussel-shell bioreactor technology functions efficiently and effectively as the material has high CaCO$_3$ content and ANC releasing sufficient alkalinity to remove Al and Fe with >99%. This well exceeds the environmental compliance targets at the Stockton site and is higher than many other similar systems. The precipitation of the major metals within the middle and upper portions of the MSB is a result of buffered pH. This leads to the formation of the characteristic iron and aluminium hydroxide layers. Below these layers pH and redox conditions transition creating an environment favourable to the formation of insoluble metal sulfides that are able to sequester the trace metals of concern, Ni, Tl and Zn at ≥%90 efficiency. MSB technology also has advantages over traditional limestone systems and other carbonate substrates, as its high neutralization capacity, coupled with high surface area and pore space, make the MSB less prone to armouring.
Although there are many benefits to implementing a bioreactor system for AMD treatment, there is a concern that increased concentrations of organic molecules may be released into the receiving environment. To date, few studies have focused on measuring these from bioreactor discharge as the principal concern is neutralizing acidity and removing metals. Fortunately, when compared to other similar substrates such as crab shell chitin, MSB material causes no significant release of organic molecules, such as NH$_4^+$ or P, which could lead to toxic effects in freshwater biota. Aside from acute toxicity, release of organic molecules may also result in other detrimental effects depending on the receiving environment. For example, increased phosphorous loading can result in eutrophication of lakes and harmful algal blooms (Correll, 1998). In terms of optimization from a geochemical perspective, the MSB only requires maintenance to continue neutralizing AMD effluent efficiently, removing metals and buffering downstream waters. Maintenance would consist of removal of sediment precipitates from the upper surface to retain the integrity of hydraulic conductivity. This would occur on a time scale of every 1-2 years based on current data.

From a biological perspective, the well-defined geochemical conditions give rise to a distinct microbial community. This community, likely dominated by chitin degradation through fermentation and hydrolysis, leads to acetate driven metabolism by environmental specialists. Within the MSB there is enough organic material in both labile and recalcitrant forms to sustain a large bacterial community. This community includes neutrophillic iron oxidizing bacteria, and sulfate reducing bacteria. These organisms such as *Flavobacterium johnsoniae*, *Sideroxydans lithotrophicus*, and *Desulfotomaculum acetoxidans* are likely the major contributors to elemental cycling within the MSB. From an operational perspective these organisms represent both a beneficial and potentially detrimental microbial community. In terms of beneficial function,
organisms such as *Desulfotomaculum acetoxidans*, promotes sulfide generation through metabolic activity. This contributes to increased alkalinity due to the generation of HCO$_3^-$, as well as promoting trapping of trace metal within biogenic sulfides. However, neutrophillic iron oxidizing bacteria, such as *Sideroxydans lithotrophicus*, present a unique issue in terms of disposal of spent AMD material. This material is rich in iron oxyhydroxides and a common disposal technique is burial and encapsulation to prevent reoxidation. However, some neutrophillic iron oxidizing bacteria are able to oxidize Fe under anoxic conditions through nitrate reduction. This may lead to significant remobilization of Fe, but needs to be further evaluated.

This study highly recommends the use of mussel shells as a substrate in passive bioreactors where available, mainly coastal regions where significant stores of mussel shells are available from the fishing industry. Continued monitoring of the full scale MSB has been green lighted and sampling will continue on a biannual basis and will allow for further evaluation of MSB technology.

**3.2 Future Research**

Although our findings have provided more information about the biogeochemical dynamics of MSB technology there are still several unknowns that require evaluation. One such unknown is long term performance. Commonly, passive treatment systems are designed to operate for many years with little to no maintenance and the full scale MSB has only been in operation for three. This study showed the need for more measurements concerning layer growth, and hydraulic conductivity over time to determine overall longevity of MSB systems. These factors play a pivotal role in determining the rate at which MSB material needs to be
replaced and desludged. These characteristics may also indirectly determine chemical alteration within the MSB and its impact on flow characteristics. Currently, the MSB is approaching hydraulic conductivity failure and removal of, or reduction in thickness of the allochthonous sediment is needed. This will not only provide an opportunity to determine the best process for this, but also to examine the behaviour of this layer during different methods of disposal. Alternatives to encapsulation may include the use of bactericides in buried material, or even reuse as an activated sludge in municipal wastewater treatment, which commonly seeks to aerate waste water. As stated previously encapsulation is the most common means of disposal, but with MSB material it may promote the activity of nitrate reducing iron oxidizing bacteria. Many phases in the bioreactor are susceptible to reductive dissolution (See Fig 2.7 B2), and increased activity from bacteria under anaerobic conditions could cause phase instability and subsequent metal release. However, the geochemical behaviour of MSB material under encapsulated conditions is only hypothetical at this point and there is a possibility that any oxidized material may transform into sulfides as a result of SRB activity. Due to these complexities, a biogeochemical investigation through simulation or sampling of encapsulated material is warranted.

As this is the only study to date that examined the microbial community in an MSB system, more data is needed to fully understand their influence on biogeochemical dynamics. One outstanding aspect is to extract mRNA for metatranscriptomic analyses. These analyses should provide direct evidence for the operational chemical pathways. Additionally, future microbial analysis will focus on whether there are shifts in the preexisting community structure as the MSB matures and biogeochemical conditions change. Collection of this data would also provide an opportunity to develop a DNA microarray specific to MSBs for gene expression
profiling. This method of evaluation would allow for evaluation of an entire suite of genes of interest to provide a definitive picture of cellular function within the MSB and how the community reacts functionally to any changes in geochemical conditions.

Lastly, establishing guidelines for transferability of the technology to other sites is a principal research goal. Currently there are two additional MSBs operating at a field scale. These systems treat AMD of a different character with different metals at varying concentrations. An examination of the biogeochemical dynamics of these systems would allow a direct comparison of MSB performance under varying conditions and provide much needed information about constraints, if any, on the use of MSB technology at additional sites. With regards to this aspect of transferability, the use of a developed microarray would allow for a simple, accurate comparison of any bioreactors installed and allow for an evaluation of their viability and any differences in functionality. Furthermore, there is the possibility of implementing MSB technology in the Great Lakes Region. There is an opportunity to test this technology, as AMD affects many waterways in Ontario (Willson, 1994; Hawley, 1977). However, there are no large commercial farms for mussel shells as there are in New Zealand, but there may be large reservoirs of similar material due to the invasive Zebra Mussel (*Dreissena polymorpha*). This invasive species was quite pervasive throughout the Great Lakes Region (Griffiths, 1991) and there may be large reservoirs that have accumulated through cleaning of hulls, as well as from natural accumulation on beaches in the region. This material may be employed in MSB, as bivalve shell structure and composition is fairly similar across species (Jacob et al., 2008). Differences may arise due to shell size and organic content depending on the origin of the shells (fresh from hulls versus weathered beach shells), but it may not be significant and is easily addressed through the addition of supplementary carbon.
References


## APPENDIX

### Table A1: Summary of Selective Extractions.

<table>
<thead>
<tr>
<th>Target Phases</th>
<th>Reagent</th>
<th>Reaction Time</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Soluble</td>
<td>Nitrogen purged Milli-Q® water</td>
<td>24 hours</td>
<td>Ribeta et al. (1995)</td>
</tr>
<tr>
<td>Bio-Available</td>
<td>0.005 M EDTA adjusted to pH 6</td>
<td>12 hours</td>
<td>Fangueiro et al. (2001)</td>
</tr>
<tr>
<td>Reducible</td>
<td>57 g/l sodium citrate dihydrate + 50 g/l sodium bicarbonate + 24 g/l L-ascorbic acid sodium salt</td>
<td>24 hours</td>
<td>Amirbahman et al. (1998)</td>
</tr>
<tr>
<td>Carbonates and Amorphous</td>
<td>1M sodium acetate solution adjusted to pH 4.5</td>
<td>24-48 hours</td>
<td>Poulton and Canfield (2005)</td>
</tr>
<tr>
<td>Amorphous Oxyhydroxides and Sulfides</td>
<td>0.5M HCl</td>
<td>1 hour</td>
<td>Heron et al. (1994)</td>
</tr>
<tr>
<td>Strong Acid Extractable</td>
<td>5M HCl</td>
<td>21 days</td>
<td>Heron et al. (1994)</td>
</tr>
</tbody>
</table>
A2. ANC Procedure

The procedure for measuring ANC has three components. The first component, referred to as a “fizz test”, was a quantitative measure of the material's reaction with acid. The fizz test required 0.5g of sample placed on a ceramic plate followed by the addition 1-3 drops of 8% HCl. The intensity of the reaction, or effervescence, is rated from 1-5 based off of intensity [Table 3].

After a fizz rating has been determined, in step 2, 2.00g of sample was added to a 250 ml Erlenmeyer flask with 20 ml of Milli-Q® water, as well as the appropriate amount and concentration of HCl determined by the fizz test [Table 3]. The sample was then reacted for a minimum of 1 hour, or until there was no visible reaction. The reaction must also be heated (80-90°C) for its duration, as well as stirred occasionally. Additionally blanks were prepared for each fizz rating used; blanks consist of the same amount of acid and water but with no sample.

The third component was addressed once the reactions were completed. After the sampled had been reacted fully with acid, it was filled to 125 ml with Milli-Q® water and the contents titrated to neutral pH using NaOH. Similar to the previous step molarity of the NaOH was determined by the fizz test rating from table 1. It should be noted that prior to titration, the pH of reactants must be measured, and fall between 1.5 and 0.8. If pH was more basic than 1.5, the fizz rating was too low and the next highest fizz rating was used. If pH was more acidic than 0.8 then too much acid was added and the sample must be reevaluated. Once these parameters were met the contents of the flask were titrated to 7.0 pH. The titration was stopped at pH 5.0 and H₂O₂ (30%) added to oxidized any ferrous iron present (Sobek et al. 1978).
Table A2: Fizz ratings and corresponding ANC parameters.

<table>
<thead>
<tr>
<th>Fizz Rating</th>
<th>0</th>
<th>1 slight reaction</th>
<th>2 moderate reaction</th>
<th>3 strong reaction</th>
<th>4 very strong reaction</th>
<th>5 very strong reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Required HCl Molarity</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Required HCl Volume (ml)</td>
<td>4</td>
<td>8</td>
<td>20</td>
<td>40</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>Required NaOH Molarity</td>
<td>0.1</td>
<td>0.1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Lower Limit for ANC</td>
<td>-</td>
<td>10</td>
<td>40</td>
<td>100</td>
<td>200</td>
<td>400</td>
</tr>
<tr>
<td>Upper Limit for ANC</td>
<td>10</td>
<td>40</td>
<td>100</td>
<td>200</td>
<td>400</td>
<td>-</td>
</tr>
</tbody>
</table>
After titration was completed, ANC values were calculated using the following formulas (ARD Test Handbook 2002, Sobek et al. 1978):

\[ \text{ANC} = \left[ Y \times \text{MHCl/ wt} \right] \times C. \]

Where:

\[ Y = (\text{Vol. of HCl added}) - (\text{Vol. of NaOH titrated} \times B) \]

\[ B = (\text{Vol. of HCl in blank}) / (\text{Vol. of NaOH titrated in blank}) \]

\[ \text{MHCl} = \text{Molarity of HCl} \]

\[ \text{wt} = \text{Sample weight in grams} \]

\[ C = \text{Conversion factor} \]

\[ C = 49.0 \text{ (calculates kg H}_2\text{SO}_4/\text{t)} \]

\[ C = 5.0 \text{ (calculates } \% \text{ CaCO}_3 \text{ equivalent)} \]

The final calculation provided a value in kg of H\textsubscript{2}SO\textsubscript{4}/Tonne and should fall within the proper range for the fizz rating [Table A2]. If values did not fall within the appropriate range the test was repeated with the fizz rating adjusted.
Figure A1: Extractable concentrations of aluminum and iron, along the vertical profile of the MSB in mg kg\(^{-1}\). Extraction targets include water soluble (top), Carbonate (middle), and bio-available (bottom).
Figure A2: Extractable concentrations of aluminum and iron, along the vertical profile of the MSB in mg kg\(^{-1}\). Extraction targets include reducible (top), Strong acid extractable (bottom).
Figure A3: Extractable trace metal concentrations in mg kg\(^{-1}\) of material, nickel, thallium and zinc, along the vertical profile of the MSB. Extraction targets include bio-available (A), Carbonate (B), as well as strong acid extractable (C). Measurements for extractions at 8 months are presented on the left half of each graph (A1, B1, C1), while measurements at 20 months are presented on the right half (A2, B2, C2). A visual representation of MSB layering can be found on the left hand side of each extraction series.
References:


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